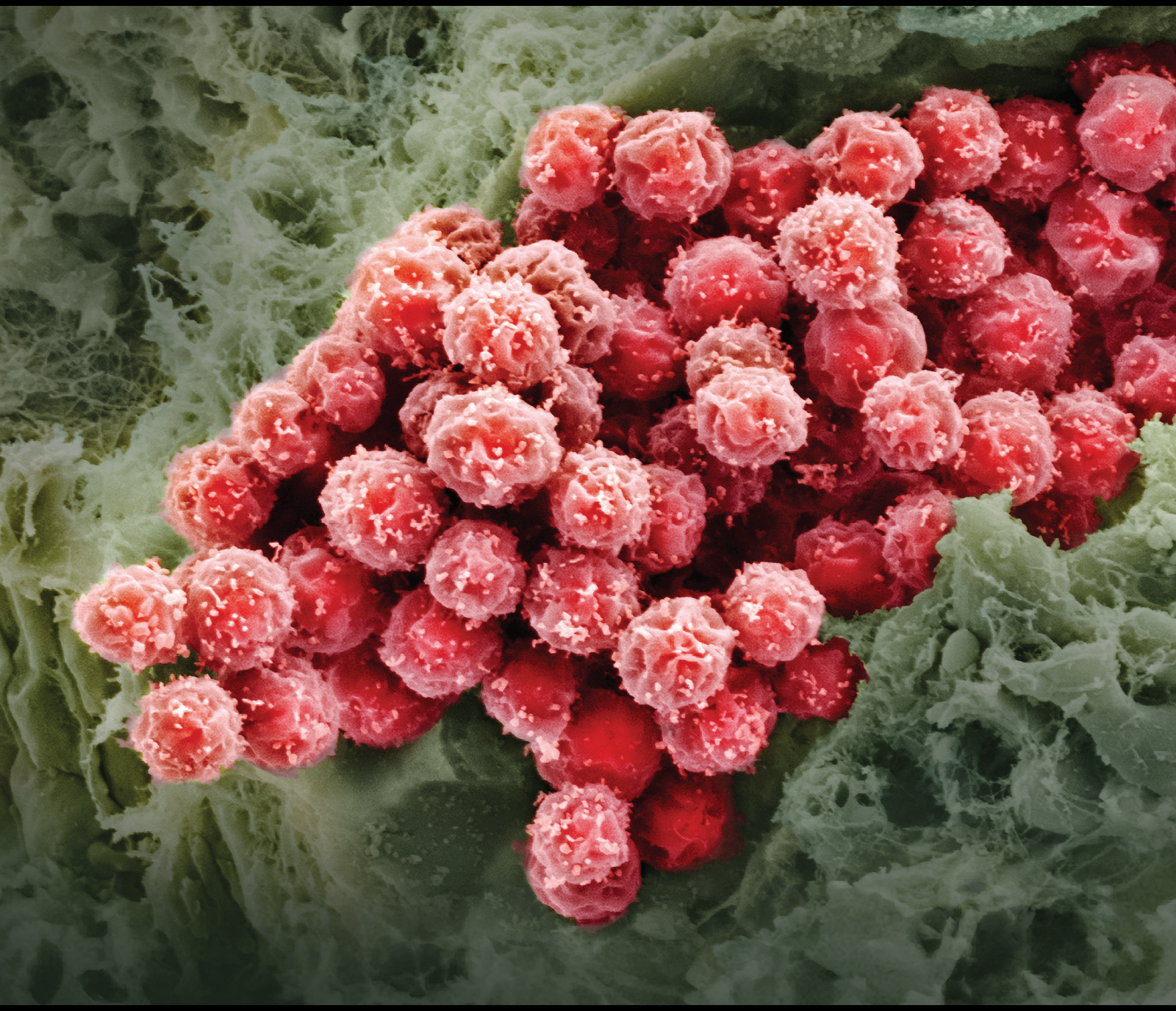
Stem Cells International

Stem Cell Differentiation and Therapeutic Use

Guest Editors: Matthew S. Alexander, Juan Carlos Casar, and Norio Motohashi



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Editorial

Stem Cell Differentiation and Therapeutic Use

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Stem cell therapy is a promising approach to cure degenerative diseases, cancer, damaged tissues, or any disease for which there are very limited therapeutic options. Stem cell therapies could potentially improve the efficiency of the human body regenerative response following an injury or insult, in addition to being a source of powerful therapeutic compounds that hold the promise of the restoration of normal function of a given tissue. The abilities to identify, isolate, expand, and differentiate stem cells have been past barriers for developing therapies in patients and treatments using stem cells have long been viewed as potential or under development. The discovery of induced pluripotent stem cells (iPSC) has encouraged many researchers in the field of stem cell biology and organized-patient groups to advocate for increased development of stem cell-based therapies and in-depth scientific knowledge.

The field of regenerative medicine and the ability to harness and manipulate stem cells to treat human diseases have been opened by a combination of many technical advances [1]. A few, among these improved technologies, should be mentioned. Whole transcriptome analyses have allowed researchers to better understand the molecular signatures of different stem cell populations in human tissues and rodent models [2]. New methods in generating novel transgenic models have allowed researchers to perform elegant lineage tracing of stem cells as they proliferate and differentiate into specific somatic lineages [3–5]. Improvements in techniques to prospectively isolate stem cell populations, manipulate their genomes, and image/track them have also greatly improved in the past decade [6–8]. Advances in large scale genetic and drug library screening have also allowed an unprecedented insight into the molecular changes and factors necessary to convert pluripotent cells into progenitors of specific lineages [9].

Last year, clinical trials using retinal pigment epithelium derived from iPS cells were performed for patients who had severe age-related macular degradation in Japan [10, 11]. These trials evaluated the safety of iPS cells generated from the recipient's own cells and they have shown that the iPS cells did not evoke any immune reactions and did not produce tumors in mice and monkeys before conducting transplants. So far, no problems or health concerns have been reported, suggesting that iPS cells constitute an additional option for stem cell-based therapies. One remaining concern is whether the engrafted iPS cells will be working as part of the regenerating tissue and whether the transplanted cells will persist for a long time. Another exciting report this year was a US-based clinical trial from Robert Lanza's group at Advanced Cell Technology, demonstrating efficacy and tolerance of human embryonic stem (ES) cell-derived retinal pigment epithelium cells transplanted into patients with Stargardt's macular dystrophy [12]. This phase 1/2 clinical trial did not show any adverse effects and an improvement in visual function in a significant number of patients receiving the transplanted cells was observed. These findings have given patients with previously untreatable degenerative disorders

hope for a potential regenerative stem-cell based treatment. The current state of development of stem cell-based therapies for different conditions is reviewed by J. F. Stoltz et al. and R. Compagna et al., in this issue. Additional potential for therapies may lie in the ability to harness the power of mesenchymal stem cells (MSCs). Mesenchymal stem cells are stem cells that developmentally originate from the embryonic mesoderm and are thought to be involved in the regeneration and tissue repair processes of several organs including the bone, vasculature, connective ligaments, and liver [13, 14]. It is this therapeutic potential of MSCs that makes them attractive for potential clinical therapies, as evident by studies of bone-marrow and tendon-derived mesenchymal stem cells of regenerative potential by M. K. Al-ani et al. in this issue. Additional work on the differentiation potential of various MSCs (such as that by D. J. Zhang et al. in this issue) into more somatic lineages highlights the progress made towards directed lineage conversion of specific stem cell populations.

Despite these promising initial studies, potential and unanticipated risks and complications or side effects might become apparent with progress of stem cell-based therapies. Continued research is fundamental for the evaluation and exclusion of potential risks before the clinical use of stem cells. It is difficult, however, to predict with certainty the risk of these therapies due to the number and complexity of the variables involved, such as type of stem or progenitor cells, their proliferation or differentiation capacity, the methods for their isolation and route of administration, the engraftment location, and others derived from the recipients' age or health condition. Rigorous preclinical experiments and safety trials are needed to address some of these issues whose design must take into account both observations from clinical experience and from animal studies including tumor formation and/or immune responses. Understanding the basic biology and differentiation potential of stem cells (as in the study of the SDF-1/CXCR4 axis as a regulator of MSCs to repair liver injury by N.-B. Hao et al. in this issue) might be an important clue to solve some of these downstream problems. It is well known that stem cells not only work as a part of tissue regeneration, but also secrete factors for maintenance and well-being of tissue homeostasis, for example, by promoting vasculature, suppressing inflammation, or accelerating cell growth. Interesting examples of these mechanisms are described in the studies by A. L. Strong et al. and D. J. Zhang et al. included in this issue.

A remaining significant hurdle for scientists and clinicians is the growing number of unregulated stem cell clinics [15]. False promises of miracle cures using stem cells have led to the burgeoning industry of stem cell tourism, where patients travel outside of countries with strong regulation of stem cell uses to countries where stem cell regulations are more lax [16]. As we move forward in advancing rational therapies for treating patients, it is important for the field to ensure patient safety and openness of sharing all clinical trial results, both positive and negative, with the public.

In this special issue, we present a collection of studies and reviews that highlight a broad variety of topics related to stem cell differentiation and potential therapeutic use. We hope that the readers will appreciate the amount of progress in addition to the challenges that have been made in the past decade towards understanding and characterizing stem cells from embryonic to adult stem cells. As is evident by the investment made by several governmental and private agencies, novel stem cell therapies are well on their way to becoming a reality for patients dealing with debilitating diseases.

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Juan Carlos Casar
Norio Motohashi

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Research Article

Study of Bone Marrow Mesenchymal and Tendon-Derived Stem Cells Transplantation on the Regenerating Effect of Achilles Tendon Ruptures in Rats

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Comparative therapeutic significance of tendon-derived stem cells (TDSCs) and bone marrow mesenchymal stem cells (BMSCs) transplantation to treat ruptured Achilles tendon was studied. Three groups of SD rats comprising 24 rats each, designated as TDSCs and BMSCs, and nontreated were studied for regenerative effects through morpho-histological evaluations and ultimate failure load. For possible mechanism in tendon repair/regeneration through TDSCs and BMSCs, we measured Collagen-I (Col-I), Col-III gene expression level by RT-PCR, and Tenascin-C expression via immunofluorescent assay. TDSCs showed higher agility in tendon healing with better appearance density and well-organized longitudinal fibrous structure, though BMSCs also showed positive effects. Initially the ultimate failure load was considerably higher in TDSCs than other two study groups during the weeks 1 and 2, but at week 4 it attained an average or healthy tendon strength of 30.2 N. Similar higher tendency in Col-I/III gene expression level during weeks 1, 2, and 4 was observed in TDSCs treated group with an upregulation of 1.5-fold and 1.1-fold than the other two study groups. Immunofluorescent assay revealed higher expression of Tenascin-C in TDSCs at week 1, while both TDSCs and BMSCs treated groups showed detectable CM-Dil-labelled cells at week 4. Compared with BMSCs, TDSCs showed higher regenerative potential while treating ruptured Achilles tendons in rats.

1. Introduction

About 30 million ligament and tendon injuries are reported annually across the globe due to lifestyle, recreation, work patterns, accidents, pharmacological agents, and degenerative biological variables such as gender, age, and genetics [1]. Anatomical studies are crucial for in-depth understanding of tendon healing and regeneration. Tendon is comprised of parallel and well organized collagen (Col) bundles, of which approximately 90% are of Col-I, while the rest of the 10% are Col-III, -IV, -V, and -VI [2]. Chronic or acute tendon injuries are primarily treated with conservative or surgical treatments, where the former is used for symptomatic relief only, is ineffective and time-consuming, but later involves the use of autografts, allografts, xenografts, and prosthetic devices [3, 4]. However, there are considerably high risks of complications such as infection, nerve damage, adhesion, and distributed skin sensibility. Therefore, it is crucial to define some innovative techniques to treat such tendon injuries.

Stem cells are undifferentiated and self-renewing cells able to differentiate into specialized cells of different types with specific functions including the biological healing process [5, 6] and include TDSCs, BMSCs, adipose-derived mesenchymal stem cells (AdMSCs), and umbilical cord blood-derived stem cells (UCB-SCs). TDSCs and BMSCs have various advantages and superiority over the other many different stem cell types such as quick proliferation, tendon repair specificity, reduced regeneration time, and superior shaped tendon formation [7]; hence we used these two stem cell types as a model in the present study. Recently various researchers reported that BMSCs may differentiate to give rise into several connective tissue types including

bone, cartilage, tendon, muscle, marrow, fat, and dermis [8–10]. BMSCs are involved to facilitate the tendon healing process intrinsically by accelerating fibroblast proliferation and modulation of certain growth factors and cytokinins [11–13]. On the other hand, TDSCs are adult stem cells residing in tendons [14], which are histologically and biochemically proven as a prime source of tendon repair [15–17]. Cheng and coworkers demonstrated that TDSCs have high potential for colony-formation compared with BMSCs [18]. It is also said that TDSCs expresses higher mRNA level of tenogenic markers- scleraxis (*Scx*), tenomodulin (*Tnmd*), and extracellular matrix (ECM) components of tendon, that is, Col-1A1, Col-1A1/Cl3-A1 ratio, and decorin (*Dcn*), than BMSCs. However, a comparison between TDSCs and BMSCs on treating tendon injury has not yet been done. We hypothesize that TDSCs are more favorable for treating Achilles tendon injuries.

In the present study, we transplanted TDSCs and BMSCs into the ruptured area of the Achilles tendon for macroscopic appearance; histomorphological analyses and biomechanical strength were observed to find the possible mechanism of the repair promotion and evaluated the cell transplantation effects of both stem cell types. The results indicate that TDSCs exhibit a better-regenerative potential when compared with BMSCs in treating ruptured Achilles tendons and could be a better alternative cell source for treating Achilles tendon.

2. Materials and Methods

2.1. Ethics Statement. Ethics Committee of Chongqing University, College of Bioengineering, and Daping Hospital Animal Experimental Center approved all experimental protocols using SD rats including collection of Achilles tendon samples.

2.2. Rats and Treatment Groups. Seventy-eight Sprague-Dawley (SD) male rats weighing 200 g obtained from the Daping Hospital's animal experimental center (Chongqing, China) were used as recipients or donors. Six SD rats, which did not undergo an operation, were the source of tendon-derived stem cells, bone marrow mesenchymal stem cells, and healthy Achilles tendons. The remaining 72 SD rats were used for Achilles tendon healing experiments. The rats were divided in to three groups: TDSC, BMSC, and nontreated group, each group 24 rats. The study was carried out at three time points 1 week, 2 weeks, and 4 weeks. Eight rats were assigned to each time point. The rats were placed in individual cages under slandered feeding system.

2.3. Cell Isolation. Six SD rats were used to isolate the various cells. The rats were sedated with pentobarbital sodium in an anesthetic chamber. Then, using 3% Fluothane in a mask, they were sacrificed. The femur and tibia, including the tendons attached to them, were dissected. BMSCs of SD rats were isolated using a modified procedure [18]. Briefly, both femur and tibia were excised, and the diaphyses were cut. The bone marrow was flushed out with DMEM-LG (Gibco) supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. Single cell suspension was generated by aspirating the bone marrow back through the syringe. The samples were then washed and centrifuged at 1000 rpm to remove the pieces of debris. The cell pellets were resuspended and expanded in a humidified incubator at 5% CO_2 and 37°C. TDSCs were isolated from rats by removal of the tendons and rinsed with PBS. The TDSCs were isolated according to the previous report [19]. Briefly, the Achilles tendons were then minced into small pieces and digested with 5 mg/mL of type I collagenase (SIGMA) at 37°C with 5% CO_2 for two hours. The undigested tissues were removed by using 70 mm nylon sieve, and the remaining cell pellets were cultured with low glucose Dulbecco's modified Eagle's medium (DMEM, Gibco) L-glutamine, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. After 12 days, the cell colonies formed and were selected for further culture. After 100% confluence, cells were subcultured, and the medium was changed every third day. For both BMSCs and TDSCs, Passage 3 (P3) cells were adopted for identification.

2.4. Cell Identification and Multidifferentiation Assays. Flow cytometry was used for identifying stem cell surface markers: CD29, CD44, and CD90 of TDSCs and BMSCs. The osteogenic, adipogenic, and chondrogenic differentiation potential of TDSCs and BMSCs were tested according to the previous report [19, 20]. After induction, Alizarin red, Oil red, and Toluidine blue staining assays were used to confirm osteogenesis, adipogenesis, and chondrogenesis, respectively.

2.5. Animal Model and Surgical Procedures. Seventy-two SD rats, weighing 200 g, provided by Daping Hospital and the Research Institute of Surgery of the Third Military Medical University were used. Prior to the study, all operations and handling procedures were approved by the hospital. The rats were divided into three groups: the BMSCs group, the TDSCs group, and the nontreated group, each group 24 rats. Three rats from each group were tested in each time point. The left hind legs of the animals were used for micro/macro observation while the right hind legs were used for measuring gene expression. Five rats from each group from each time point were used for biomechanical evaluation. The rats were anesthetized with pentobarbital sodium in an anesthetic chamber and then with 3% Fluothane in a mask. In aseptic conditions, 10 mm longitudinal incision of the right and left hind limb was made directly over the Achilles tendon (Figures 1(a) and 1(b)). A segment from the middle part of the Achilles tendon was cut using a surgical blade 5 mm from the calcaneal insertion site. Clinical sutures were used to suture the incision, and iodine was directly applied (Figure 1(c)). The TDSCs and BMSCs were labeled with CM-DiI (C7000, Invitrogen), after that the donor TDSCs (1×10^6/0.1 mL DMEM) or BMSCs (1×10^6/0.1 mL DMEM) were injected around the Achilles tendon of each rat with a syringe (Figure 1(d)). During the recovery period, the rats were placed in individually sterilized cages under standard feeding system. At the end of each time point, the rats were sacrificed by over dose of ether anesthesia.

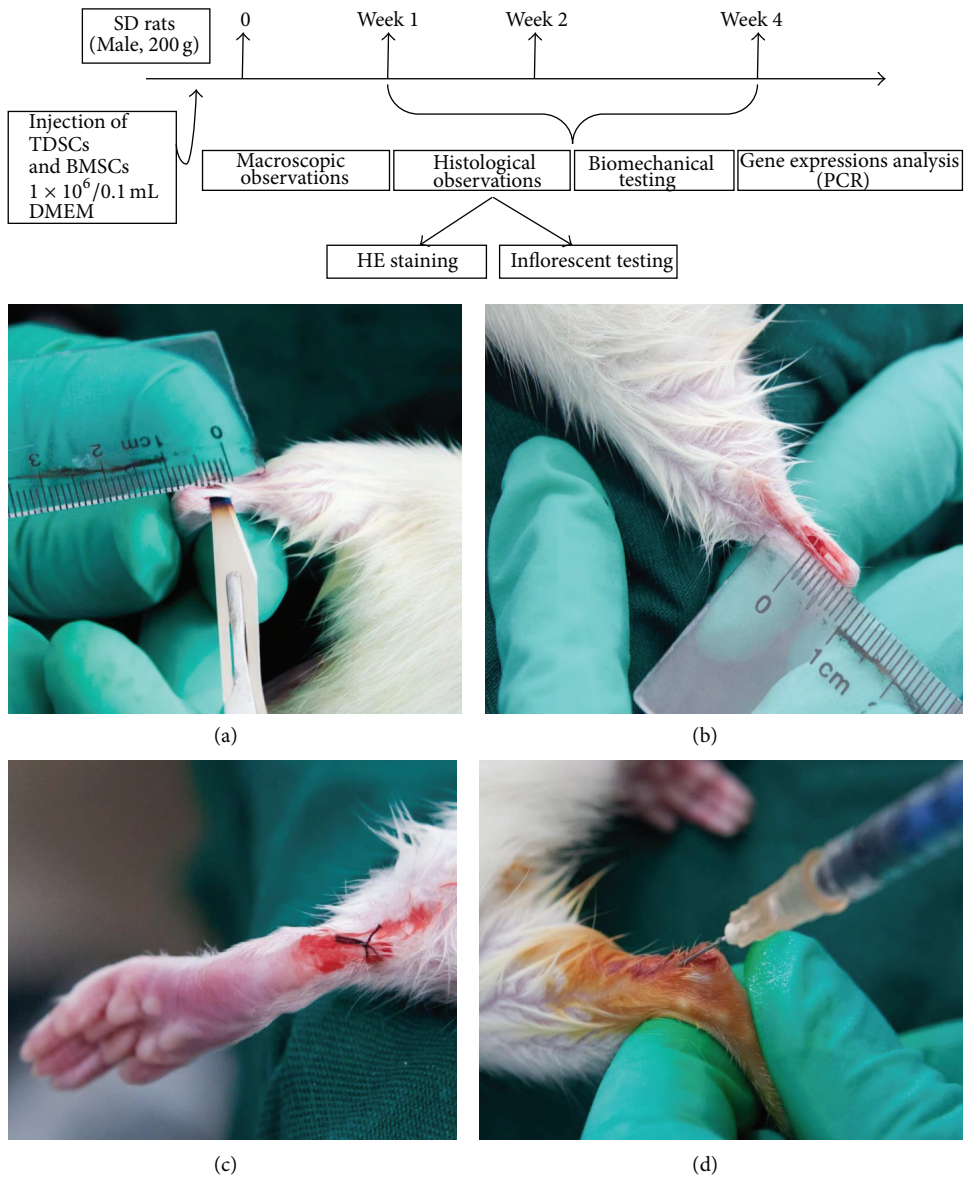


Figure 1: Experimental protocol. A complete transverse incision was made 10 mm from the calcaneal insertion of the Achilles tendon ((a) and (b)). Clinical suture was used to suture the skin and iodine was directly applied (c). TDSCs (1×10^6/0.1 mL DMEM) or BMSCs (1×10^6/0.1 mL DMEM) were injected in the Achilles tendon with use of a syringe (d).

2.6. Macroscopic Assessment. At the end of each time point, the rats were sacrificed, and the treated legs were removed for macroscopic observation. The appearance of the regenerated tendons was observed and compared to that of the nontreated group.

2.7. Histological Evaluation. The treated rats were sacrificed, and the Achilles tendon between the calcaneus and musculotendinous junction was harvested at each time point. The tendon was immersed in 4% PFA overnight, dehydrated, and embedded into optimal cutting temperature compound (OCT). The specimens were cut into 10 μm sections by freezing microtome (Leica CM1900) and stained with hematoxylin and eosin (HE) for histological evaluation.

2.8. Biomechanical Testing. Five rats from each group were used for biomechanical testing as follows: the Achilles tendon between the calcaneus and musculotendinous junction resected at 1 week, 2 weeks, and 4 weeks after incision. The proximal and distal ends of the Achilles tendon were fixed securely in serrated grips and mounted on to a mechanical testing machine (Instron). With 100 N load cell capacity, the Achilles tendon was pulled at a constant speed of 10 mm/min until rupture. The data was recorded with software (Win Test'7).

2.9. Quantitative (RT) Polymerase Chain Reaction (qPCR). We examined the expression of Col-III, Col-I, and GAPDH in every time point. Total RNA was extracted using the total

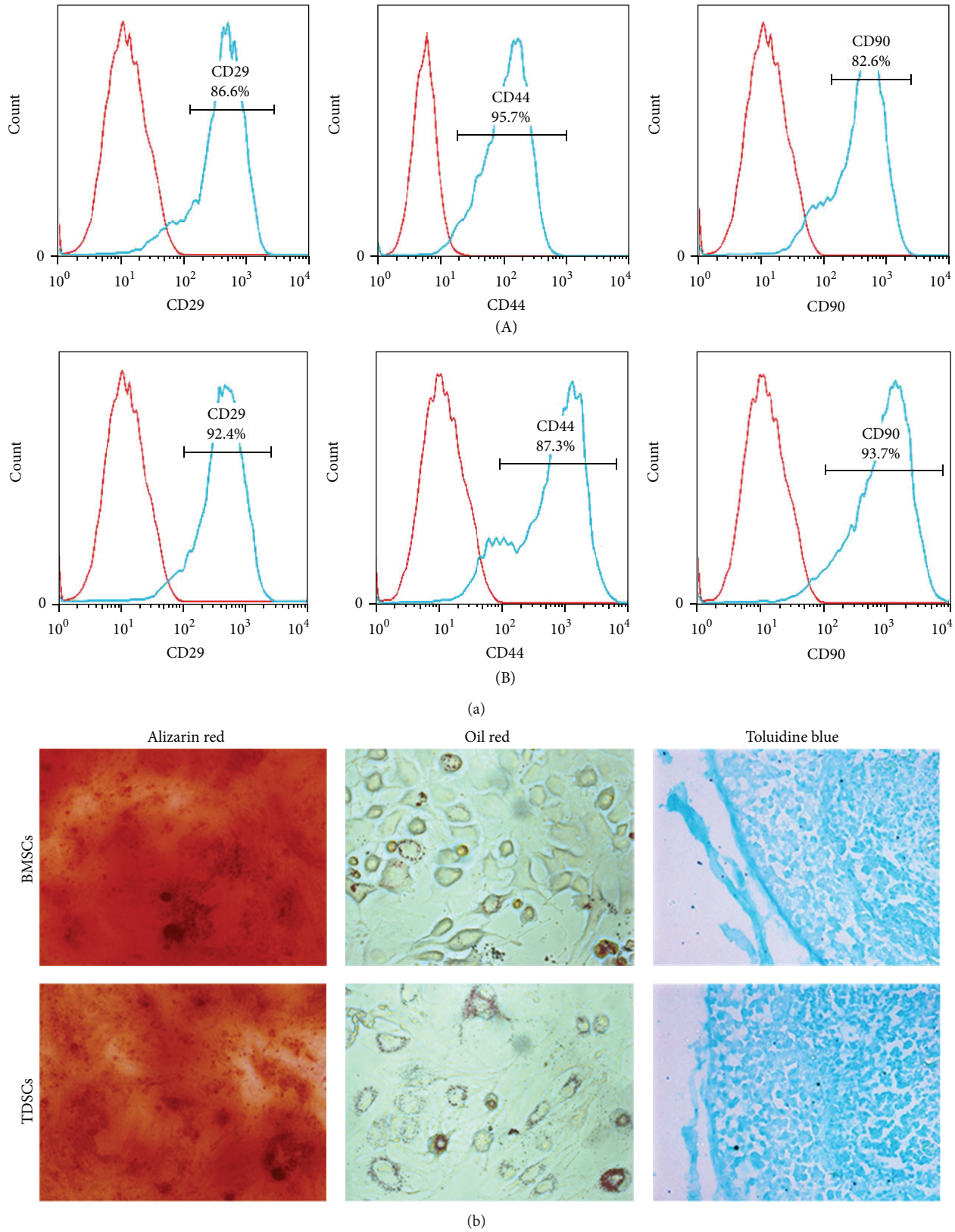
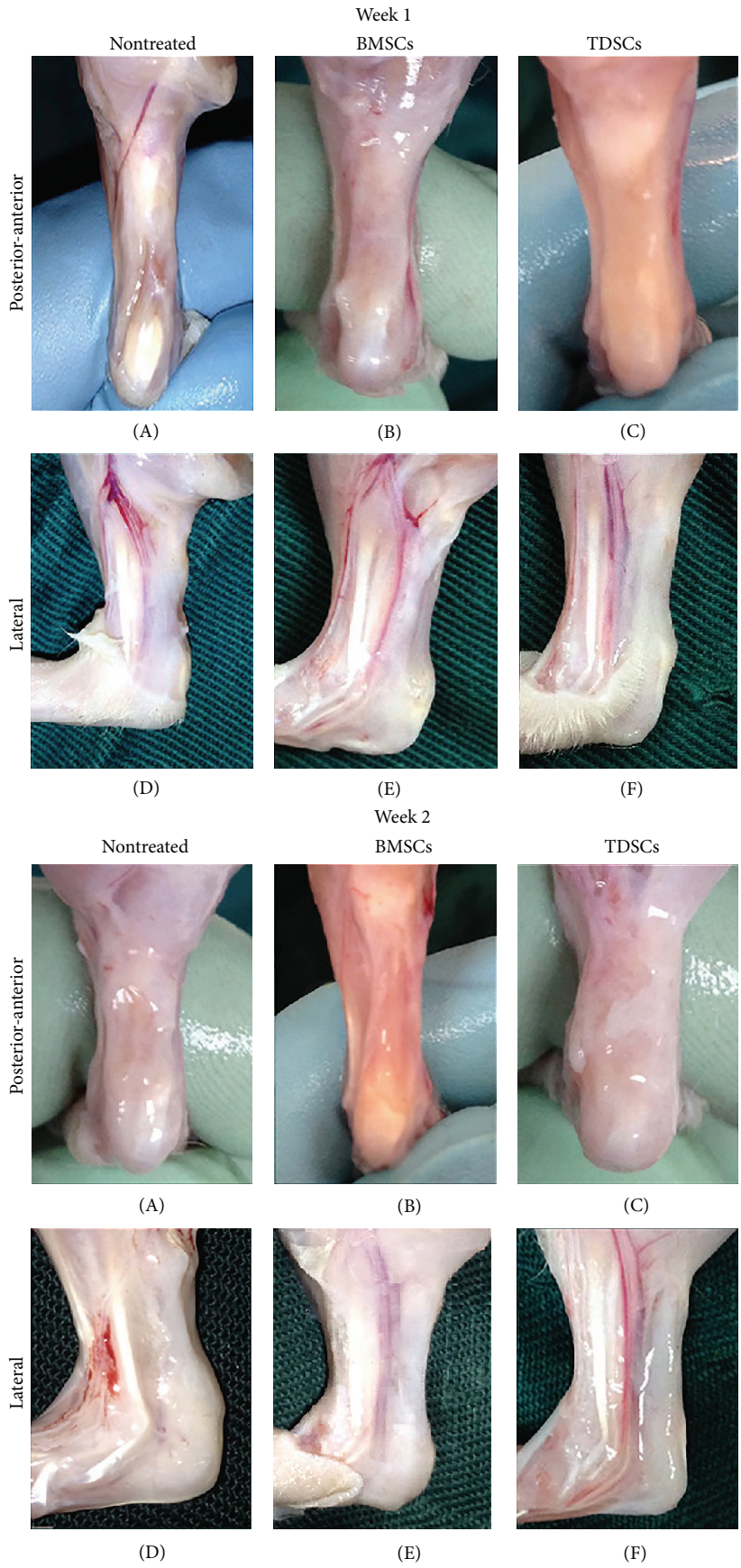


Figure 2: Cell identification of TDSCs and BMSCs. (a) Stem cell surface markers of FCM: (a)-(A) TDSCs, (a)-(B) BMSCs. (b) Multidifferentiation of TDSCs and BMSCs: osteogenesis (Alizarin red); adipogenesis (Oil red); chondrogenic (Toluidine blue).



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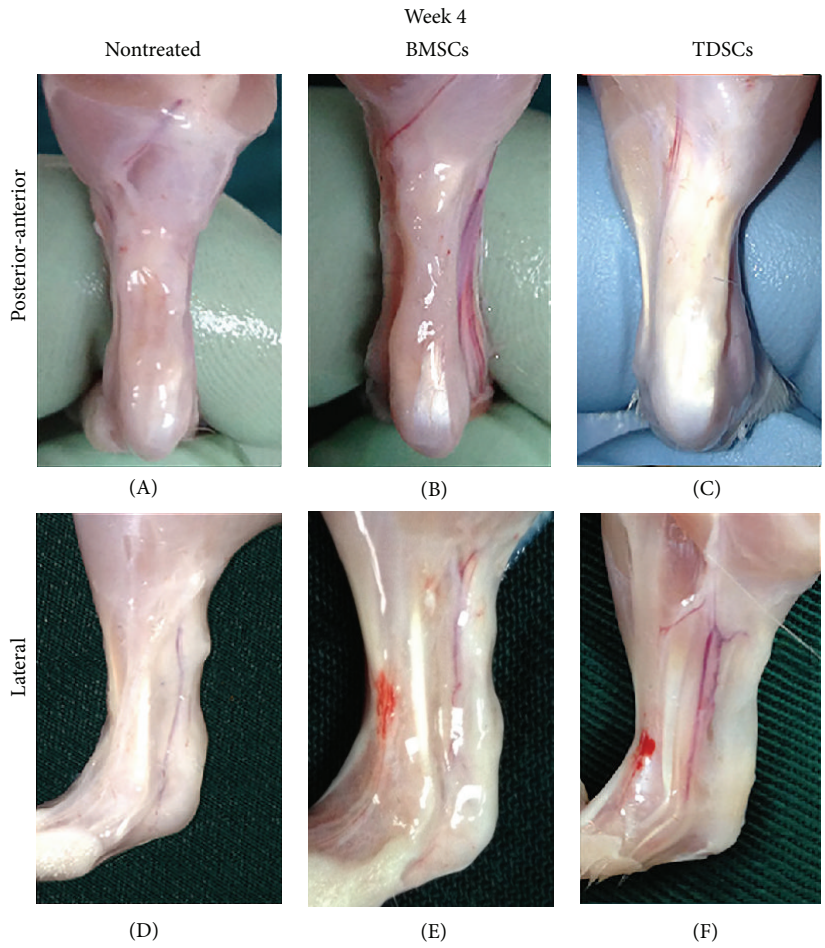

Figure 3: Macroscopic findings at 1 w, 2 w, and 4 w after surgery. The posterior-anterior appearance of the Achilles tendon in the nontreated group (A), the BMSCs group (B), and the TDSCs group (C). The lateral appearance of the Achilles tendon in the nontreated group (D), BMSCs group (E), and TDSCs group (F).

RNA extraction kit (Bioteke Corporation) according to the manufacturer's instructions. RNA was subjected to reverse transcription to complementary DNA (cDNA) using the First Strand cDNA kit (Thermo Scientific Rt-First Strand cDNA Synthesis kit, K1622). PCR conditions were 65°C for 5 min, then 42°C for 60 min, and termination at 70°C for 5 min. The products were stored at 80°C. Total cDNA for each sample was amplified in a final volume of the reaction mixture containing SsoAdvanced SYBR Green qRT-PCR supermix (Bio-Rad number 1725264) ready-to-use reaction cocktail and specific primers for Col-I: Forward: AAGGTGACAGAGGCATAAAG, Reverse: GGAAGCTGAAGTCATAACCA And Col-III: Forward: CATGATGAGCTTTGTGCAAT, Reverse: CTGCTGTGCCAAAATAAGAG. The cycling conditions were the denaturation at 95°C for 30 sec, 39 cycles at 95°C for 5 sec, optimal annealing temperature for 20 sec, 72°C for 30 sec, and 60°C to 95°C with a heating rate of 0.1°C/s. The CFX 96 Real-Time PCR Detection System (Bio-Rad) was used to record the results. The relative expression level of the gene of interest normalized to GAPDH was analyzed according to the $2^{-\Delta\Delta ct}$ Method.

2.10. Immunofluorescent Assay. The treated Achilles tendon of each group was collected in week 1 and week 4 and performed frozen sections. All the sections were immune stained with Tenascin-C (1 : 100, Abcam) primary antibody, followed by Alexa Fluor 488 dye-labeled secondary antibody. DAPI (Roche) was used to stain cell nuclei and observed under the immunofluorescent microscope to check the cells transplantation regenerative processes.

2.11. Statistical Analysis. All the data are expressed as means ± standard deviations. Statistical analysis was performed with one-way ANOVA, followed by LSD test for comparison between two groups (Origin Lab Origin V 8.0 Software). A P value of <0.05 was considered significant.

3. Results

3.1. Study of Stem Cell Markers for the Confirmation and Differentiation Ability of TDSCs and BMSCs. Flow cytometry analysis was done to identify the stem cells. Both TDSCs and BMSCs were tested positively for CD 29, CD 44, and

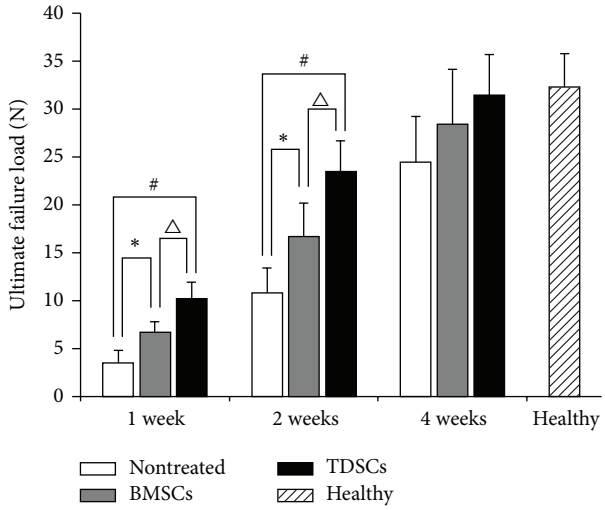

Figure 4: Results of biomechanical testing. The ultimate failure load in the nontreated group, the BMSCs group, and the TDSCs group at 1 w, 2 w, and 4 w after surgery. Healthy indicates the ultimate failure load in a normal Achilles tendon at thirteen weeks of age. $^{*}P < 0.05$ and $^{\#}P < 0.05$ compared with control and $^{\triangle}P < 0.05$ compared with BMSCs were considered significant (data represents mean ± SD, $n = 5$).

CD 90, which are indicative of stem cell surface markers (Figure 2(a)). It confirmed that the cells we isolated from the Achilles tendon and bone marrow were stem cells. Multidifferentiation capacity is one of the universal characteristics of stem cells. The differentiation analyses showed that the adipogenesis (Oil red), chondrogenesis (Toluidine blue), and osteogenesis (Alizarin red) of the isolated TDSCs and BMSCs were emerged after induction (Figure 2(b)). Hence, based on the results of cell identification analyses, it became assured that the isolated stem cells were TDSCs and BMSCs.

3.2. Week-Wise Macroscopic Assessment of Morphological Changes in Repaired Achilles Tendon. The skin was sutured with a clinical suture to inject TDSCs (1×10^6 /0.1 mL DMEM) and/or BMSCs (1×10^6 /0.1 mL DMEM) in the Achilles tendon using a sterile syringe. After transplantation of the TDSCs and BMSCs, changes in appearance on the treated area were analyzed at weeks 1, 2, and 4 after surgeries. At week 1, the defect area appeared clearly in the nontreated group (Figures 3(A) and 3(D)), while the BMSCs group revealed some connective tissue in the treated area (Figures 3(B) and 3(E)). The TDSCs group revealed a good start of growth in connective tissue around the treated area (Figures 3(C) and 3(F)). On approaching week 2, the growth of connective tissue was stunted while the defected place area was obvious in the nontreated group, which remained as such during week 1 (Figures 3(A) and 3(D)). On the other hand, BMSCs treated group appeared better than week 1 group, and TDSCs showed the best growth in connective tissue at the treated area (Figures 3(C) and 3(F)).

At week 4, the TDSCs group appeared significantly different than the other groups and displayed a complete Achilles tendon with a normal appearance in the posterior-anterior and lateral views (Figures 3(C) and 3(F)). The BMSCs group showed obvious connective tissue growth with a little transverse notch appearance clearly in the posterior-anterior and lateral views (Figures 3(B) and 3(E)). The nontreated group showed no tissue growth at all (Figures 3(A) and 3(D)).

3.3. Biomechanical Testing. The tensile strength of the repaired Achilles tendon in various study groups was tested by the ultimate failure load method. The ultimate failure load in the TDSCs group was considerably higher (10.2 N) than that in BMSCs (6.7 N) and nontreated groups (3.5 N) at week 1 after incision ($P < 0.05$). The failure load of TDSCs and BMSCs was increased 1.9-fold ($^{\#}P < 0.05$) and 0.9-fold ($^{*}P < 0.05$), respectively, where the former has 0.5-fold higher change ($^{\triangle}P < 0.05$). The ultimate failure load at week 2 after incision was significantly higher for the TDSCs (23.5 N) than the BMSCs (16.5 N) and nontreated (10.8 N) by 1.2-fold ($^{\#}P < 0.05$) for TDSCs and the BMSCs group by 0.5-fold ($^{*}P < 0.05$). The failure load of TDSCs exceeded 0.5-fold ($^{\triangle}P < 0.05$) versus BMSCs. At week 4, the ultimate failure load of the TDSCs group again showed a higher value (30.2 N) than the BMSCs group (28.45 N) and the nontreated group (25.3 N). It is important to note that among these three groups, BMSCs were also higher than the nontreated group. However, there was no statistically obvious difference between TDSCs and BMSCs. In addition, at week 4 after surgery, the ultimate failure load of TDSCs and BMSCs reached nearly that of a healthy (Figure 4).

3.4. Histological Study of the Healing Achilles Tendon. The histological analyses of Achilles tendon sections were made and gone through hematoxylin and eosin staining for the nontreated group, the BMSCs group, and the TDSCs group, at three time points (weeks 1, 2, and 4) (Figure 5). Dense connective tissue was observed in the TDSCs and BMSCs treated groups at week 1 after surgery. For the TDSCs group, visible longitudinal fibrous tissue had already emerged along with well-organized cell structures not seen in other two groups. At week 2 after surgery, both TDSCs- and BMSCs-treated and particularly TDSCs-treated tendons exhibited more ECM deposition and obvious longitudinal fibrous tissue than that of nontreated tendons with a greater number of spindle-shaped cells aligned/organized along the longitudinal (tensile) axis of the tendon. A similar trend was observed at week 4 after surgery TDSCs given improved tendon status than BSMCs, and hence both TDSCs and BSMCs showed a spindle-shaped morphology distributed along the longitudinal fibrous tissue of the tendon. On the contrary, the cells of the loose and thin longitudinal fibrous tissue that began to appear in the nontreated group made little organization with higher vascularization. In order to examine the transplanted, labeled TDSCs in the excised Achilles tendon, frozen sections were prepared and analyzed by fluorescent microscopy. The CM-DiI positive cells (red) were detectable around the tendon at week 4 after transplant (Figures 6(a) and 6(b)), indicating that those transplanted cells were still alive and may participate in the process of regeneration.

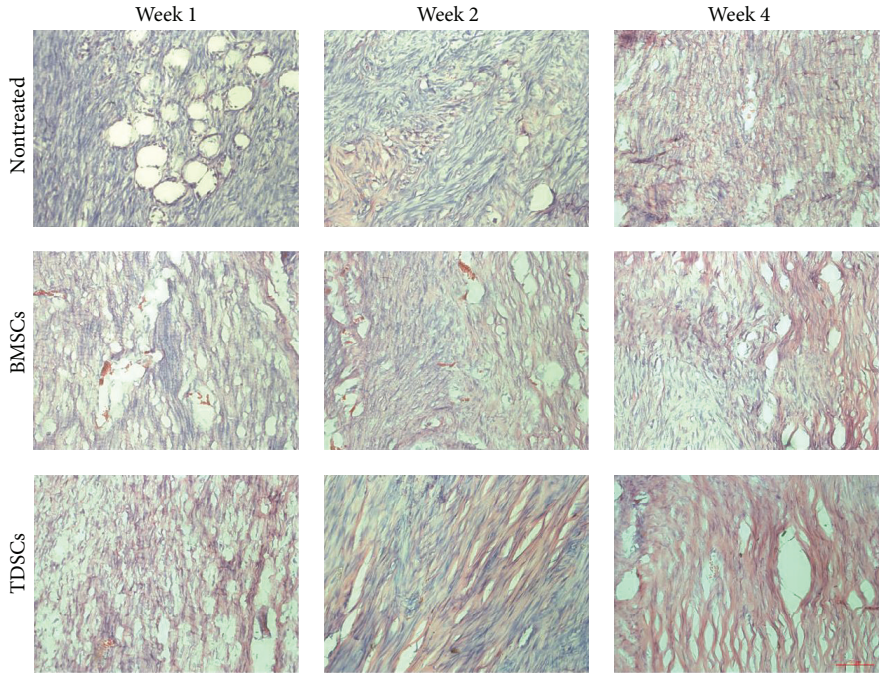

Figure 5: Histological analysis of the Achilles tendon: HE staining of the nontreated group, the BMSCs group, and the TDSCs group, at three time points (1 week, 2 weeks, 4 weeks). Bar: 200 μm.

3.5. Collagen I and Collagen III Gene Expression Analysis. To detect host ECM (collagen) deposition conditions, quantitative real-time PCR was performed to investigate rat-specific Col-I and Col-III (Figure 6(a)). At the first week, TDSCs and BMSCs Col-I gene expression levels were upregulated up to 6.2-fold ($P < 0.05$) and 4.2-fold ($P < 0.05$), respectively. The levels of Col-III were upregulated by 4.2-fold ($P < 0.05$) in TDSCs and 2.2-fold ($P < 0.05$) in BMSCs compared with nontreated group. In addition, the expression of Col-I and -III was higher in the TDSCs group than the BMSCs group and it showed significant differences ($P < 0.05$). At week 2 after surgery, Col-I gene expression levels were upregulated by 4.1-fold ($P < 0.05$) and 2.3-fold ($P < 0.05$) in the TDSCs and BMSCs groups, respectively. In addition, in both TDSCs and BSMCs the Col-III levels were upregulated by 2.3-fold ($P < 0.05$) and 1.2-fold ($P < 0.05$), respectively, compared with nontreated group. In addition, the expression of Col-I and -III was higher in the TDSCs group compared with the BMSCs group and it showed significant differences ($P < 0.05$). At week 4 after surgery time, TDSCs Col-I level was higher than BMSCs; it showed a significant difference, but only the TDSCs Col-I gene expression level showed a significant difference and was upregulated by 1.5-fold ($P < 0.05$) compared with nontreated group, and by 1.1-fold ($P < 0.05$) in the BMSCs-treated group (Figure 6(a)). Additionally, Col-III in all groups showed almost the same expression level (Figure 6(a)). It is obvious that both TDSCs and BMSCs have the ability to boost the ECM gene expression. But herein we observed that TDSCs compared to BMSCs have more agility to trigger the genes involved in expression of ECM and accelerated tendon repair many folds.

3.6. Immunofluorescent Assay of Injured Achilles Tendon followed by TDSCs and BMSCs Transplantation. The organization by immunofluorescence staining found that, after 1 week of implantation TDSCs and BMSCs in the injured Achilles tendon, cells were found in many parts of the distribution of CM-Dil labelled (Figure 6(b)). Following implantation of TDSCs and BMSCs around the injured Achilles tendon, a large portion with Tenascin-C staining was detected in both treated groups (Figure 6(b)), where TDSCs treated group of mouse showed higher expression of Tenascin-C than the BMSCs group. In addition, 4 weeks after implantation TDSCs and BMSCs were still able to detect CM-Dil labelled cells (Figure 6(c)). In short, the results suggest that in the early postimplantation TDSCs and BMSCs can promote the expression of Tenascin-C. Additionally, the implanted cells can survive for at least 1 month in the Achilles tendon injury and are involved in the tendon reconstruction.

4. Discussion

Achilles tendon rupture accounts for about 35% of all tendon injuries due to low blood supply and low metabolic activity of tendon fibroblastic cells, in addition to these low healing potentials seen in the ruptured tendons. Previous studies reported TDSCs to be immune-privileged cells having potential for allogeneic transplantation [2, 14, 16], which led to cell banking and can be used during emergencies. Tissue regeneration depends primarily on the rate of proliferation and differentiation of endogenous stem cells to produce a high amount of ECM. It has been reported that TDSCs have higher colony-formation ability, proliferate rapidly, and

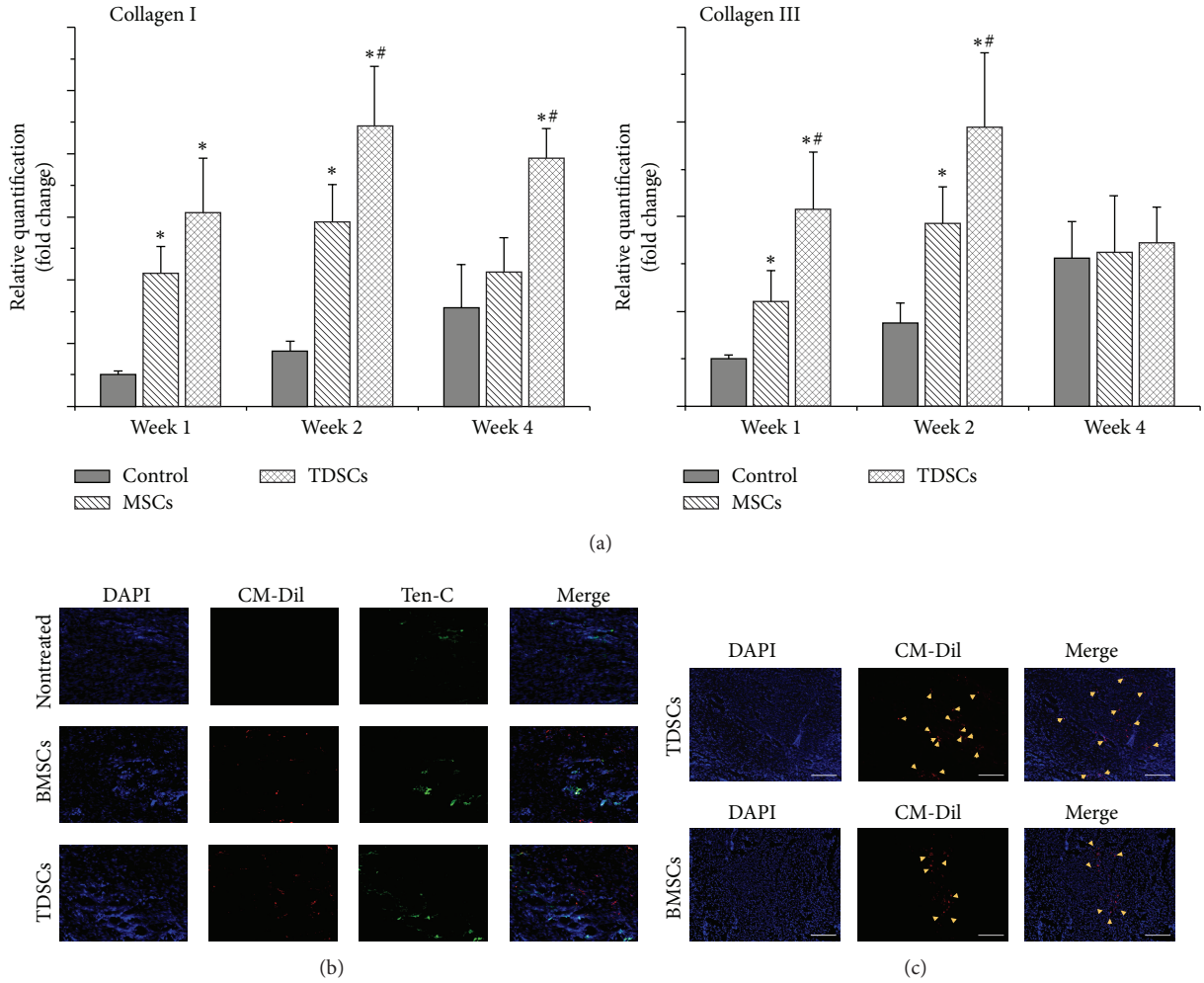

Figure 6: The analysis of tendon-related ECM expression. (a) Rat-specific gene expression analysis of tendon-related ECM genes: collagen I and collagen III. The gene transcript levels were relative to GAPDH and normalized to nontreated group. $^{*}P < 0.05$ means compared with the control group, $^{\#}P < 0.05$ means compared with BMSCs group were considered significant (data represents mean ± SD, $n = 3$). (b) Tenascin-C immunofluorescent testing in the injured Achilles tendon. (c) Cell tracking of TDSCs and BMSCs after transplant at 4 weeks: the nuclei were stained by DAPI (blue spots); the CM-Dil was red spots. Bar: 200 μm.

possess some universal stem cell characteristic compared to BMSCs depending on the age and origin of the stem cells [17, 19–21]. Studies also show that TDSCs express higher mRNA level of tenogenic markers, scleraxis, tenomodulin, and ECM components of tendon compared to BMSCs [14, 17, 22]. By using mouse model Bi et al. [14] found that, compared to BMSCs, TDSCs have more ability to express higher mRNA level for Sox9, Comp, Runx2, and Scx, while in humans TDSCs express increased level of tenomodulin (TNMD) compared to human BMSCs does.

TDSCs as compared to BMSCs are thought to be a potent therapeutic cell treasure, which take an active part in proper and enhanced musculoskeletal repair including tendon repair [21, 23]. TDSCs showed high potential for chondrogenic and osteogenic differentiation compared to BMSCs and hence at appealing candidate for tendon-bone junction regeneration [21]. Keeping in view the superiority of DMSCs over other stem cell lines, in our study, we chose two different stem cells, namely, TDSCs and BMSCs, and transplanted them into the Achilles tendon injured area of rats. After transplantation, the animals were allowed to heal for four weeks. Macroscopic appearance, histomorphology, and biomechanical strength were used to evaluate animal performance and tissue integrity throughout the healing process. At four weeks into the experiment, the treated (TDSCs and BMSCs) groups showed better results than the nontreated group. In the early stages of regeneration, TDSCs showed a prompt stimulatory effect on tissue remodeling, in both macro/micro appearance and biomechanical strength. At one week after transplantation small changes were observed; regenerated tissue was covering the treated region in the TDSCs and BMSCs groups while there was no visible connective tissue in the injured area of the nontreated group. At two weeks, the TDSCs were better in macro/micro appearance than the other two groups. Four weeks after transplantation, the histological evaluation of TDSCs detected more fibroblastic cell presence arranged in

parallel rows and positive collagen fiber in the treated area as seen in the TDSCs group (Figure 5), and the Achilles tendon displayed almost normal appearance (Figures 3(C) and 3(F)).

The higher mechanical strength can be explained by the increasing production of collagen. Higher mechanical strength suggests that the cell transplantation promotes the organization and synthesis of the various ECM components responsible for the structural and functional repair of tendon tissue during different stages of healing. The ultimate failure load in the TDSCs group was considerably higher than that of the BMSCs group and the nontreated group. At one week and two weeks, TDSCs rapidly improved biomechanical strength in the early stages of healing. However, after four weeks, the ultimate failure load of the TDSCs group still showed better results than other groups but was not significantly different. In addition, the TDSCs and BMSCs groups showed a higher value of reached almost the healthy (Figure 4).

In short, we supposed that, after cell transplantation, TDSCs are the first to adapt to the microenvironment in the ruptured Achilles tendon. Because of the fast proliferation and high affinity of tendon niches, TDSCs might differentiate rapidly into the functional tenocytes to synthesize a greater amount of ECM for remodeling.

In addition, we investigate the possible mechanism of the repair promotion by cell transplantation and test it by three experiments, RT-PCR to check the collagen expression, immunofluorescence to analyze the Tenascin-C protein expression, and CM-Dil to locate the stem cell. Initially the gene expression of both Col-I and Col-III genes were upregulated after transplantation of TDSCs and BMSCs in Achilles tendon, and TDSCs showed a higher enhancing effect than BMSCs. We considered that, in the short time since the injury, the host needs a lot of cells to concentrate into the wound. After cell transplantation, TDSCs and BMSCs quickly joined in the regenerative process and revealed a high gene expression level of collagen. However, at 4 weeks, the stimulatory effect of BMSCs for collagen type I gene expression was gone. In contrast, TDSCs still showed the enhanced effect, which means TDSCs provide continuous stimulation of collagen type I for connective tissue formation in the injured area. In addition, at week 4, all the enhancement of Col-III gene expression in the TDSCs and BMSCs was gone. Because of the complicated signal mechanism of the microenvironment after four weeks, Col-III synthesis might not play the most important role in the regenerative process. The explanation may be that Col-III is more important during the earliest stages of tendon healing, because it can rapidly form crosslinks and stabilize the precarious repair site but less so in the late stages of tissue remodeling [15, 22].

Tenascin-C is reported as an important ECM protein in providing elasticity to the musculoskeletal tissues [15, 24]. This feature is of great importance in the degenerative and regenerative processes where the normal biomechanical environment of the musculoskeletal tissues is disturbed by injury. In our study, we found that, in the early stage, both TDSCs and BMSCs implantation groups can promote Tenascin-C protein synthesis in the Achilles tendon ruptured location, and even former was observed with high protein expression than the latter group.

The present study led us to speculate that stem cells transplantation promotes regenerative processes, and TDSCs are the first to adapt within the microenvironment in ruptured Achilles tendon. In addition, we found the CM-Dil labeled transplanted cells were detectable around the tendon at week 4 after surgery. It can be interpreted that the transplanted cells were still alive and were involved in the tendon remodeling process quickly and efficiently as compared to BMSCs and nontreated group, hence proven to be the best choice.

5. Conclusion

This study provides evidence that TDSC and BMSC transplantation improves the healing potential of ruptured Achilles tendon in rats. In addition, TDSCs exhibited a better regenerative potential when compared with BMSCs in treating ruptured Achilles tendons and may be a better alternative cell source for treatment during Achilles tendon injuries.

Conflict of Interests

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

Acknowledgments

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Review Article

Mesenchymal Stem Cells for Cardiac Regenerative Therapy: Optimization of Cell Differentiation Strategy

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With the high mortality rate, coronary heart disease (CHD) has currently become a major life-threatening disease. The main pathological change of myocardial infarction (MI) is the induction of myocardial necrosis in infarction area which finally causes heart failure. Conventional treatments cannot regenerate the functional cell efficiently. Recent researches suggest that mesenchymal stem cells (MSCs) are able to differentiate into multiple lineages, including cardiomyocyte-like cells in vitro and in vivo, and they have been used for the treatment of MI to repair the injured myocardium and improve cardiac function. In this review, we will focus on the recent progress on MSCs derived cardiomyocytes for cardiac regeneration after MI.

1. Introduction

As the leading cause of mortality, cardiovascular disease is a major problem of global public health. Among cardiovascular diseases, coronary heart disease (CHD) is the main disease type causing the majority of deaths. At present, the treatment of CHD mainly includes medicine, percutaneous coronary intervention (PCI), and operation. To some extent, these treatments could improve myocardial ischemia and heart failure symptoms. Although the surgery operations make the occlusion artery unobstructed again, the damage to myocardial wall is irreversible. The current pharmacological and surgical measures are limited to palliative effects. Shortage in donor hearts and high cost are hindering the prevalence of heart transplantation. In 2001, Orlic et al. [1] transplanted autologous bone marrow mesenchymal stem cells (BMSCs) into mouse damaged heart and found these stem cells mostly differentiated into cardiomyocytes. This important discovery guided the scientists and clinicians to engage in plenty of researches on stem cells transplantation to treat myocardial infarction (MI). Significant progress has been made in the MSC research field, such as cell culture condition and technique of inducing differentiation in vitro [2, 3]. The differentiated myocardial cells from stem cells provide a promising perspective to cell treatment on cardiac diseases [4–6].

Stem cells include embryonic stem cells (ESCs) and adult stem cells (ASCs), commonly holding two major capabilities of self-renewal and differentiation. ASCs can be isolated from different adult tissues and can be differentiated into a variety of cell types [7]. As a kind of ASCs, mesenchymal stem cells (MSCs) have been described in nearly all postnatal tissues or organs, including umbilical cord blood [8, 9], placenta [10–12], and bone marrow [13], among others. MSCs represent an infrequent progenitor population with multiple differentiation potentials [14–19]. They are able to differentiate into several mesenchymal lineages, such as cartilage, muscle, vascular endothelial cells, and epidermic cells [20, 21]. With the advantage of autologous transplantation which avoids the immune rejection and ethical concerns, MSCs have great application prospect in personalized treatment of cardiovascular diseases [22–24].

2. The Induction Approaches of Cell Differentiation In Vitro and In Vivo

Currently, the major methods to induce myocardial cell from BMSCs include biochemistry induction, myocardial microenvironment induction, and genetic modification (Figure 1).

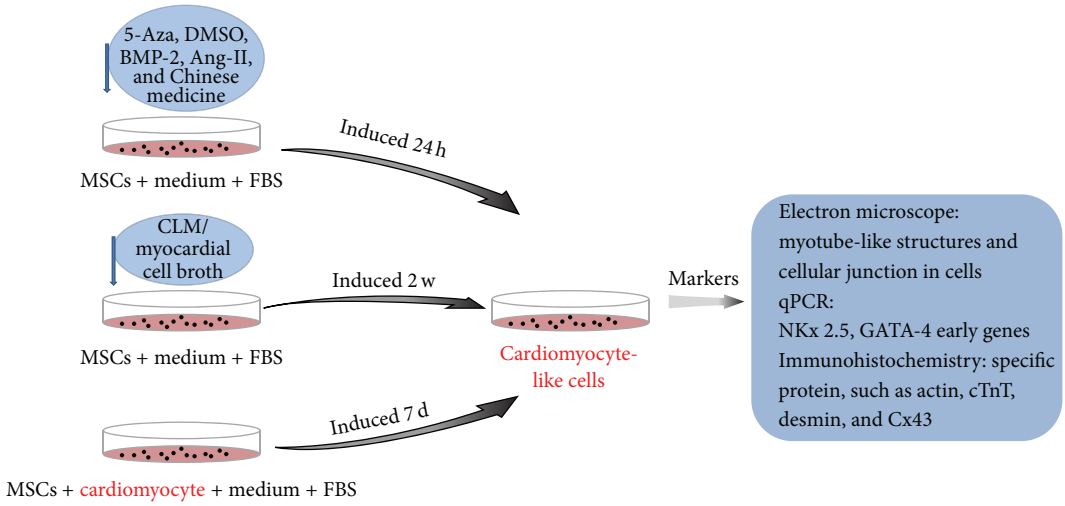

Figure 1: The diagram for the induction and identification of cardiomyocyte-like cells. MSCs cultured in medium supplemented with 5-Aza, DMSO, and BMP-2 will be induced to cardiomyocyte-like cells 24 h later. MSCs incubated in CLM/myocardial cell broth will differentiate to cardiomyocyte-like cells after 2 w. MSCs cocultured with cardiomyocyte will differentiated to cardiomyocyte-like cells 7 d later. The identification methods consist of morphology detection and molecular marker analysis.

2.1. Biochemical Substance

2.1.1. 5-Azacytidine (5-Aza). 5-Aza, a chemical analogue of cytidine, is generally known as a demethylation pharmaceutical that can induce MSCs differentiation into cardiomyocyte-like cells by activating some dormant genes through demethylation [37]. In 1995, Wakitani et al. [25] first reported the successful isolation and culture of MSCs in vitro. After a 24-hour incubation with 5-Aza, they could observe myotube-like structures and cardiac-specific proteins expression in 7–10 d. These results showed that BMSCs could differentiate into cardiomyocyte-like cells with 5-Aza supplement, laying the foundation for BMSCs differentiation into cardiomyocyte-like cells. In 1999, Makino et al. [26] and others induced the immortalized BMSCs differentiation with 5-Aza. They observed myotube-like structures after 1 week, spontaneous beating after 2 weeks, and synchronous contraction after 3 weeks. The differentiated BMSCs not only expressed cardiac-specific proteins but also exhibited biological and electrophysiological characteristics of myocardial cells. Fukuda [38] found that the myocardial cells induced by 5-Aza had two kinds of action potentials. One comes from sinus nodal cells, and the other one might come from ventricular myocytes. Jaquet et al. [39] first separated human MSCs (hMSCs) for in vitro culture and incubated these hMSCs with 10 μmol/L 5-Aza. The immunocytochemistry showed that 80% hMSCs expressed smooth muscle actin in two weeks, indicating these hMSCs might be differentiated into other muscle cells. Although 5-aza is the most commonly used chemical inducer, the differentiation efficiency is low, mainly due to the potential toxicity of 5-Aza and fat deposit in the cytoplasm which induce cell death. All the inducer applied in BMSCs differentiation are listed in Table 1.

2.1.2. Bone Morphogenetic Protein-2 (BMP-2). As a multifunctional glycoprotein, BMP-2 contributes to regulating of a wide variety of cell functions, including cell growth, differentiation, and apoptosis, among others [40]. Several studies have shown that the BMP-2 expression is initiated in early embryonic development [41]. BMP-2 plays a fundamental role in directed differentiation of cardiac stem cells and the development of embryonic heart through regulating the expressing of some cardiac transcription factors [42]. He Qizhi and Haijie [27] found that BMP-2 could also induce BMSCs differentiation into cardiomyocyte-like cells in vitro. Recent studies have shown that the roles of BMP-2 in gene expression of cardiogenic factors and cardiac differentiation from BMSCs were mediated by three molecular pathways: Smads, P38-MAPK, and PI-3K/Akt [43–45].

2.1.3. Angiotensin-II (Ang-II). Ang-II is capable of stimulating the proliferation of vascular smooth muscle cells [46] and fibroblast [47]. By regulating the signal of MAPK [48] and tumor growth factor (TGF) [49–51] and their consequent pathways, Ang-II can induce BMSCs to differentiate into cardiomyocyte-like cells. Xing et al. [28] induced BMSCs differentiation with Ang-II in vitro. After 4-week induction, the cells exhibited morphological characteristics of myocardial cells with cTnI expression and showed muscle wire-like structure under the electron microscope.

2.1.4. DMSO. DMSO was proved to induce mouse P19 cells to differentiate into beating myocardial cells [29, 52–55]. DMSO plays a critical role in increasing the expression of prodynorphin and dynorphin B at the transcriptional level. It turns on both GATA4 and Nkx2.5 expressions, and then it recruits α-MHC and ventricle-specific cardiac myosin light chain-2 (MLC-2v) to form a functional compound [56]. Another study also showed that DMSO could mediate the releasing of calcium from intracellular stores in sarcoplasmic reticulum. Elevation of calcium concentration may play an important role in the induction of cell differentiation [57].

Table 1: The inducer for BMSC differentiation.

Inducing condition	Year	Researcher	Culturing duration after induction	Detection marker
5-Aza	1995	Wakitani et al. [25]	24 h	7–10 days observing myotube-like structures and expressing cardiac-specific protein
	1999	Makino et al. [26]	24 h	1 week: myotube-like structures, 2 weeks: spontaneous beating, and 3 weeks: synchronous contraction, expressing cardiac-specific protein and exhibiting biological and electrophysiological characteristics of myocardial cells
BMP-2	2005	He Qizhi and Haijie [27]	24 h	The expression of Nkx2.5, GATA-4, cTnT, and CX43 increasing
Ang-II	2012	Xing et al. [28]	24 h	Expressing cTnI after 4 weeks, exhibiting morphological characteristics of myocardial cells, and being seen as muscle wire-like structures under the electron microscope
DMSO	1999	Skerjanc [29]	>6 d	Spontaneously beating cardiac myocytes after 6 days
Panax notoginseng saponins	2006	Yang et al. [30]	24 h	2 weeks: beginning to express MHC and more apparent after 4 weeks
Sal B	2007	Chen et al. [31]	24 h	Expression of NKX2.5 GATA-4 mRNA enhanced and peaked at 7 days; the expression of α-actin appeared at 14 days
Icariin	2008	Shao-Ying [32]	24 h	28 days, weakly expressing GATA-4, Nkx2-5, combining with 5-Aza enhance its induction
Astragaloside	2007	Xian et al. [33]	24 h, 48 h, and 72 h	4 weeks detecting cardiac-specific protein desmin, cTnI, α-MHC, and β-MHC, no significant difference in induction rate with different length
Microenvironment in vivo	2002	Toma et al. [34]		hMSCs could differentiate into myocardium and express myocardium specific protein in left ventricular microenvironment of SCID mice—cTnT and phosphoprotein regulating Ca-ATP activity at sarcoplasmic reticulum.
CLM	2005	Yuan et al. [35]	7 d	7 days, the cells growing well, expressing α-actin and cTnT
Coculturing with cardiomyocytes	2003	Rangappa et al. [36]	48 h	hMSCs coculturing with cardiomyocytes at a 1 : 1 ratio, expressing contractile proteins and cardiac specific genes, MHC, and beta-actin

2.1.5. Traditional Chinese Herb. Traditional Chinese herb can effectively induce stem cells differentiation into myocardial cells without any toxic or side effect [58]. Several studies [30, 59, 60] indicated that MSCs supplemented with by notoginsenoside in vitro could differentiate into cardiomyocyte-like cells. The morphologic features and characteristic markers of these cells were consistent with cardiomyocytes. Additional research [61] claimed that glucocorticoids released from myocardial tissue could induce BMSCs to migrate and differentiate into endothelial cells. There are several other traditional Chinese medicine inducers which also can drive MSCs to myocardial cells, such as Dan phenolic acid B [31], icariin [32], and astragaloside [33, 62].

2.2. Myocardial Microenvironment

2.2.1. Myocardial Microenvironment In Vivo. Derived from the embryonic mesoderm, MSCs exhibit multiple differentiation potentials into mesoderm groups such as bones, cartilages, and myocardium under suitable conditions. Toma and his colleagues [34] reported that the transplanted hMSCs could successfully differentiate into myocardium and express

myocardium specific proteins after cell transplantation into left ventricle of SCID mice. The myocardium specific proteins cTnT and phosphoprotein could regulate Ca-ATP activity in sarcoplasmic reticulum.

2.2.2. Myocardial Microenvironment In Vitro

(1) Cardiomyocyte Lysis Medium (CLM). Yuan et al. [35] successfully initiated MSCs differentiation into cardiomyocyte-like cells using cardiac specific cell lysate, generated from primary myocardial cells. Cao et al. [63] induced hMSCs differentiation into cardiac myocytes with the minipig's cardiomyocyte lysate. These derived cardiomyocytes expressed cTnT, Cx43, and CD31. They also induced hMSCs differentiation with 5-Aza and differentiated cardiomyocytes expressed cTnT and Cx43, but not CD31. It is indicated that some compositions of CLM could also promote the differentiation from MSCs to endothelial cells which might help create basic conditions for revascularization.

(2) The Supernatants of Cultured Cardiomyocytes. Multiple evidence showed that BMSCs cultured in the media supplemented with myocardial cell culture supernatants could differentiate into cardiomyocyte-like cells [64]. Wang et al. [65] found that 10%, 20%, 30%, 40%, and 50% supernatants of the cardiomyocytes groups were used in induction of BMSCs, without morphological change. The expressions of a-SMA, β-actin, and troponin-T were significantly higher in 10%, 20%, 30%, 40%, and 50% supernatant of cardiomyocytes groups than those in control group, and the most significant percentage was 30%. Li et al. [66] found that the concentrations of insulin-like growth factor-1 (IGF-1), platelet-derived growth factor (PDGF), and fibroblast growth factor (FGF) in the supernatant of cardiomyocytes culture were significantly higher than that in BMSCs culture. Their results indicated that IGF-1, PDGF, and FGF in the supernatant of cardiomyocytes may have capability to induce BMSCs to differentiate into cardiomyocyte-like cells, and insulin-like growth factor may serve as the main cytokine.

(3) Coculture with Myocardial Cells. After coculturing GFP labeled rat MSCs with the cardiomyocytes in different proportions for 7 days, He et al. [67] successfully detected the cardiac specific proteins expression and action potential. Rangappa [36] and others indicated that the induction efficiency of MSCs cocultured with myocardial cells is obviously higher than those cultured alone. Through investigation on the structure of the gap conjunction between cardiomyocytes, Plotnikov and his colleagues claimed that direct contraction of cells was very important during the differentiation procedure [68–70].

2.3. Genetic Modification. In recent years, genetic modification has become a novel induction strategy which can converse BMSCs into myocardial cells in the molecular level. By inducing one or several key genes to activate cardiac gene networks, BMSCs could obtain cardiac differentiation. Several key transcription factors including Nkx2.5, GATA4, and TBX5 are expressed in the early cardiac development and regulate the expression of many cardiac structural proteins which are irreplaceable to the development of heart [71–75]. Recently, Jamali et al. found that exogenous Nkx2.5 gene expression could induce P19 cells to differentiate into cardiomyocyte-like cells alone [76]. Furthermore, with exogenous expression of Nkx2.5, the P19CL6 could differentiate into myocardial cells earlier and more efficiently when supplied with DMSO [77].

3. Identification of Successful Cardiac Differentiation from MSCs

BMSCs can differentiate into cardiomyocytes through the induction of chemicals, cytokine, and simulated cardiac microenvironment. The differentiated cells were polygonal or star-shape under the microscope. The ultrastructure and filament in the cytoplasm were observed by transmission microscope and cardiac specific cellular junction existing between cells.

First, we can detect the expression of cardiac marker genes Nkx 2.5 and GATA-4 by qPCR. Tissue-specific transcription factor GATA-4 and homologous nucleoprotein Nkx2.5 are two early markers of cardiac precursor cells, which play an important role in early cardiac development [78].

Second, we can test myocardial cell specific proteins including actin, cTnT, desmin, and Cx43 by immunofluorescence technique. Actin is the cytoskeletal proteins of the muscle cells, which is expressed in skeletal and cardiac muscle, and plays an important role in maintaining myofibrillar morphology and signal transmission in the sarcomere [79–81]. Cardiac troponin-T (cTnT) is only expressed in the myocardium, thus being a specific protein in the identification of myocardial cells [82, 83]. Desmin is the intermediate filament protein in muscle with 476 amino acids. It not only connects the adjacent myofibrils, but also connects myofibrils, nucleus, cytoskeleton, and organelle. Furthermore, desmin plays important roles in signal transduction [84]. Additional research [85] showed that desmin was involved in cell signal transduction and gene expression regulation which are closely related to left ventricular remodeling. Cx43 mainly exists in the atrial and ventricular muscle and participates in the formation of gap junctions. It composes three kinds of special structure of intercalated disc with intermediate junction and desmosome. Gap junctions mediate electrical and chemical coupling between adjacent cardiomyocytes, through forming the cell-to-cell pathways for orderly spread of the wave of electrical excitation responsible for a functional syncytium [86]. The expression of Cx43 in MSCs after induction indicates that myocardial cells own the morphological basis of the intercalated disc structure formation. It provides the material basis for the rhythmic systolic and diastolic motion. Cx43 maintains electrical activity and synchronization of systolic and diastolic functions which are very important to keep on myocardial function.

Adult cardiomyocytes show complicated electrophysiological characteristics. It has been shown that ion-channel proteins are expressed differently during differentiation. Two kinds of ion-channel proteins are expressed in early phase of sustained calcium current (Ica-L) and transient outward

potassium current (Ito), but myocardial cells in later period of differentiation express all ion current: voltage dependent Na current (INa), delayed rectifier K current (Ik), inward rectifier K current (Ik1), muscarinic receptor agonist inward rectifier K current (IKAch), and the pacemaker current (If).

Additional research [2, 87–90] suggested that action potential consists of sinus node-like action potential, atrial muscle-like action potential, and ventricular-like action potential. These cells have the longer action potential duration and platform period, the smaller resting potential, and a pacemaker current slowly depolarizing in late diastole. The early cardiomyocytes express pacemaker-like cells action potential derived from two ion currents (Ica-L, Ikto), whereas the late cardiomyocytes, such as the atrial and ventricular muscle cells, express three action potentials-a 75 mV resting potential, maximum action potential, and the overshoot rate [87].

4. MSCs-Based Clinical Therapy for MI

The most important aim of the basic researches of the MSCs is to serve the clinical treatment. MSCs, which have the ability to differentiate into cardiomyocyte-like cells, endothelial cells, and smooth muscle cells, become one of the most popular cells in MI treatment area. The cardiomyocyte-like cells can be differentiated from the MSCs in vitro with inducement of several external induction factors. These cardiomyocyte-like cells can be transplanted into MI patient and direct contact myocardial cells which provide a microenvironment of the induction of the MSCs into myocardial cells. As a result, it can help to repair the infracted heart muscles better.

4.1. Safety and Efficacy Evaluation of Stem-Cell Based Therapy. In 2001, the first case of the autologous stem cell transplantation for acute MI in clinical trials was carried out by Dr. Strauer who is a medical scientist from Dusseldorf of Germany [91]. A total of 1×10^7 autologous stem cells were transplanted into infracted artery by catheter during percutaneous coronary angioplasty. After 10 weeks, it was shown that the intracoronary autologous stem cell transplantation for acute MI was safe and feasible through myocardial single photon emission computed tomography, echocardiogram, and nuclein ventriculography. At present, it is also verified that stem cell transplantation for ischemic heart disease treatment is safe and preliminary effective via clinical trials of REPAIR-AMI [92], MAGIC Cell-3-DES [93], BOOST [94], PROTECT-CAD [95], and so on.

4.2. The Suitable Transplant Time after MI. After MI, several factors are unfavorable for the survival of transplanted cells such as a large number of inflammatory cell infiltration, ischemical reperfusion injury, and microcirculatory disturbance. Meanwhile, a series of cell factors including stromal cell derived factor, vascular endothelial growth factor, and hepatocyte growth factor are upregulated, which is good for the aggregation, proliferation, and differentiation of transplanted cells toward the infarction area. Therefore, when to transplant is an important factor which affects the survival of transplanted cells and curative effect. If the transplantation is too early, a lot of transplanted cells will die due to the adverse local microenvironment. On the contrary, if it is too late, the transplanted effect is limited because of irreversible myocardial injury and formed ventricular remodeling.

There are several clinical trials carrying out the stem cell transplantation in different points of time. Comparing with the transplantation 1 hour after MI, the amount of survival cells are much less than that after 1-2 weeks, and the improvement of left ventricular function and reduction of the scar area is also lower [96]. The transplantation in 24 hours is not able to improve cardiac systolic function but can reduce the infarction area [97]. The research of REPAIR-AMI demonstrated that the BMSCs treatment in 4 days after MI is not beneficial but can improve cardiac systolic function in 4–8 days after infarction.

In 2009, MYSTAR trial first adopted the injection of autologous MNCs via both myocardial and coronary arteries to treat the MI patient. The LVEF of these patients was less than 45%. The transplant curative effect is measured by the differences between LVEF of early stage of AIM (3–6 weeks) and that of advanced stage (3-4 months).

4.3. The Dose of Transplant Cells after MI. The dosage of stem cells used to treat MI varied enormously between different investigations. In 2002, Ghostine et al. [98] injected 5×10^4 cells by intramyocardial delivery system. Fukushima et al. [99] injected 5×10^6 GFP-expressing skeletal myoblasts by either retrograde intracoronary or intramyocardial routes. As an urgent problem, researchers are pitching great effort in exploring the optimal transplanted cell dosage.

4.4. Delivery Route of Transplant Cells

4.4.1. Intracoronary (IC) Artery Injection. MSCs are infused to injured sites by percutaneous artery injection into coronary artery. This approach ensures the higher dose of transplanted MSCs to infarction and its surrounding region at the first time [100]. In post-AMI study, this "homing" phenomenon about migration of cells into cardiomyocytes is only found in intracoronary injection instead of intravenous injection. This method is a common clinically practiced approach [101], but there are security issues. In patients with coronary artery obstructions, MSCs need to be infused by retrograde coronary venous (RCV) delivery system. Vicario et al. [102] and Yokoyama et al. [103] also provide correlated data in this area.

4.4.2. Surgical Intramyocardial (IM) Injection. At present, most studies recommend transplanting stem cells by epicardial puncture under open-heart surgeries like CABG [104–106] or thoracoscopic. Intramyocardial injection has been the most accurate and direct approach for injecting stem cells to MI region of the heart. For its advantage of targeting localized myocardium, this method avoids many complex issues such as homing of the transplanted cells. The biggest drawback of IM injection is the invasive procedures, and the injection site is likely to cause cardiac arrhythmia and systemic embolization [107].

4.4.3. Intravenous (IV) Infusion. Without heart surgery and catheterization, intravenous injection of stem cells is a simple and least invasive delivery route. In an experimental model of acute MI, heart function was improved significantly by peripheral intravenous injection of EPCs or BMSCs [108], but a lot of transplanted cells remained outside of the myocardium [109]. This limited the clinical application.

4.4.4. Tissue Engineering Technology. Tissue engineering technology is a novel strategy to improve the efficacy of cell engraftment. MSCs are cultured on biological materials such as a hydrogel, 3D scaffold to form monolayer cells with better cell-to-cell adhesion. This enables direct tissue transplants and minimizes loss of cells [110]. The engrafted sheet survived on ischemic myocardium and grew to a thick stratum including some newly formed vessels and cardiomyocytes [111]. The technology creates an excellent environment which is suitable for MSCs survival, proliferation, and differentiation.

4.5. Assessment of Various Cell Delivery Methods. In order to detect the cell viability and repair effect of BMSCs delivered via different route, tracing technology and cardiac ultrasound are applied. Hou et al. [112] traced cells via radioactively labelling to evaluate the efficacy of cell engraftment. They reported 11 (surgical injection), 2.6 (coronary artery), and 3.2% (coronary venous) of them being retained, respectively. Lee et al. [113] dual labeled the stem cells with HSVttk reporter gene and iron oxide particles for PET imaging of cell viability and MR imaging of cell location. They applied cardiac ultrasound and electrocardiogram to validate its therapeutic potential for MI. The improved ventricular function was measured via ejection fraction and stroke volume. With increases in advanced technology on stem-cells based therapy, the evaluation of efficacy of MSCs engraftment will be more perfect and powerful.

5. Conclusion

In summary, the methods to induce BMSCs differentiation into cardiomyocyte-like cells include biochemical drug induction in vitro, such as 5-aza, BMP-2, AngII, DMSO, and various herbs. Chemical inducers are known to have the possible toxicity. Even under the best concentrations and optimal inducing time, chemical inducers may lead to cell death. Due to their toxicity and undesirable effect, chemical inducers are not able to be used in clinical translation. Furthermore, myocardial microenvironment could affect BMSCs differentiation. Thus, creating culture conditions that more closely mimic cardiac environment is a good idea, such as cardiomyocyte lysate, culture medium of myocardial cells. Therefore, differentiation methods with the myocardial microenvironment will be more prospective. A study [114] has shown that the inducing rate of culture medium of myocardial cells is not as good as CLM. The evidence indicated that some soluble substances contributing to inducing BMSCs differentiation could be released by cardiomyocytes. However, these substances could not be released until myocardial cells are broken [114, 115]. Because MSCs interact with cardiomyocytes by paracrine and autocrine after directly coculturing with cardiomyocytes, there are physical stimulations such as electrical activity and mechanical traction between MSCs and cardiomyocytes [68–70]. Thus, among various induction methods, CLM and direct coculturing with myocardial cells may be more feasible. It will be expected that application of both CLM and coculturing will further boost the MSCs differentiation into cardiomyocytes.

Human bone marrow mesenchymal stem cells have wide application prospect because it can be obtained autologously and have no immune rejection. Furthermore, it is easy to culture in vitro and can be induced to differentiate into myocardial cells through many ways. However, there are still several problems needed to be addressed. The specific pathways and the regulation mechanism of hMSCs differentiation into cardiomyocyte-like cells are still not clear. More studies are needed to determine optimal infusion dosage, timing with different induction method. To satisfy the clinical usage, it needs to ameliorate the conditions of induction and to further improve the differentiation efficiency. However, the clinical translation of stem-cell based therapy is a more complex process, and its efficacy needs to be fully investigated in a larger sample size and evaluated in a great quantity of preclinical experiments.

Therefore, what is needed in the stem cell research is the investigation of the best/safest cell type and improvement after clinical treatment for MI. The convincing researches need more considerations, well-conceived plan, and rigorous experiments. It is convinced that with the deepen of the research and the improvement of technology, the application perspective of BMSCs transplantation to treat MI will be extremely bright.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Han Shen and Ying Wang contributed equally to this work.

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Research Article

Modifications of Human Subcutaneous ADMSC after PPARγ Activation and Cold Exposition

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Mesenchymal stem cells are a diverse population of cells with a wide range of potential therapeutic applications. In particular, cells from adipose tissue have the distinction of being easily accessible and contain a lot of stem cells. ADMSCs can be induced to mature adipocyte and activate the energy expenditure upon treatment with total PPARγ agonists. Additionally these cells may respond to cold by activating the thermogenic program. In the present study, we determined the effect of partial agonism of PPARγ and temperature reduction on phenotype and metabolic activity of ADMSCs from human adipose subcutaneous tissue. We found that adipocytes differentiated with total and partial agonists of PPARγ and exposed to 31°C are able to respond to cold significantly increasing the expression of thermogenic proteins such as UCP1, PGC1α, and CITED1, a marker of beige phenotype. Additionally, we found that adipocyte cells subjected to cold had a reduction in triglycerides and increased adiponectin levels. These data confirm the promising role of ADMSCs as a treatment for metabolic disorders since it is possible to induce them to mature adipocytes and modulate their phenotype toward a cell with high-energy expenditure and metabolic beneficial effect.

1. Introduction

Stem cells derived from adult tissue have acquired an important role in regenerative medicine as well as a model to determine the origin of chronic nontransmissible diseases [1]. Mesenchymal cells derived from adipose tissue have a special connotation because they are easily obtained and have a great capability to transform [2, 3]. Due to this competence of autorenewal and differentiation in cellular types such as osteoblasts, myocytes, chondrocytes, and adipocytes, ADMSCs (adipose-derived mesenchymal stem cells) are being introduced in a number of therapies of tissue regeneration [4, 5]. A possible therapeutic application of ADMSCs is a phenotypic modulation to treat obesity, a disease considered pandemic with multiple metabolic and cardiovascular repercussions [6]. Since therapeutical resources to treat obesity are scarce, the use of ADMSCs as a model of obesity treatment has become a plausible alternative [7–9]. The capacity of ADMSCs to acquire a determined phenotype depends on specific transcription factors. Activation of PPARγ, a nuclear receptor, which may be activated by specific ligands, can differentiate ADMSCs towards adipocytes. We have previously observed that both total PPARγ agonism with Rosiglitazone and partial agonism with Telmisartan can differentiate unipotent mice cells 3T3-L1 towards adipocytes [10]. However, little is known about partial activation of PPARγ in adipocytes differentiation of ADMSCs [11]. It has recently been observed that it is possible to obtain more active calorigenic adipose cells from human adults, a fact only observed in newborns and inferior mammals that have brown adipocytes [12]. In this contest the adipose cells in adults may be white, brown, and beige. The origin of brown adipose cells is closer with muscle cells, whereas beige adipocytes maybe in part arise from white adipose cells or have their own mesenchymal origin [12–14]. In fact, continuous stimulation of PPARγ receptors is capable of activating the beige phenotype from ADMSCs obtained from human tissue. Even in mice, total agonist of PPARγ is necessary to induce beige phenotype [15, 16]. Additionally, adipose cells can be more active in the production of heat after temperature reduction. Exposure to cold activates the sympathetic nervous system for the liberation of norepinephrine, which acts on β-adrenergic receptors, and triggers a signaling pathway that activates the transcription of genes involved in thermogenesis and energy expenditure

[17, 18]. Nevertheless, chronic cold exposition may induce thermogenesis and browning of WAT fat, through alternate pathway that may include activation of macrophages and activation of adenosine A2a receptors [19, 20]. From the metabolic point of view, cold generates weight reduction and improves the metabolism of triglycerides by increasing lipids uptake in the brown adipocyte tissue and modulates the expression of some adipocytokines as adiponectin [21].

We use ADMSCs from subcutaneous human tissue to investigate whether pharmacological stimulation with total and partial agonist of PPARγ can activate traits of beige adipocytes as well as to respond to cold activating the thermogenic program. Additionally, we studied the metabolic effect on the levels of triglycerides and the activation of adiponectin under the circumstances described above.

2. Material and Methods

Samples of subcutaneous fat were obtained from 5 female patients, with average age of 31 ± 4 (20 to 40) years, who underwent abdominoplasty. Patients presented a BMI (body mass index) between 23 and 25 kg/m^2. Additionally, donors were not under any treatment with drugs 3 months prior to taking the sample, hence not presenting with any kind of disease.

Lipid profile, carbohydrates metabolism, and thyroid function were within normal levels. Patients received detailed information regarding the objective of the study and signed the informed consent. The project was approved by the ethics committee from Universidad de La Sabana.

2.1. Cell Cultures. ADMSCs were isolated from 30 g of subcutaneous abdominal adipose tissue collected during surgery. Fat samples were washed using PBS and all fibrous material and visible blood vessels were removed. After the samples were digested with 250 U/mL collagenase type I, 20 mg/mL BSA, and 60 μg/mL gentamicin in PBS for 90 minutes at 37°C in agitation. After the digestion the samples were centrifuged at 200 g for 10 minutes and pellet was suspended in a solution of erythrocytes lysis composed of 154 mm ammonium chloride (NH_4Cl), 5.7 mm monobasic potassium phosphate (K_2HPO_4), and 0.1 mM EDTA pH 7.3 for 10 min. This mixture was filtered on a nylon mesh of 150 μm pore, followed by centrifugation at 200 g for 10 min. Then the cell pellet was suspended in growth medium consisting of DMEM/F12 plus 10% fetal bovine serum and gentamicin 50 μg/mL at a density of 10,000 cells/cm^2. After 24 hours the cells were washed and induced to proliferation in medium PM4 (DMEM/F12, 2.5% fetal bovine serum, 1 ng/mL of basic fibroblast growth factor, 10 ng/mL epidermal growth factor, and insulin 8.7 μM) up to 100% confluence and subjected to differentiation into mature adipocytes. Cells were identified with the marker CD34 by flow cytometry.

2.2. Differentiation and Quantification of Triglyceride. Human mesenchymal stem cells were differentiated into adipocytes using a mixture of 66 nM insulin, 1 nm triiodo-L-thyronine, 10 μg/mL transferrin, 0.5 mm isobutylmethylxanthine, dexamethasone 100 nM, 1 μM of Rosiglitazone, or 50 μM of Telmisartan as PPARγ agonists in DMEM/F12 for 72 hours. Subsequently, the medium was changed to basal preadipocytes medium containing equal concentrations of insulin, triiodo-L-thyronine, and transferrin alone for 10 days, changing the medium every 3 days. Adipocyte differentiation was observed by staining with Oil Red O, previously fixing mature cells with 10% formaldehyde in PBS for 15 m at 37°C; the solution of ORO in isopropanol was then added for 2 hours at room temperature. It was subsequently removed and washed with water to remove residual dye. To quantify triglyceride, 1 mL of isopropanol was added for 5 min, to distain the fat deposits. Absorbance was measured at 510 nm wavelength and the relative value of triglycerides was determined.

2.3. Cold Induction. ADMSCs were cultured in 6-well boxes, induced to adipogenic differentiation, and maintained for 10 days at 37°C in basal differentiation medium. 18 hours before cold induction, mature adipocytes were washed with PBS and the basal differentiation medium was changed. Subsequently, cells were subjected to 31°C during 4 hours in an incubator with 5% CO_2. After this time, the adipocytes were stained with ORO for quantification of triglyceride and other lysates for total protein extraction. Respective controls were cells under similar conditions of differentiation maintained at 37°C.

2.4. Western Blot Analysis. Total protein differentiated adipocytes from subcutaneous fat were obtained by using RIPA buffer (Abcam, Cambridge, MA, ab156034) and 1 μg Protease Inhibitor (Roche Diagnostic, Mannheim, Germany). They were then quantified using the Bradford method to work with a concentration of 50 μg of protein. It was followed by denaturation at 95°C that was conducted to subject the extracts to electrophoresis in polyacrylamide gel. Subsequently the product of electrophoresis was transferred to PVDF membrane pretreated with 100% methanol for 2 min.

Blocking of the membrane was performed with PBS-T (1X PBS and Tween 20 0.1%) and nonfat milk at 5%. Then it was incubated with the respective antibodies to detect thermogenic proteins: rabbit anti-PGC-1α (peroxisome proliferator-activated receptor gamma coactivator 1-alpha), 1 : 1000 dilution (Abcam, Cambridge, MA, ab54481), rabbit anti-UCP-1 (uncoupling protein 1), dilution 1 : 1000 (Abcam, Cambridge, MA, ab155117), and mouse anti-CITED1 (Cbp/p300-interacting transactivator with Glu/Asp-rich carboxy-terminal domain) (Abcam, Cambridge, MA, ab87978). Secondary antibodies against rabbit IgG-HRP for UCP1, PGC-1α and a dilution of 1 : 2000 and 1 : 3000, respectively (Abcam, Cambridge, MA, ab6721). Furthermore, mouse IgG-HRP (Abcam, Cambridge, MA, ab6728) was used for CITED1 at a dilution of 1 : 2000. In addition, the expression of adipocytokines as FABP4 (fatty acid binding protein 4) was detected using a rabbit antibody anti-FABP4 (Abcam, Cambridge, MA, ab92501) at a dilution of 1 : 2000 and adiponectin (Abcam, Cambridge,

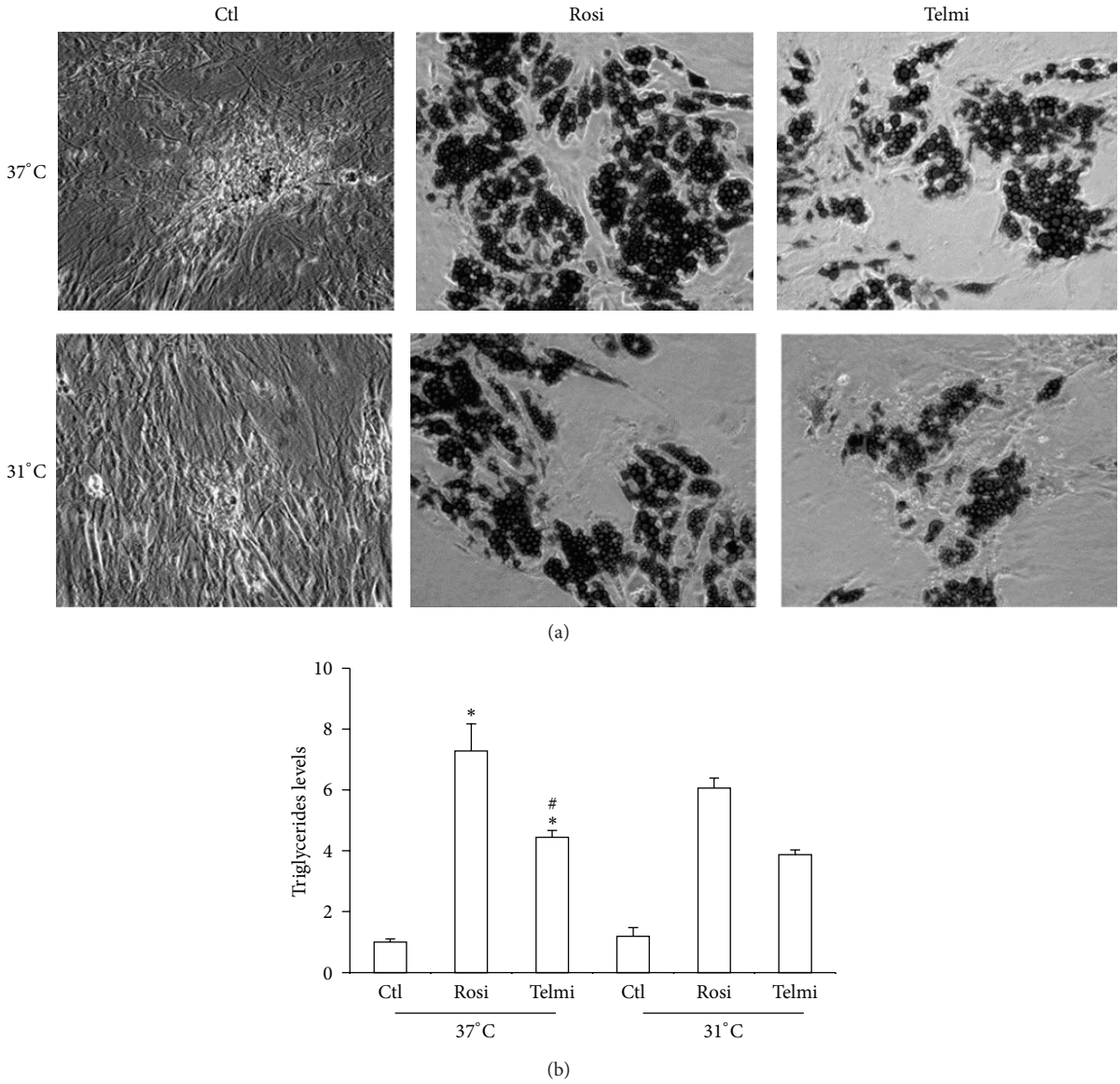

Figure 1: Effect of cold on triglyceride levels in differentiated human adipocytes. (a) ADMSCs were obtained from human subcutaneous adipocytes differentiated and induced for 10 days with 1 μM of full agonist (Rosiglitazone) and 50 μM partial agonist (Telmisartan). Cells were maintained at 37°C or exposed to 31°C for 4 hours and after, staining was performed with Oil Red O. (b) Quantification of triglyceride levels is expressed as relative values. Statistical analysis was performed using ANOVA test. Data are expressed as mean ± SD of 3 independent experiments performed in triplicate. Differences were considered statistically significant at $P < 0.05$. Rosi (Rosiglitazone), Telmi (Telmisartan). ∗Differences between triglyceride levels of treatments with PPAR agonist in relation to control (induction of differentiation with basal medium). # indicates differences found between treatments.

MA, ab75989) at a dilution of 1 : 3000, and as secondary antibody rabbit IgG-HRP was used at 1 : 5000. Detection was performed by chemiluminescence according to the instructions of Luminata Crescendo Kit (EMD Millipore, Darmstadt, Germany). Images were captured and analyzed using myECL Imager (Thermo-Scientific, Waltham, MA). Quantitative analysis was performed by densitometry using my Image Analysis program in 3 independent experiments. Results were subjected to the test of variance analysis (ANOVA) and differences were considered statistically significant when the value of the mean with standard error was $P < 0.05$.

2.5. Treatment of Adipose Cell with IL4. In order to know the effect of IL4 on thermogenesis, ADMSCs obtained from 3 female patients were grown in 6-well boxes and differentiated as described above. Mature adipocytes were washed with PBS and treated with IL4 10 ng/mL (PeproTech, Rocky Hill, NJ) in 0.1% BSA. Cells were immediately subjected to 31°C or 37°C for 4 hours. The respective controls were cells treated only with BSA. Total proteins were obtained to perform western blot analysis and were incubated with the respective antibodies for adiponectin and PGC-1α. The experiments were performed in triplicate. Quantitative analysis was performed as described above.

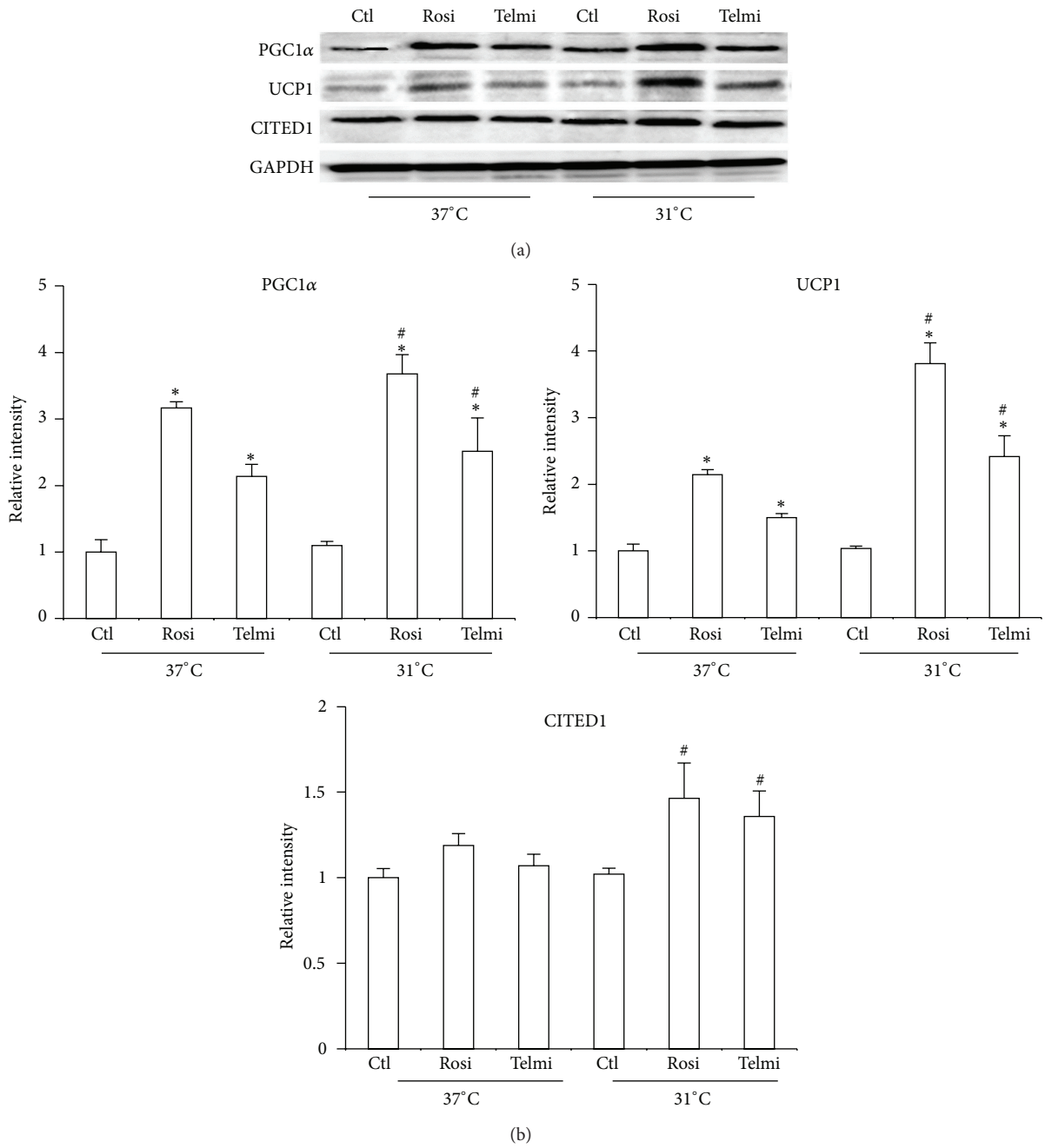

Figure 2: Effect of cold and full and partial agonists in the adipocyte phenotype. (a) Differentiated adipocytes were obtained and treated as described in Figure 1; total protein was isolated for detecting expression of PGC1α, UCP1, and CITED1 using western blot. (b) Relative intensity levels were determined by densitometry of bands. Analyses were performed using ANOVA test. Data are expressed as mean ± SD and differences were considered $P < 0.05$. ∗Differences between total and partial agonist in relation to control. # indicates meaningful differences observed after 4 hours of treatment with agonists after exposure to cold in comparison with adipocytes treated in equal conditions at 37°C. Data were normalized with GAPDH.

3. Results and Discussion

Stem cells derived from adipose tissue (ADMSCs) are a useful tool for the study of different types of diseases. We obtained ADMSCs from subcutaneous adipose tissue of healthy women as a model to assess the effect of adipocyte differentiation with different PPARγ agonists and also to observe the influence of temperature on the development of thermogenic and metabolic markers. After establishing the conditions for differentiation and culture we obtained ADMSCs that were identified by flow cytometry using the CD34 marker [22, 23].

The effect of PPARγ agonist was initially studied, whether partial or total, to induce differentiation of ADMSCs into

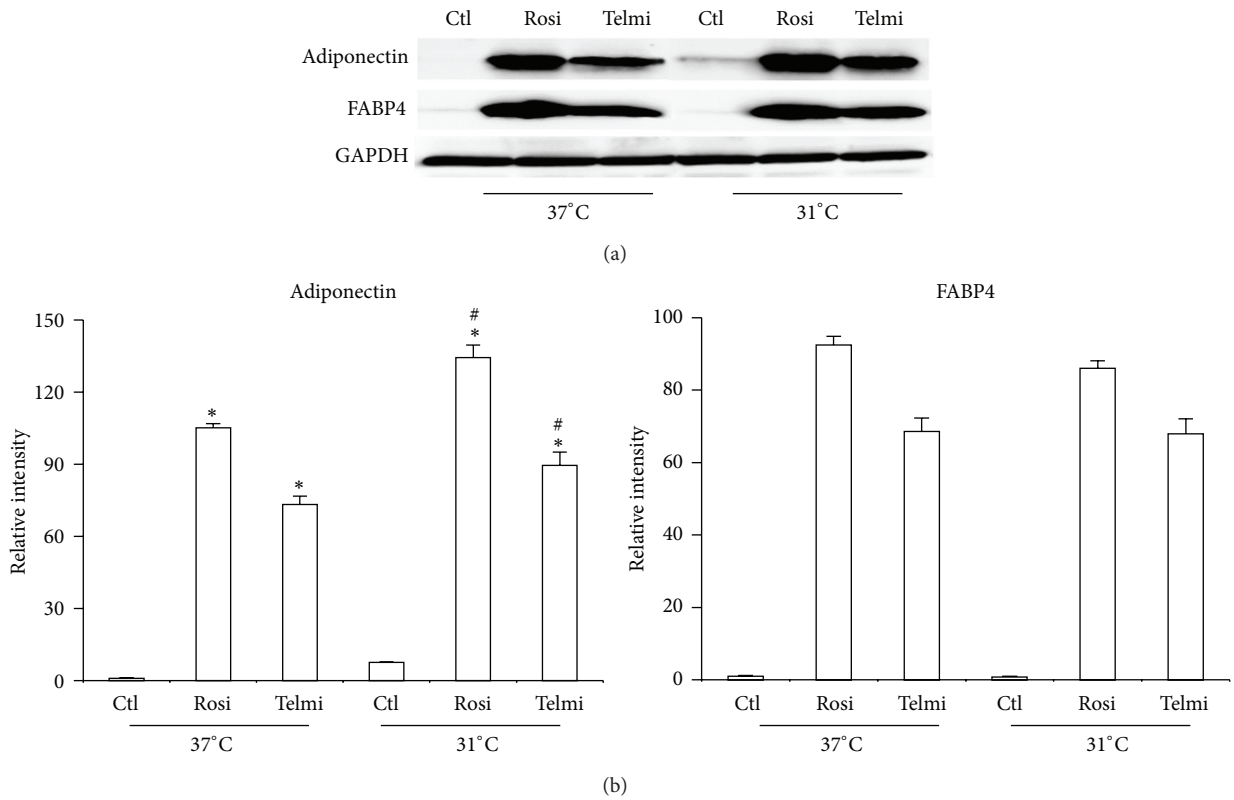

Figure 3: Effect of cold in the levels of adipocytokines. (a) The differentiated adipocytes were obtained and treated as described in Figure 1; total protein was isolated for detecting expression of adiponectin and FABP4 by western blot. (b) Relative intensity levels were determined by densitometry of bands. Analyses were performed using ANOVA test. Data are expressed as mean ± SD and differences were considered significant with $P < 0.05$. ∗Differences between total and partial agonist in relation to control. # indicates meaningful differences observed after 4 hours of treatment with agonists after exposure to cold in comparison with adipocytes treated in equal conditions at 37°C. Data were normalized with GAPDH.

adipocytes. After 10 days of differentiation it was observed that cells were capable of storing triglycerides using both agonists (Figure 1). This observation makes the important role of this nuclear receptor in the process of differentiation of the adipose cells evident. In the present study both total and partial PPARγ agonists induced triglyceride accumulation and differentiation of ADMSCs to mature adipose cells. We have considered the evaluation of a partial agonist of PPARγ because it has been shown that excessive fat accumulation and retention of liquids are some of the undesirable effects of total PPARγ agonists. It is for this reason that selective agonists may have a better therapeutic efficacy [24, 25]. When cells were subjected to a temperature drop, in both cases a reduction in the accumulation of triglycerides was observed (Figures 1(a) and 1(b)). Previous studies in animal models have shown that cold can possibly have a lipolytic effect on fat cells, increasing transporters of fatty acid and specific enzymes such as lipase lipoprotein [26]. It was recently observed that the reduction in temperature can steadily increase expression of higher heat production genes [17]. An important event observed in this study is the increase in markers of energy expenditure triggered by the two types of agonists. Even though it was previously observed that Rosiglitazone may increase PGC-1α and UCP1 levels [27], this is the first evidence of the effect of a PPARγ partial modulator in the activation of these markers (Figure 2). It was additionally observed that temperature reduction may increase the production of markers of energy expenditure, more evident in the case of Rosiglitazone as compared to Telmisartan.

The reduction in temperature has been shown to have a physiological impact on the activation of thermogenic markers of adipose cells. In some animal studies it was found that cold can increase thermogenesis markers and possibly adipose cells have a functional change [28]. In this work, human adipocyte cells underwent a temperature reduction from 37°C to 31°C, keeping it during 4 hours. After being at 31°C during this period, the cells increased the expression of thermogenesis markers as UCP1 and PGC-1α (Figure 2). The activity of the sympathetic nervous system that stimulates β-adrenergic receptors may be important for thermogenesis activation in response to cold. Our data show that both partial and total PPARγ agonist may modify the expression of protein involved in energy expenditure and strikingly the cells can respond to cold increasing the thermogenesis [17]. Hence it is possible that, besides sympathetic nervous system mediation, some additional factors may be important for the phenotypic induced by cold. It has been recently

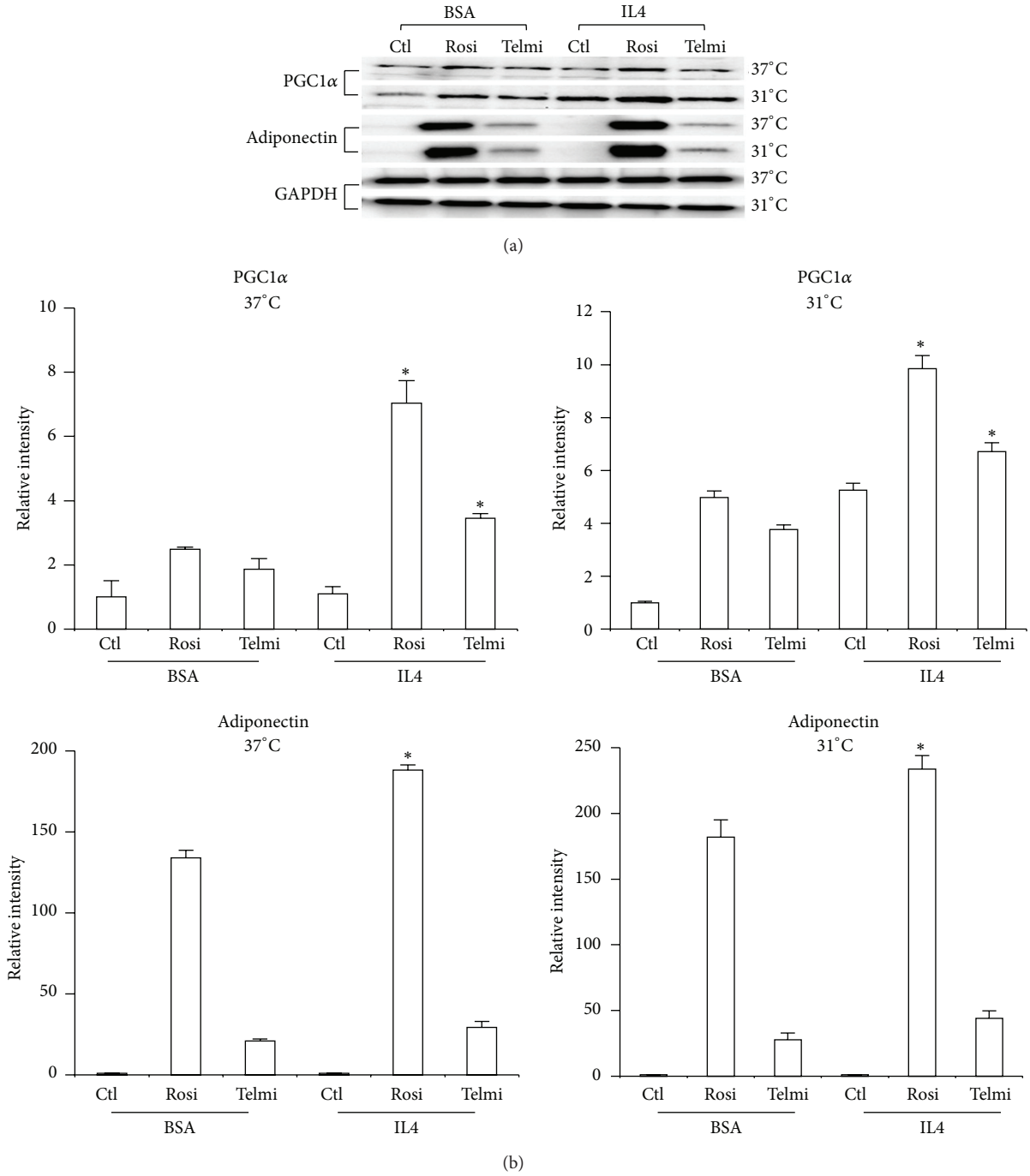

Figure 4: Effect of IL4 in the levels of adipocytokines. (a) The differentiated adipocytes were obtained and treated as describe before; total protein was isolated for detecting expression of adiponectin and PGC-1α by western blot. IL4 was added to cells during a period of 4 hours and at the same time cells were exposed to 37°C or 31°C. (b) Relative intensity levels were determined by densitometry of bands. Analyses were performed using ANOVA test. Data are expressed as mean ± SD and differences were considered significant with $P < 0.05$. ∗The differences between the cells treated with IL4.

demonstrated that activation of adenosine receptor or IL4 production is probable factors that mediate this functional change at low temperatures. However, the mechanism by which cold can modify the activity of adipocytes is still to be elucidated [20].

In comparison to effect of temperature over energy expenditure, the cold consequence on adipocytokines expression has been poorly studied. In the present study, we observed an increase in adiponectin expression in cells that were differentiated by both PPARγ agonists (Figure 3(a)).

This observation together with the decrease of the amount of triglycerides as well as an increase in the thermogenic markers of adipose cells directs us to believe that WAT cells from ADMSCs have browning process towards beige adipocytes. It is important to highlight the first occasion in which a reduction in temperature has a beneficial change in adiponectin production by adipose cells derived from ADMSCs, both at baseline and after therapy PPARγ agonists (Figures 3(a) and 3(b)).

Recently a new population of regulatory cells that mediate cold response was observed in mice alternatively activated (type 2/M2) macrophages. When activated by eosinophils via IL4 and IL13 signaling, M2 macrophages are recruited to subcutaneous WAT and secrete catecholamines to activate WAT browning [20, 29, 30]. We treated the differentiated adipose cells from ADMSCs with IL4 during a period of 4 hrs at 37°C and 31°C. IL4 increased the expression of adiponectin and PGC-1α and temperature reduction had synergistic effect with IL4 (Figures 4(a) and 4(b)). This leads us to make the hypothesis that IL4 is an important factor in the process of browning of scWAT from ADMSCs.

4. Conclusion

In the present study we demonstrate that partial activation of nuclear receptors PPARγ may determine a change of ADMSCs lineage to adipocyte. Additionally, it was observed that both the reduction of temperature and the IL4 might have a protective metabolic effect from obesity.

Conflict of Interests

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

Acknowledgments

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Research Article

Rat Nasal Respiratory Mucosa-Derived Ectomesenchymal Stem Cells Differentiate into Schwann-Like Cells Promoting the Differentiation of PC12 Cells and Forming Myelin *In Vitro*

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Schwann cell (SC) transplantation as a cell-based therapy can enhance peripheral and central nerve repair experimentally, but it is limited by the donor site morbidity for clinical application. We investigated weather respiratory mucosa stem cells (REMSCs), a kind of ectomesenchymal stem cells (EMSCs), isolated from rat nasal septum can differentiate into functional Schwann-like cells (SC-like cells). REMSCs proliferated quickly *in vitro* and expressed the neural crest markers (nestin, vimentin, SOX10, and CD44). Treated with a mixture of glial growth factors for 7 days, REMSCs differentiated into SC-like cells. The differentiated REMSCs (dREMSCs) exhibited a spindle-like morphology similar to SC cells. Immunocytochemical staining and Western blotting indicated that SC-like cells expressed the glial markers (GFAP, S100β, Galc, and P75) and CNPase. When cocultured with dREMSCs for 5 days, PC12 cells differentiated into mature neuron-like cells with long neurites. More importantly, dREMSCs could form myelin structures with the neurites of PC12 cells at 21 days *in vitro*. Our data indicated that REMSCs, a kind of EMSCs, could differentiate into SC-like cells and have the ability to promote the differentiation of PC12 cells and form myelin *in vitro*.

1. Introduction

Schwann cells (SCs) are myelin-forming glial cells in peripheral nervous system (PNS), and they have been reported to support nerve regeneration in both PNS and central nervous system (CNS) by forming myelin and providing various neurotrophic factors and molecular anchors [1–4]. In PNS, end-to-end anastomosis has been recommended as the primary choice for the treatment to acute transection injury of nerve tissue. When the gap between two damaged nerve ends is too large to be sutured, autologous nerve grafts are usually required [5]. Limited supply of autologous nerve tissue and the morbidity of the donor site are challenges to the neurologists. In CNS, spinal cord injury, representing common CNS damage and notorious for its life-long disability complication to the patients, is still lacking effective cure method both scientifically and therapeutically [6]. Tissue engineering techniques which provide seed cells for nerve injury could be an alternative repair strategy. Successful tissue engineered regeneration of nerve tissue have been reported using Schwan cells, a variety of cell carriers and cytokines and growth factors, which may open a way for therapeutic cure of damaged nerve tissue [7–16].

However, serious concerns for using SCs to tissue-engineering nerve tissue are the invasive approaches to collect SC-donor tissue and the difficulties to culture expand them [17]. Therefore, expansion and induction of stem cells *in vitro* are considered a promising tool to overcome the practical and ethical concerns of tissue transplantation, and actively searching for an appropriate source of cells has been the primary focus of nerve tissue engineering field.

Mesenchymal stem cells (MSCs) originating from mesoderm may be an alternative cell source for the generation of nerve tissue due to their multipotent differentiation properties. Some reports show that bone marrow-derived mesenchymal stem cells (BMSCs) and adipose-derived

mesenchymal stem cells (ADSCs) can transdifferentiate into SCs [12, 18]. However, the neurogenic potential of MSCs is weaker when compared with those of stem cells derived from neural tissue, as they originate from the mesodermal layer [19, 20]. Although it has been shown that neural stem cells (NSCs) isolated from the brain of new born Sprague Dawley (SD) rats could differentiate into SC-like cells, human NSCs are difficult to be widely used in clinical practice because of the difficulties to obtain from allogeneic tissue sources and unavailability of autologous sources [21, 22].

Ectomesenchymal stem cells (EMSCs), which are pluripotent cells capable of self-renewal and differentiation into multiple cell types, are derived from the neural crest during embryonic development [23, 24]. During the embryonic development ectomesenchyme contributes to the formation of craniofacial structures. Nasal septum mucosa is composed of olfactory mucosa in the upper portion and respiratory mucosa in its lower part although they both arise from the embryonic ectoderm layer. In adult mammals, stem cells present in human nasal mucosa and can be induced to differentiate into neuron like cells [25]. Our previous work demonstrated that respiratory mucosa adjacent to the olfactory mucosa contains a population of EMSCs [26]. We also found that respiratory mucosa stem cells (REMSCs) were more amenable to differentiate into neural or glial cell compared to bone marrow-derived MSCs after a short period of neural induction culture [27]. Therefore, it is postulated that the REMSCs may have the potential to differentiate into SC-like cells.

In the present study, REMSCs were isolated, expanded, and identified as EMSCs and induced to differentiate into SC-like cells. The ability of promoting the differentiation of PC12 cells and forming myelin was also assessed *in vitro*.

2. Material and Methods

2.1. Cultivation of REMSCs and PC12 Cells. This study has been approved by the IACUC of Jiangsu University. The cultivation and isolation of REMSCs were performed as previously reported by Liu et al. [26] with minor modification. In brief, adult SD rats were anaesthetized with intraperitoneal injection of pentobarbital sodium (0.05 g/kg). Middle third of nasal septum was dissected, washed in DMEM/F-12 (DF12) (Gibco, USA) three times, and then cut into small pieces and digested with 0.25% trypsin (Gibco, USA) in phosphate buffered solution (PBS, Gibco, USA) for 25 min at 37°C. The tissue/cells suspension was placed into a 25 cm^2 flask in growth medium that is DF12 supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA) and cultured at 37°C in 5% CO_2 and 95% air with saturated humidity. The growth of the cells was recorded daily by digital camera connected to a phase-contrast microscope (Zeiss, Observer, A1). The medium was changed every 72 hours. When adherent cells had migrated from the explants and reached 80% confluent, generally one week in culture, cells were suspended with 0.05% trypsin-EDTA and reseeded in new culture flasks at 5×10^3 cells/cm^2 in the same growth medium for observation. Cells at their fourth passage were used for all of the characterization studies.

PC12 is a cell line derived from a pheochromocytoma of the rat adrenal medulla. This cell line is commonly used as a model system for neuronal differentiation in a culture set-up [28]. PC12 cells were cultured at 37∘C and 5% CO2 in DF12 medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, USA).

2.2. Induction of Rat REMSCs into SC-Like Cells. REMSCs at their 4th passage were used to differentiate into SC-like cells. SC-like cells differentiation medium (SCDM) was DF12 medium supplemented with 10% FBS, 5 ng/mL platelet-derived growth factor-AA (PDGF-AA; PeproTech, USA), 10 ng/mL bFGF (PeproTech, USA), 5 μM forskolin (Sigma, USA), and 200 ng/mL heregulin (HRG, PeproTech, USA). Cells were incubated for 10 days in SCDM with fresh medium added every 72 hours.

2.3. Coculture SC-Like Cells with PC12 Cells In Vitro. The ability of induced SC-like cells to promote the differentiation of PC12 cells was determined by examining their interaction with PC12 cells and the myelin-forming ability of SC-like cells. The PC12 cells were dissociated and replated at a density of 500 cells/cm^2 in dishes in DF12 plus 10% FBS. After 24 hours, 4 groups of cocultures were established: group A: PC12 cells were cultured alone in DF12 plus 10% FBS; group B: PC12 cells were cultured in SCDM; group C: PC12 cells and 5000 cells/cm^2 REMSCs were cocultured in DF12 plus 10% FBS; group D: PC12 cells and 5000 cells/cm^2 SC-like cells were cocultured in SCDM. These 4 groups were cultured for 5 days and the mediums were replaced every 48 hours.

To observe the interaction of the two types of cell, SC-like cells were infected with green fluorescent protein (GFP) recombinant adenovirus, and PC12 cells were labeled by CM-Dil (Invitrogen, USA). GFP recombinant adenovirus was amplified in HEK 293 cell line. After REMSCs were cultured in differentiation medium for 1 week, GFP virus was added to infect the REMSCS at 100MOI for 24 hours. CM-Dil was added to PC12 cells culture medium at working concentration (1 μM); PC12 cells were incubated in the culture medium with CM-Dil for 5 minutes at 37°C and then for an additional 15 minutes at 4°C. After the PC12 cells had been labelled, they were washed with PBS and resuspend in fresh medium for coculture with SC-like cells.

2.4. Western Blotting. Western blot analysis was used to detect the expression of P75, GFAP, CNPase, S100β, SOX10, nestin, vimentin, and CD44 by SC-like cells and REMSCs and the expression of NF-H, Synapsin II, GAP-43, and PSD-95 by PC12 cells. Total protein was extracted with RIPA buffer (10 mM Tris–HCl pH 7.8, 150 mM NaCl, 1% Nonidet P40, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 8 M urea, 10 lg/mL aprotinin, and 1 mM PMSF). Protein concentration was determined by a bicinchoninic acid kit. Equal amounts of protein (50 μg) from each sample were loaded onto 8% polyacrylamide gels, separated by 10% polyacrylamide gel electrophoresis, and electrophoretically transferred to PVDF membrane (Millipore, USA). Five% nonfat dry milk was used to block nonspecific binding of antibody. The membranes

were incubated overnight at 4°C with either rabbit anti-P75 (1 : 500, Abcam, England), mouse anti-GFAP (1 : 400, Santa Cruz, USA), mouse anti-CNPase (1 : 500, Abcam, England), rabbit anti-S100β (1 : 1000, Abcam, England), rabbit anti-SOX10 (1 : 1500, Abcam, England), rabbit anti-vimentin antibody (1 : 1000, Abcam, England), rabbit anti-CD44 (1 : 500, Boster, China), anti-nestin antibody (1 : 500, Santa Cruz, USA), mouse anti-NF-H (1 : 300, Santa Cruz, USA), rabbit anti-Synapsin II (1 : 300, Santa Cruz, USA), mouse anti-GAP-43 (1 : 300, Santa Cruz, USA), rabbit anti-NGF (1 : 1000, Abcam, England), or rabbit anti-PSD-95 (1 : 500, Abcam, England) antibodies. The membranes were then incubated with HRP-conjugated goat anti-mouse or goat anti-rabbit secondary antibodies (1 : 1000, BioLegend, USA) for 1 hour. Membranes were treated with ECL chemiluminescent substrate (Millipore, USA) for 1 minute and developed by exposure to a cooled CCD camera (Sage Imaging System). Quantification of detected bands was performed by densitometry using ImageJ software.

2.5. Immunofluorescent Staining. Cells were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100/1% BSA in PBS. The primary rabbit anti-nestin antibody (1 : 300), rabbit anti-Vimentin antibody (1 : 200), rabbit anti-SOX10 (1 : 1000), rabbit anti-CD44 (1 : 200), anti-PSD-95 (1 : 1000), and anti-NF-H (1 : 300) were used to stain REMSCs for identification of EMSCs phenotype. The primary mouse anti-GFAP (1 : 300), rabbit anti-P75 (1 : 200), rabbit anti-S100β (1 : 300), rabbit anti-GALC (1 : 200, Santa Cruz, USA), and rabbit anti-CNPase (1 : 200) were used to stain SC-like cells for identification of SC phenotype. These cells were incubated at 4°C overnight with secondary antibodies including CY3-conjugated goat anti-mouse IgG (1 : 300, BioLegend, USA) and CY3-conjugated goat anti-rabbit IgG (1 : 300, BioLegend, USA) diluted in 1% BSA/PBS for 2-3 h at room temperature. Nuclei were labeled with Hoechst 33342 (Sigma, USA). The stained cells were examined with an inverted fluorescent microscope (Zeiss, Observer, A1, Germany).

2.6. Analysis of Neurite Outgrowth of PC12 Cells. After the PC12 cells were cocultured with SC-like cells infected with GFP or REMSCs infected with GFP for 5 days, morphological analysis and quantification of neurite bearing cells were performed under a fluorescent microscope as described previously [29, 30]. More than 100 cells in at ten randomly selected fields were counted and the cells with neurites greater than or equal to the length of its cell body were positive for neurite outgrowth. The positive cells were counted and expressed as a percentage of the total cells in each field. The neurite length was also measured for all the cells positive for neurite outgrowth in a field by tracing the longest length neurite. Average maximal neurite length per neurite-bearing cell in each field was calculated and data from the ten fields in each dish was designated as one experiment. The neurite length of neurite-bearing cells was measured by ImageJ software (NIH) [31] and recorded. These coculture experiments were repeated three times and analyzed independently.

2.7. Myelination Capacity of SC-Like Cells. PC12 cells were dissociated and replated at a density of 500 cells/cm^2 in a culture dish and cultured in DF12 supplemented with 10% FBS. After 24 hours, SC-like cells were seeded at a density of 5000 cells/cm^2 with PC12 cells and the medium was replaced with SCDM. As a control, the other two groups were designed: SC-like cells cultured alone, and REMSCs seeded with PC12 cells. The medium was changed every 72 hours. After 7 days in culture, the cells were fixed in 2% glutaraldehyde and then evaluated by scanning electron microscopy (Hitachi-S4800, Japan). After 21 days in culture, cells were fixed in 2% glutaraldehyde in sodium cacodylate buffer at 4°C for 24 hours, then fixed with 1% osmium tetroxide and 1% uranyl acetate, and embedded in epon. Ultrathin sections (50–70 nm) were cut and mounted on Formvar-coated slot grids. The ultrastructure of these cells was observed with transmission electron microscopy (Philips-Tecnai 12, Netherlands).

3. Statistical Analysis

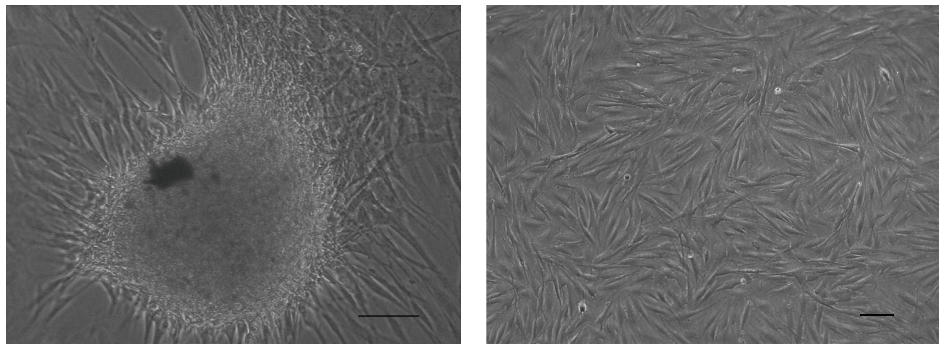
Data were obtained from three separate experiments described above and present as mean ± SEM. One-way analysis of variance (ANOVA) with Dunnett's *T*3 test and Student's *t*-test was used to analyze the data. Values of $P < 0.05$ were considered to be statistically significant.

4. Results

4.1. Characteristics of REMSCs. After 5 days, adherent cells migrated from the explants and formed colonies (Figure 1(a)). REMSCs at 4th passage appeared as fibroblastic-like cells and proliferated rapidly on plastic plates (Figure 1(b)). Immunofluorescent staining showed almost all of the REMSCs expressed neural crest cell markers such as SOX10 (87.6 ± 0.7%), nestin (90.8 ± 0.8%), vimentin (92.2 ± 0.8%), and CD44 (88.1 ± 0.8%) (Figures 2(a)–2(d)).

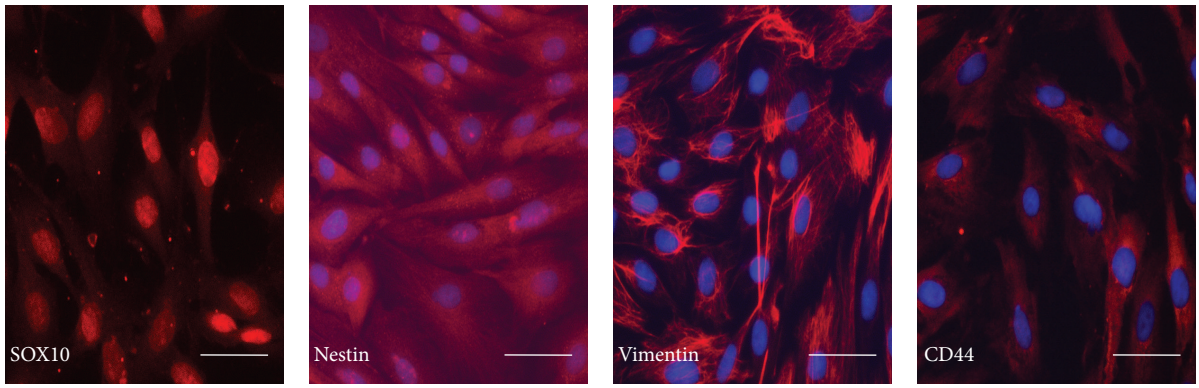
4.2. Differentiation into SC-Like Cells. REMSCs were treated with SCDM containing a mixture of glial cell growth factors for 10 days. The morphology of SC-like cells and the expression of the SC proteins such as GFAP, S100β, Galc, CNPase, and P75 were examined. After inductive differentiation, rat REMSCs changed from fibroblast-like morphology to spindle shape that seems to be more elongated than before, and these cells could continue to proliferate (Figure 3(a)). Immunofluorescent staining showed that about 79 ± 1.2% of the differentiated cells were positive for GFAP (Figure 3(b)); 81.7 ± 1.0% of the differentiated REMSCs were positive for P75 (Figure 3(c)); 89.2 ± 1.6% of the differentiated REMSCs were positive for S100β (Figure 3(d)); 84.9 ± 0.9% of the differentiated REMSCs were positive for Galc (Figure 3(e)), 84.6 ± 1.9% of the differentiated REMSCs were positive for CNPase (Figure 3(f)).

To further confirm the immunofluorescent staining results, Western blot analysis was used to examine the expression of glial specific markers and neural crest markers (Figure 4). β-Actin was used as a loading reference.



(a) (b)

Figure 1: Cultivation and expansion: (a) primary cultured REMSCs at 5 days; cells migrated from the explants and attached to the culture plate. (b) Rat REMSCs at 4th passage demonstrated large and flat cell morphology. Bar: 50 μm for all pictures.



(a) (b) (c) (d)

Figure 2: Labeling of neural crest cell markers on REMSCs: those markers were positively stained for SOX10 (a), nestin (b), vimentin (c), and CD44 (d). Nuclei were labeled with Hoechst 33342 (blue) except for SOX10. Bar: 20 μm for all pictures.

The expression level of each protein was expressed as the ratio of the expression level of the marker protein to β-actin. The expression levels of GFAP, CNPase, P75, and S100β in SC-like cells were more pronounced compared to REMSCs ($P < 0.01$) (Figures 4(a) and 4(c)). In addition, Figures 4(b) and 4(d) showed the downregulation of nestin, vimentin, and CD44 proteins in SC-like cells ($P < 0.01$). However, the level of SOX10 was similar in REMSCs and SC-like cells ($P > 0.05$) (Figures 4(b) and 4(d)).

4.3. Functional Analysis of SC-Like Cells. To detect the ability of SC-like cells to induce the differentiation of PC12 cells and form myelin structures with the neurites of PC12 cells, we cocultured SC-like cells with PC12 cells which are neuron-like cells. PC12 cells were labeled with CM-Dil (Red); SC-like cells or REMSCs were infected with GFP virus (Green). PC12 cells in group A (PC12 cells cultured alone) and group B (PC12 cells cultured in SCDM) were round red and have few neurites (Figures 5(a) and 5(b)). In group C (PC12 cells and REMSCs were cocultured in DF12 medium), short neurites could be detected (Figure 5(c)), while, in group D (PC12 cells and SC-like cells were cocultured in SCDM), PC12 cells grew with long neurites (Figure 5(d)). Compared with group A ($3.2 \pm 0.4\%$), the percentage of positive neurite-bearing cells was significantly increased to $38.0 \pm 2.3\%$ ($P < 0.01$) and $57.9 \pm 2.6\%$ ($P < 0.01$), respectively, in group C and group D, but group B ($3 \pm 0.3\%$) ($P > 0.05$) had no significant difference (Figure 5(e)). Also, the percentage of positive neurite-bearing cells in group D significantly increased compared with group C ($P < 0.01$) (Figure 5(e)). Likewise, compared with group A (10.1 ± 0.5 μm), the length of the longest neurite significantly increased to 72 ± 3.7 μm ($P < 0.01$) and 223 ± 7.5 μm ($P < 0.01$), respectively, in group C and group D (Figure 5(f)). There was no significant difference between group A and group B (9.7 ± 0.4 μm) (Figure 5(f)). To further investigate the differentiation of PC12 cells, Western blotting was used to examine the expression levels of NF-H, Synapsin II, GAP-43, and PSD-95 in PC 12 cells. As shown in Figure 6(a), compared with group A, the level of GAP-43, NF-H, Synapsin II, and PSD-95 significantly ($P < 0.01$) increased in group C and group D. There was significant difference between group C and group D in NF-H, Synapsin II, PSD-95, and GAP-43 ($P < 0.05$). Immunofluorescent staining showed that differentiated PC12 cells in group D expressed NF-H ($35.3 \pm 0.42\%$) and PSD-95 ($56.7 \pm 0.47\%$) (Figures 6(b) and 6(c)). The level of NGF was examined as well. SC-like cells

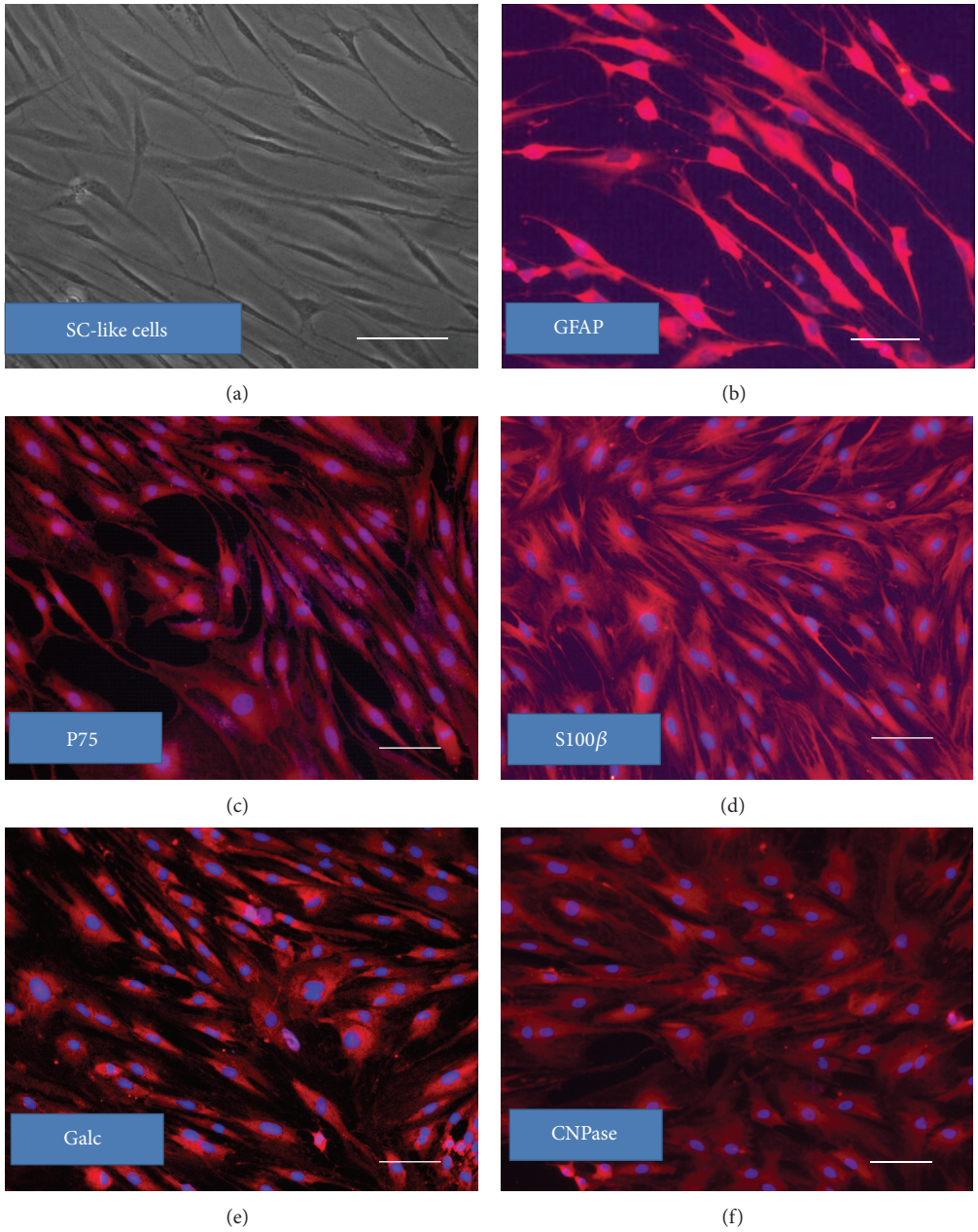


FIGURE 3: SC-like cells differentiated from REMSCs demonstrated typical Schwann cell morphology and were labeled with related cell surface markers. After 10 days, cells appeared typical bipolar and spindle-like Schwann cell phenotype (a), immunofluorescent staining showed that differentiated cells were stained positively with GFAP (b), P75 (c), S100β (d), Galc (e), and CNPase (f). Nuclei were labeled with Hoechst 33342 (blue). Bar: 50 μm for all pictures.

were strongly positive for NGF expression as compared with REMSCs (Figure 7). To assess the myelination capacity of SC-like cells, PC12 cells were cultured with SC-like cells or REMSCs. Consisting with the morphology observed under phase contrast microscopy, observation under scanning electron microscopy (SEM) also shows that SC-like cells were bipolar and spindle-like shaped (Figure 8(b)). When PC12 cells were cocultured with REMSCs the neurites were shorter (Figures 8(c1) and 8(c2)) and, in contrast, when cocultured with SC-like cells for 7 days, the neurites of PC12 cells were longer and grew along with SC-like cells (Figures 8(d1) and 8(d2)). After 21 days of coculture, transmission electron microscopy (TEM) showed that SC-like cells could form myelin sheath with neurites (Figure 9(c)). On the contrary, REMSCs could not form myelin structures with PC12 neurites (Figure 9(b)), and REMSCs could not form myelin structures without neurites (Figure 9(a)).

5. Discussion

Previous studies have shown that REMSCs could form neurospheres in neurosphere-forming condition and differentiate

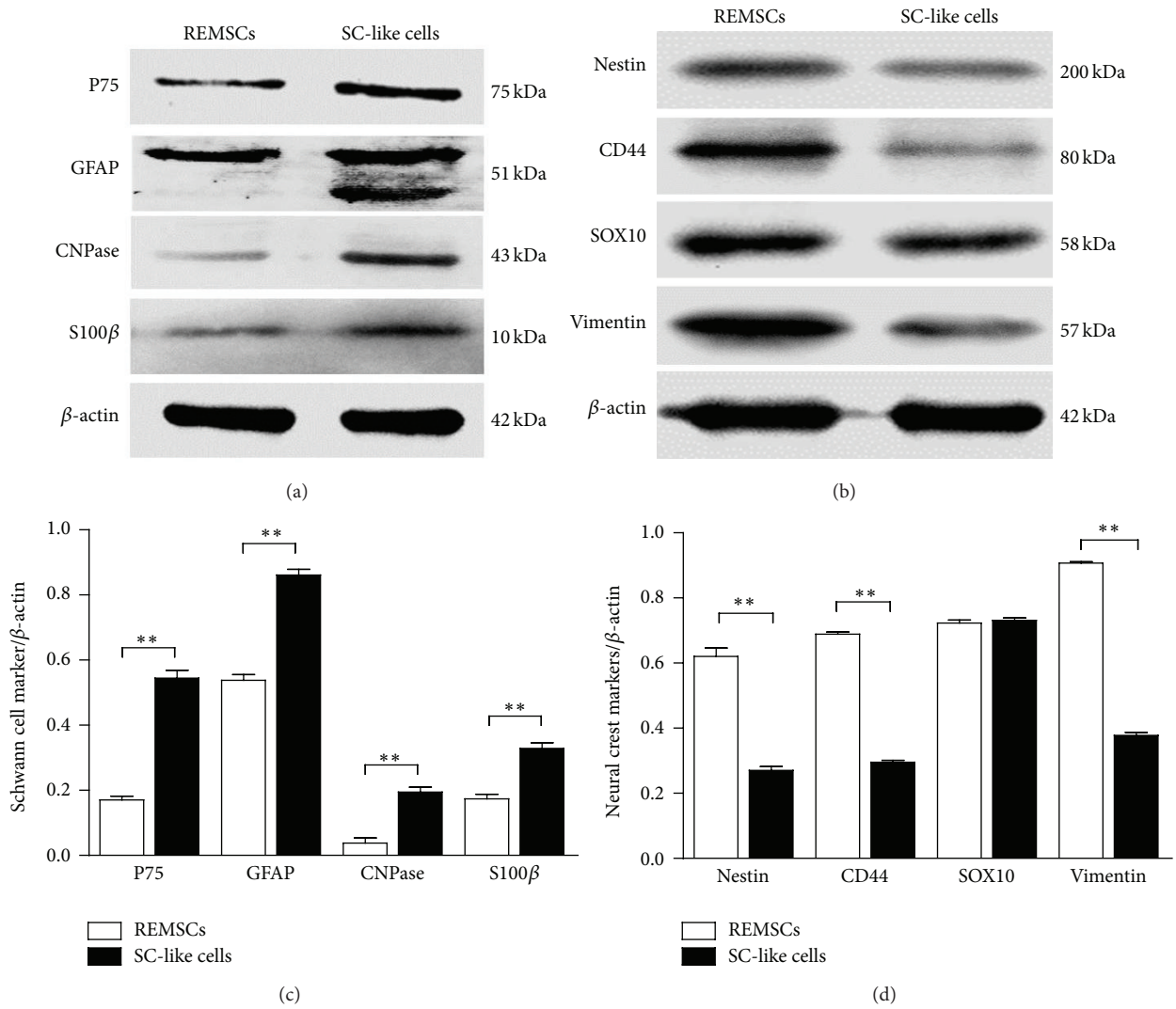


Figure 4: Western blotting showed the upregulation of expression of Schwann cell markers and the downregulation of expression of neural crest markers except for SOX10. Expressions of Schwann cell markers including GFAP, CNPase, P75, and S100β by REMSCs and SC-like cells were shown in (a); expressions of neural crest markers including SOX10, nestin, vimentin, and CD44 by REMSCs and SC-like cells were shown in (b); the experiments were replicated three times, and β-actin was used as a loading control. Quantitation of each marker was calculated using morphometric analysis with ImageJ software. Each bar showed the ratio of the expression level of marker protein to β-actin (c, d). The data were presented as mean ± SEM of three independent experiments. $^{**}P < 0.01$ represented significant differences when compared between RMSCs and SC-like cells.

into neurons [32]. However, whether REMSCs could differentiate along a Schwann cell lineage is still unknown. Here, our results show that differentiated REMSCs have similar morphological and phenotypic characteristics as Schwann cells, and more importantly, differentiated REMSCs possess myelin-forming ability, which is the most important function of Schwann cells.

EMSCs originate from the neural crest during embryonic development and contribute to the formation of craniofacial structures [33]. In the head region, neural crest-derived stem cells can be found in a number of organs and tissues [34–40]. Recently, respiratory mucosa cells isolated from adult human inferior turbinate are reported to be multipotent neural crest-derived stem cells [41]. Our previous studies have shown that REMSCs expressed MSCs markers such as CD90, CD45, and CD105 can different into neuron-like cells and osteoblasts [25, 27]. A study from Goldstein and colleagues also shows that nasal stem cells derived from septum can form neurosphere and give rise to neuronal-like cells under differentiation conditions [32]. In the current study, immunofluorescent staining of REMSCs showed that most cells express neural crest markers including nestin, vimentin, and SOX10 (Figure 2). Nestin and vimentin are regarded as a marker of neural stem cells and expressed in neural crest cells [42–46]. SOX10 plays a role early in development when it is present in the neural crest cells, and it is the only transcription factor needed for the generation of glial cells from crest cells during the embryonic development [47].

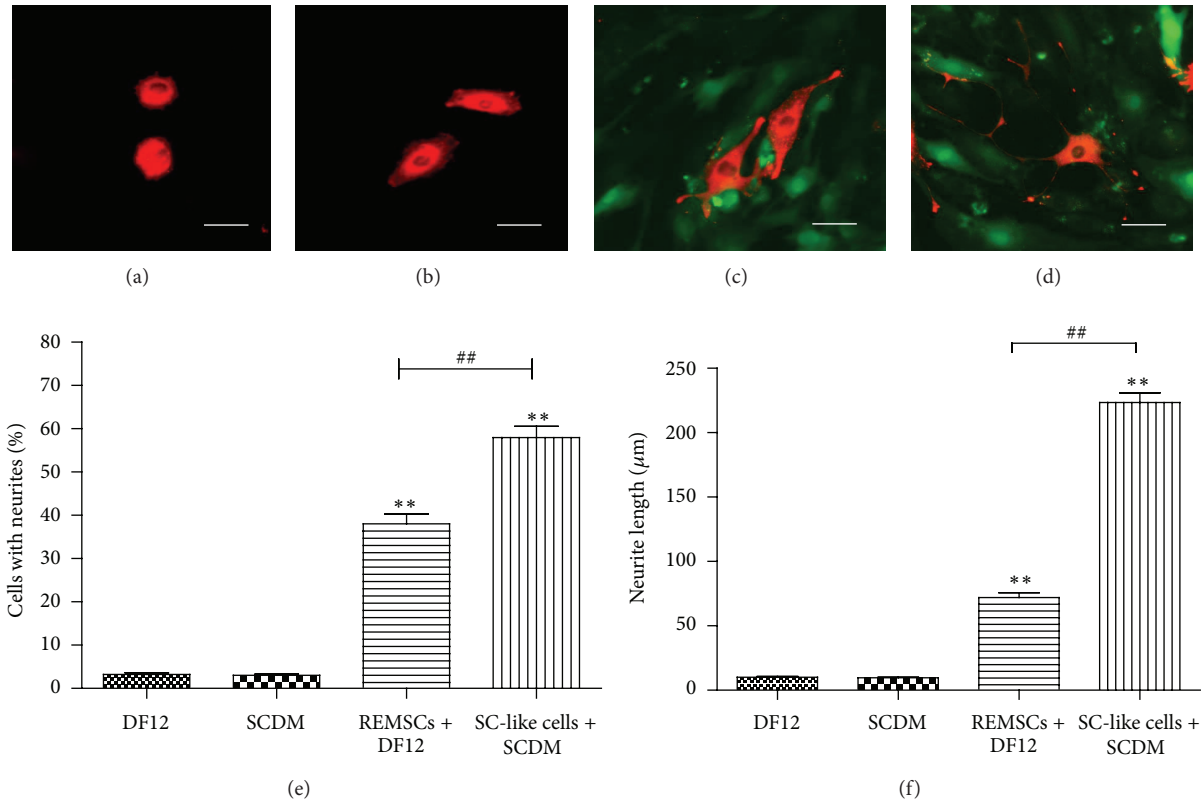


Figure 5: Neurite outgrowth by PC12 cells was observed in different conditions. PC12 cells were labelled by CM-Dil (red) and SC-like cells or REMSCs were infected with GFP virus (green). (a) PC12 cells were cultured alone in DF12 medium. (b) PC12 cells were cultured in SCDM alone. (c) PC12 cells were cocultured with REMSCs in DF12 medium. (d) PC12 cells were cocultured with SC-like cells in SCDM. PC12 cells with few neurites in (a) and (b) were round red. PC12 cells with neurites were observed in (c) and (d), and the cells in (d) showed morphological phenotype of mature neuron. The percentage of cells with neurites (e) and the length of neurites (f) were shown in the two bar graphs, respectively. Data were presented as mean ± SEM from three independent experiments. $^{**}P < 0.01$ represent significant differences compared between group C or group D and group A; $^{\#\#}P < 0.01$ represent significant differences compared between groups C and D. Bar: 50 μm for all figures.

Meanwhile, these cells also express CD44 that is the marker of premigratory and migratory cranial neural crest [48]. The coexpression of nestin, vimentin, SOX10, and CD44 provides strong evidence that REMSCs originate from neural crest and could be able to differentiate into glial cells and neuron-like cells.

It has been reported that stem cells from a variety of tissue sources were able to differentiate into SC-like cells [12, 18, 49]. Nestin-positive BMSCs have been observed to differentiate along the glial cell lineage [50, 51]. Minor percentile of adipose-derived stem cells was identified with positive nestin labeling and able to differentiate into SC-like cells [18, 52]. Also, it has been demonstrated that the glial formation potentials of MSCs derived from bone marrow and adipose may be explained by the presence of crest-derived cell subpopulation [53]. In addition, Labat et al. also found BMSCs originating from neural crest in the peripheral blood mononuclear cells, which simultaneously expressed the mesoderm markers and the neural ectoderm markers [54]. It suggests that ectomesenchymal-derived stem cells may exist in multiple tissues. However, the amount of the crest-derived cells in those tissues is too small to obtain sufficient SCs for effectively clinical application. The current study shows that almost all the REMSCs derived from nasal septum were nestin-positive and SOX10-positive. In agreement with our findings, REMSCs derived from human inferior turbinate also expressed neural crest markers [41]. Those results together suggest that REMSCs may have stronger potential to differentiate into SC-like cells than other source-derived stem cells.

To investigate the ability of REMSCs to differentiate into SC-like cells, REMSCs were cultured in a differentiation medium (HRG, FSK, PDGF-AA, and bFGF) which is previously used to induce Schwann cells from MSC and ADSC [18, 49, 55]. After 5 days, the REMSCs demonstrated elongated-spindle morphology. Western blot showed the downregulation of nestin, vimentin, and CD44 (Figures 4(b) and 4(d)). However, the level of SOX10 of SC-like cells is similar with REMSCs. SOX10 has been reported to be a neural crest marker [42]. Furthermore, SOX10 is important and expressed at all stages of Schwann cell development and works both independently and synergistically with other transcription factors to regulate Schwann cells specific loci [56–59]. In our study, western blot analysis showed that the level of SOX10

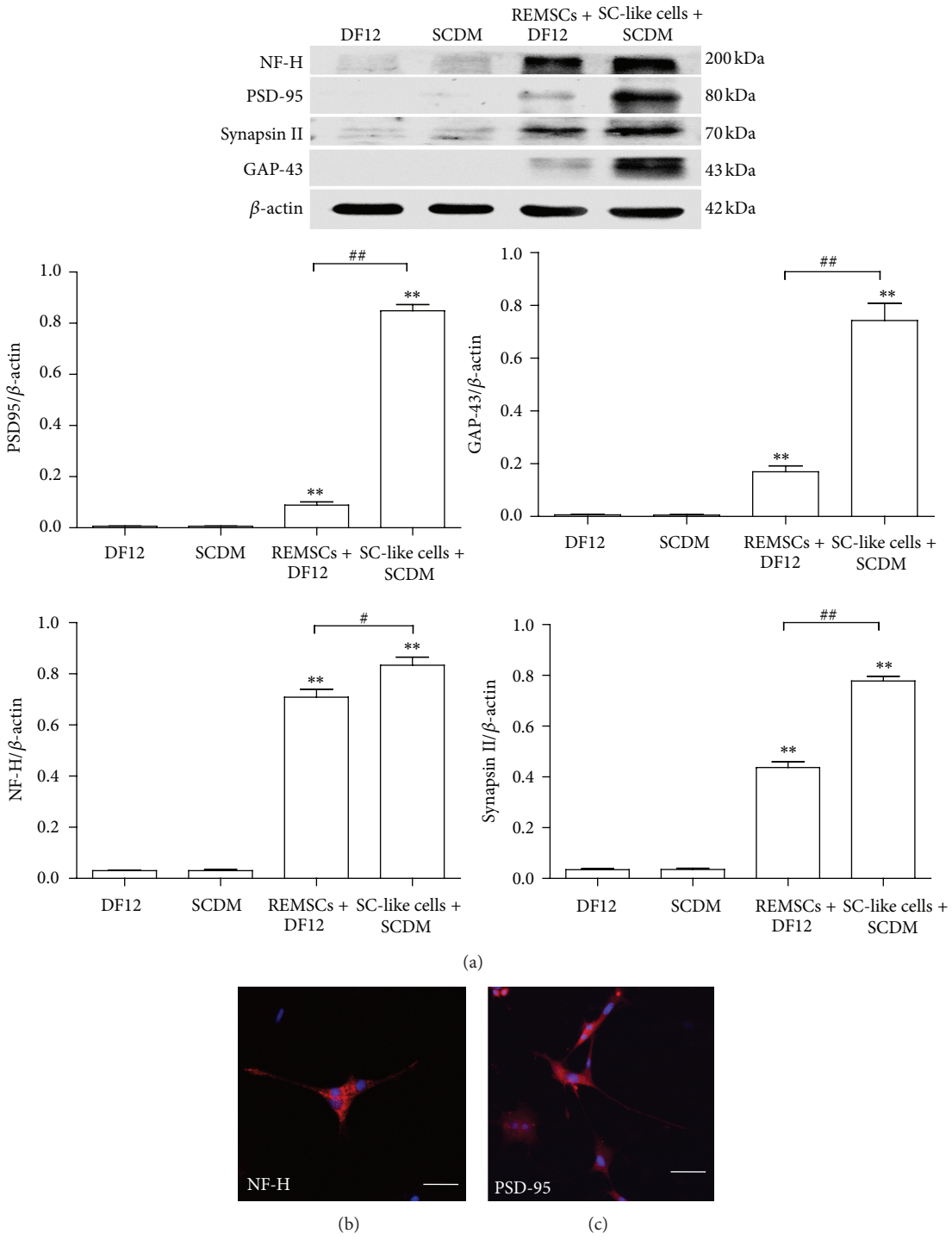


FIGURE 6: Western blot and immunofluorescent labeling indicated that SC-like cells promoted the differentiation of PC12 cells into mature neuron-like cells. (a) Neural cell markers including NF-H, GAP-43, PSD-95, and Synapsin II were detected in PC12 cells after being cultured for 5 days for all four groups, which were PC12 cells treated with DF12 medium, PC12 cells treated with SCDM, PC12 cells treated with DF12 medium and REMSCs, and PC12 cells treated with SC-like cells and SCDM. β-Actin was used as a loading control. The experiments were replicated three times and a representative blotting was shown. Each bar showed the ratio of marker protein to β-actin. The data were presented as the mean ± SEM of three independent experiments. $^{**}P < 0.01$ represent significant differences compared with group A; $^{\#\#}P < 0.01$ represent significant differences compared with group C. Immunofluorescent staining showed that differentiated PC12 cells in group D expressed NF-H (b) and PSD-95 (c). Bar: 50 μm for all pictures.

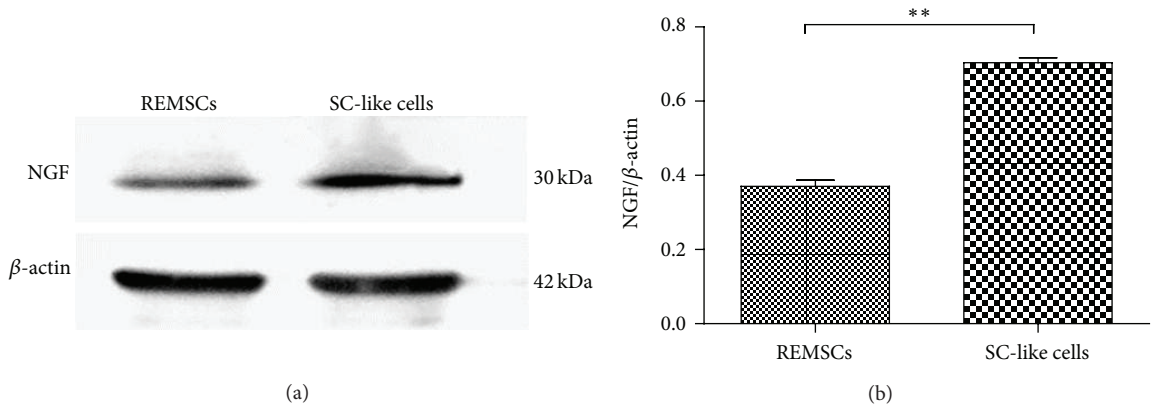


Figure 7: Expression levels of NGF by REMSCs and SC-like cells were observed and quantitated by Western blot. Significant differences between REMSCs and SC-like cells were observed ($^{**}P < 0.01$). Data were presented as mean ± SEM from three independent experiments.

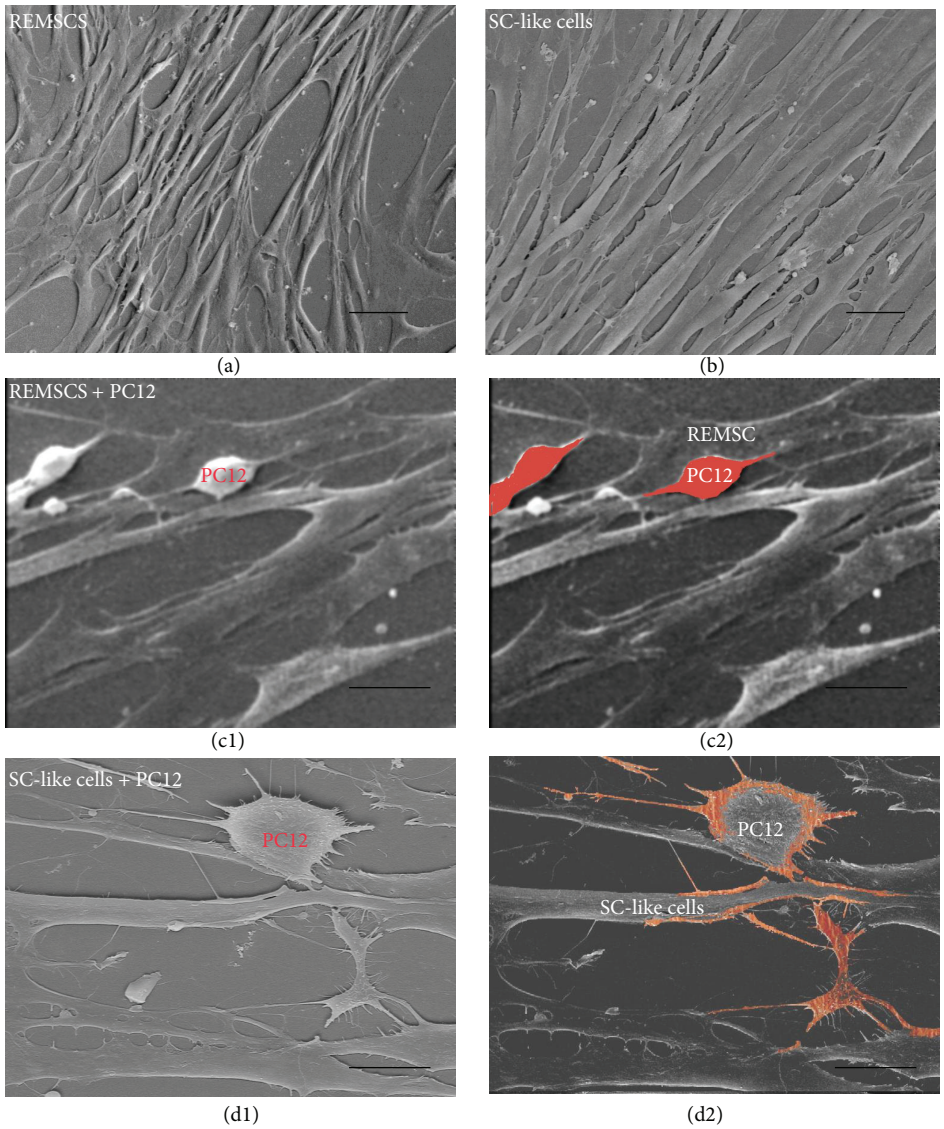
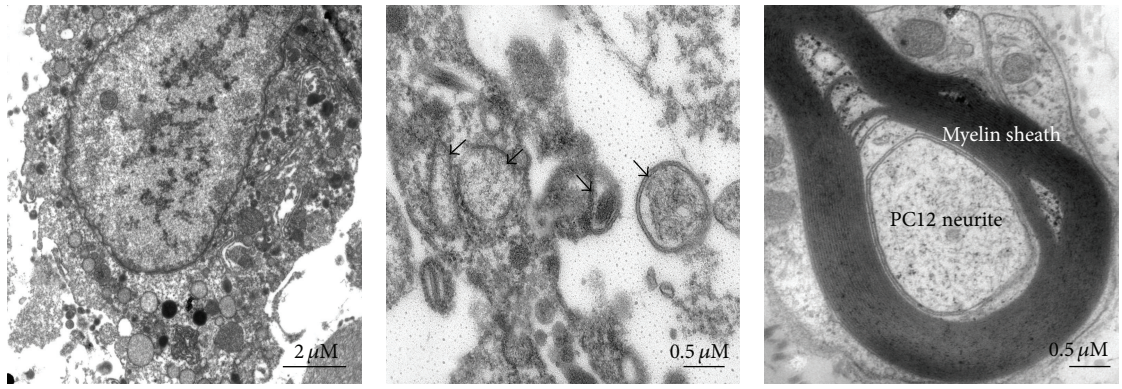


Figure 8: Morphology of REMSCs, SC-like cells, and PC12 cells cocultured with REMSCs or SC-like cells was observed under SEM. REMSCs cultured in DF12 medium (a) and SC-like cells cultured in SCDM (b), PC12 cells (marked with red) cocultured with REMSCs in DF12 medium (c1, c2), and PC12 cells (marked with red) cocultured with SC-like cells in SCDM (d1, d2). The imaging showed that the neurites of PC12 cells in (d1, d2) were longer than that in (c1, c2), and the neurites grew along with SC-like cells. Bar: 20 μm for all pictures.



(a) (b) (c)

Figure 9: TEM observation showed that SC-like cells formed myelin sheath covering the neurites of PC12 cells (c). Myelin structures were not found when SC-like cells were cultured alone without PC12 cells (a). REMSCs could not form myelin structures with PC12 cells, and the arrows indicated that there was no myelin structure around the neurite. Bar: 2 μm (a), 0.5 μm ((b) and (c)).

was similar in both REMSCs and SC-like cells. We speculate that SOX10 may play a role in the differentiation of REMSCs. Immunofluorescent staining of dREMSCs showed that most cells expressed Schwann cell markers such as GFAP, P75, and S100β (Figure 3). Similar results were also reported in previous studies [12, 18, 49]. Interestingly, SC-like cells expressed Galc which is a specific cell-surface antigenic marker for oligodendrocytes in culture [60]. Furthermore, these SC-like cells expressed CNPase (Figure 3), which was expressed in oligodendrocytes and Schwann cells. CNPase is regarded as marker for myelin-forming cells and photoreceptors for some neurons in long-term culture [61]. CNPase is both membrane bound and linked to microtubules, and it is the third most abundant myelin protein in the CNS, representing 4% of CNS myelin proteins [62]. Overexpression mutations show that CNPase plays a role in myelin compaction [63, 64]. Therefore, CNPase is considered to be a marker for the cells to produce myelin. These results suggested that the SC-like cells have the capability of myelination.

Evidence of morphological and phenotypic characteristics may not be enough to justify that the function of SC-like cells is similar to Schwann cells. It has been demonstrated that SC can induce the differentiation of PC12 cells and form myelin sheath with PC12 neurites [12]. Here, we tested the function of SC-like cells by being cocultured with PC12 cells. When cocultured with SC-like cells for 5 days, PC12 cells extended neurites and the percentage of cells with neurites significantly increased compared with that in coculture with REMSCs (Figure 5). Western blotting showed that SC-like cells promoted the expression level of NF-H, GAP-43, PSD-95, and Synapsin II in PC12 cells (Figure 6(a)). Immunofluorescent staining showed that differentiated PC12 cells expressed NF-H and PSD-95 (Figures 6(b) and 6(c)). NF-H provides stability to developing axonal neurites, and it is related to the stages of axonal outgrowth [65]. Also PSD-95, which is a membrane-associated guanylate, is the main scaffolding protein in the excitatory postsynaptic density [66]. All these results suggested that SC-like cells could promote the differentiation of PC12 cells. NGF is considered to play an important role in the differentiation of PC12. When PC12 cells are treated with NGF, PC12 cells extend neurites and form synapse structure and neurite network, differentiating into neuron-like cells [67]. In the current study, Western blotting showed that the expression level of NGF of SC-like cells was higher than that of REMSCs (Figure 7), which indicates that SC-like cells may promote the differentiation of PC12 cells by expression of NGF.

Myelin-forming ability is very important to SC cells. It is reported previously that SC-like cells induced from BMSCs and ADSCs can form myelin sheath *in vitro* [12, 52]. SC-like cells from REMSCs may have the ability to form myelin *in vitro* and *in vivo*. According to the "carpet crawler" model, myelination starts by the spreading of a membrane sheet along the neurite before it makes a turn and moves underneath the growing sheet [68]. We observed that SC-like cells grew along with the neurites of PC12 cells at 7 days (Figure 8) and formed myelin structures with neurites at 21 days (Figure 9(c)). However, myelin structures were not detected in SC-like cells cultured without PC12 cells and REMSCs cultured with PC12 cells. A previous study has demonstrates that Schwann cells become myelinating or nonmyelinating depending on the signal from axon [47]. Whether SC-like cells from REMSCs could form myelin *in vivo* is still a question to be answered in future studies though.

MSCs have been demonstrated to be safe, as they do not form tumor after transplantation [69]. Studies have shown that mouse embryonic stem cells (MESCs) would form teratocarcinomas when injected into immunodeficient mice [70]. Also, when injected into human embryonic stem cells (hESCs) in severe combined immunodeficient mice, hESCs could generate primitive, undifferentiated tumors [71]. On the contrary, Sieber-Blum found that epidermal neural crest stem cell grafted into the adult spinal cord does not form tumors [72]. Similarly, Stefan Hauser showed that neural crest stem cell from adult human inferior turbinate was not able to create teratoma [41]. Those data may collectively indicate the safety of using REMSCs and may suggest that transplantation of REMSCs could be an alternative cell-based therapeutic

strategy for neurotissue engineering and neurodegenerative diseases. However, large scale preclinical and clinical studies on its safety are needed before any clinical application.

6. Conclusion

REMSCs isolated from nasal septum are able to differentiate into SC-like cells which have similar morphological, phenotypic characteristics, and function with Schwann cells. These findings may suggest that transplantation of REMSCs could be an alternative cell-based therapeutic strategy for neurotissue engineering and neurodegenerative diseases.

Abbreviations

SC:	Schwann cell
EMSCs:	Ectomesenchymal stem cells
MSCs:	Mesenchymal stem cells
BMSCs:	Bone marrow-derived stem cells
ADSCs:	Adipose-derived stem cells
REMSCs:	Respiratory mucosa stem cells
SC-like cells:	Schwann-like cells
DF12:	DMEM/F-12
bFGF:	Basic fibroblast growth factor
PDGF-AA:	Platelet-derived growth factor-AA
dREMSCs:	Differentiated REMSCs
GFAP:	Glial fibrillary acidic protein
Galc:	Galactosylceramidase
FBS:	Fetal bovine serum
NGF:	Nerve growth factor
GFP:	Green fluorescent protein
SCDM:	Schwann-like cells differentiation medium
MESCs:	Embryonic stem cells
hESCs:	Human embryonic stem cells.

Conflict of Interests

The authors declare no conflict of interests.

Acknowledgments

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Research Article

Acute Lymphoblastic Leukemia Cells Inhibit the Differentiation of Bone Mesenchymal Stem Cells into Osteoblasts In Vitro by Activating Notch Signaling

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The disruption of normal hematopoiesis has been observed in leukemia, but the mechanism is unclear. Osteoblasts originate from bone mesenchymal stem cells (BMSCs) and can maintain normal hematopoiesis. To investigate how leukemic cells inhibit the osteogenic differentiation of BMSCs and the role of Notch signaling in this process, we cocultured BMSCs with acute lymphoblastic leukemia (ALL) cells in osteogenic induction medium. The expression levels of Notch1, Hes1, and the osteogenic markers Runx2, Osteopontin (OPN), and Osteocalcin (OCN) were assessed by real-time RT-PCR and western blotting on day 3. Alkaline phosphatase (ALP) activity was analyzed using an ALP kit, and mineralization deposits were detected by Alizarin red S staining on day 14. And then we treated BMSCs with Jagged1 and anti-Jagged1 neutralizing Ab. The expression of Notch1, Hes1, and the abovementioned osteogenic differentiation markers was measured. Inhibition of the expression of Runx2, OPN, and OCN and reduction of ALP activity and mineralization deposits were observed in BMSCs cocultured with ALL cells, while Notch signal inhibiting rescued these effects. All these results indicated that ALL cells could inhibit the osteogenic differentiation of BMSCs by activating Notch signaling, resulting in a decreased number of osteoblastic cells, which may impair normal hematopoiesis.

1. Introduction

Acute lymphoblastic leukemia (ALL) cells arise from the malignant proliferation of lymphoid precursors and occupy the bone marrow niche. Such niches, or bone marrow microenvironments, are known to regulate hematopoietic stem cell (HSC) survival, proliferation, and differentiation and thus play a crucial role in normal hematopoiesis. The malignant proliferation of leukemic cells disrupts normal bone marrow niches and creates abnormal microenvironments [1, 2], impairing normal hematopoiesis. In addition, these microenvironments are more favorable for leukemia stem cells because they support abnormal hematopoiesis [3] and mediate drug resistance [4]. However, the mechanisms underlying the leukemic cell-related disruption of bone marrow microenvironments are poorly understood.

Osteoblasts are an important part of the endosteal niche and play an essential role in the regulation of normal HSCs [5]. Osteoblastic cells can stimulate HSC expansion, maintain quiescence, and promote HSC mobilization. In addition, bone progenitor dysfunction can induce myelodysplasia [6], and even a single genetic change in osteoblasts can induce leukemogenesis [7]. These results demonstrate the important role that osteoblasts play in HSC regulation.

Bone mesenchymal stem cells (BMSCs) are recognized as bone marrow stroma stem cells and can differentiate into multiple cell lineages, including osteoblasts, adipocytes, and chondrocytes. The ultimate differentiation of BMSCs depends on signals from neighboring cells, and Notch signaling plays a critical role in cell differentiation during and after embryogenesis [8]. There are four Notch receptors (Notch1–4) and five known ligands (Jagged1 and 2 and

Table 1: The primary characteristics of ALL children at diagnosis.

Sample number	Sex	Age (months)	% of BM blast	Immunophenotype	Cytogenetics
1	F	44	98	common B-ALL	46XX
2	F	75	98.5	common B-ALL	46XX
3	M	133	97.5	T-ALL	46XY
4	M	82	86.5	common B-ALL	46XY
5	F	37	99	Pre-B	46XX
6	M	13	94.5	Pre-B	46XY

M, male; F, female.

Delta-Like1, 3, and 4), which are single-pass transmembrane proteins [9]. Notch-ligand interactions contribute to maintenance and renewal of adult tissues, such as the skin, the hematopoietic system, and the central nervous system. In the bone marrow, Notch signaling can maintain the stemness of BMSCs by suppressing osteoblast differentiation [10]. Meanwhile, the constitutive expression of the Notch1 intracellular domain impairs osteoblast differentiation and enhances adipogenesis in stromal cell cultures [11]. In addition, the activation of Notch signaling in osteoblasts causes osteopenia [12]. The abnormal activation of Notch pathways not only determines cell differentiation but also causes tumors. The oncogenic role of Notch signaling in T-cell malignancies has been well defined, and some B-cell malignancies express high level of Notch receptors and their ligands Jagged1 [13, 14]. However, whether abnormalities of Notch signaling in leukemia affect the differentiation of BMSCs to osteoblasts is unclear.

We hypothesized that leukemic cells can alter BMSCs differentiation via the activation of Notch signaling, resulting in a decreased number of osteoblastic cells, which may impair normal hematopoiesis. To test this hypothesis, we cocultured leukemia cells and BMSCs in vitro and observed the effect of leukemic cells on the osteogenic differentiation of BMSCs. Furthermore, we investigated whether Jagged1-induced Notch1 signaling played a key role in this process.

2. Materials and Methods

2.1. Patient Characteristics and Specimens. BM samples from 63 children with newly diagnosed ALL were recruited for this study. The diagnosis of ALL was based on morphology, cell immunophenotype, and cytogenetic analysis. The presence or absence of invasion osteoclasia or osteoporosis was determined by computerized tomography (CT) scans. The mRNA expression of Jagged1 was assessed by real-time RT-PCR. The study was approved by the institutional ethics committee of the Affiliated Children's Hospital of Chongqing Medical University in accordance with the Declaration of Helsinki, and written informed consent was obtained from patients and/or their legal guardians.

2.2. Cell Culture. BM samples from six children with newly diagnosed ALL and three healthy volunteers who donated bone marrow for transplantation were obtained for cell culture. The primary characteristics of these children in this study are presented in Table 1. Bone marrow mononuclear cells (BMNCs) were isolated by density gradient centrifugation (Lymphocyte separation medium, TBD, Tianjin, china) within 6 hours of sampling. Adherent cells were removed by plastic adherent culture and the remaining BMNCs were immediately used for the laboratory research. The BMNCs from healthy volunteers were cultured in DMEM/F12 (Gibco, USA) supplemented with 10% FBS (Gibco, Australia) in a 5% CO_2-in-air incubator at 37°C. After 48 hours of adhesion, nonadherent cells were collected and stored in liquid nitrogen until use. The adherent cells were maintained in culture, with the medium being replaced every 2-3 days. Once the cultures reached 80–90% confluence, BMSCs were recovered by the addition of a 0.25% trypsin solution. All the experiments were performed with BMSCs harvested between the third and sixth passages.

2.3. Coculture. BMSCs were cultured alone or cocultured with ALL cells at a 1/10 ratio for 3 or 14 days to study the osteogenic differentiation of BMSCs. The cells were cultured in osteogenic induction medium, which consisted of growth medium supplemented with 0.1 mM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma), and 50 mM vitamin C (Sigma). The expression of Notch1, Hes1, and the osteogenic markers Runx2, Osteocalcin (OCN), and Osteopontin (OPN) was assessed by real-time RT-PCR and western blotting on day 3. The ALP activity was analyzed using an ALP kit, and mineralization deposits were assessed by Alizarin red S staining on day 14.

2.4. Activation and Inhibition of Notch Signing in BMSCs. The recombinant rat Jagged1-Fc fusion chimera (R&D Systems) was dissolved in phosphate-buffered saline (PBS) at 10 μg/mL and immobilized in flat-bottom 96-well plates overnight at 4°C, according to the manufacturer's protocol. Human IgG-Fc (R&D Systems) was used for the control. BMSCs were seeded in plates coated with Jagged1 or IgG-Fc at 10^4 cells/well. After 2 days of culture, the medium was replaced with osteogenic induction medium, and the BMSCs were cultured for 3 or 14 days.

BMSCs were cocultured with ALL cells in osteogenic induction medium containing anti-Jagged1 neutralizing Ab (10 μg/mL, GeneTex, USA) or vehicle (PBS) for 3 or 14 days. The expression of Notch1, Hes1, and the osteogenic markers Runx2, OCN, and OPN was assessed by real-time RT-PCR and western blotting on day 3. The ALP activity was analyzed

Table 2: Primers sequences for real-time RT-PCR analysis.

Gene	Length	Annealing temperature (°C)	Sequence
OCN	81	60.7	GGTGCAGCCTTTGTGTCCA
			GGCTCCCAGCCATTGATACA
OPN	81	63	GGCCGAGGTGATAGTGTGGTT
			AGCATCAGGGTACTGGATGTCA
Hes1	313	63.5	AAAATGCCAGCTGATATAATGGAG
			GGTCTGTGCTCAGCGCAGCCGTCA
Notch1	76	63.5	CGGGTCCACCAGTTTGAATG
			GTTGTATTGGTTCGGCACCAT
Runx2	101	62.3	TTATTCTGCTGAGCTCCGGAA
			AACTCTTGCCTCGTCCACTCC
Jagged1	164	60	GCTGCCTTTCAGTTTCGC
			CGCCCGTGTTCTGCTTCA
GADPH	114	54	CCACATCGCTCAGACACCAT
			GGCAACAATATCCACTTTACCAGA

using an ALP kit, and mineralization deposits were assessed by Alizarin red S staining on day 14.

2.5. Alkaline Phosphatase Activity and Alizarin Red Staining. ALP activity was detected using an ALP kit (Nanjing built Technology Co. Ltd, Nanjing, China) according to the manufacturer's protocol. Alizarin red S staining was used to visualize the mineralization deposits of BMSCs after different treatments on day 14. The ALL cells were removed, and the BMSCs were washed with cold PBS. They were then fixed in 10% formalin for 1 hour and stained with 2% Alizarin red S.

2.6. Real-Time Polymerase Chain Reaction (RT-PCR) Analysis. Total RNA was extracted using TRIzol reagent (Ambion, USA) and reverse-transcribed using the PrimeScript RT reagent Kit (TaKaRa, Japan). The mRNA expression of the genes encoding Jagged1, Notch1, Hes1, Runx2, ALP, OPN, OCN, and the housekeeping gene GAPDH was determined using the SYBR Green master mix (TaKaRa, Japan) on CFX 96 real-time PCR machine (BIO-RAD). The PCR conditions were as follows: 94°C for 30 s for the initial step; 39 cycles of 94°C for 5 s and the appropriate annealing temperature for 30 s; and extension in the last cycle for 5 s. The target expression was normalized to GAPDH and relative to a calibrator (control group). The relative expression was calculated using the formula $2^{(-\Delta\Delta Ct)}$. The primer sequences are listed in Table 2.

2.7. Western Blotting. BMSCs undergoing different treatments were washed with PBS and lysed in ice-cold lysis buffer with a protease inhibitor cocktail. Total protein and nuclear fractionation were performed using a whole protein or nuclear extraction kit (KENGEN Biotechnology, Nanjing, China). Equal amounts of protein (50 μg) were fractionated by SDS-PAGE, transferred to polyvinylidene difluoride membrane (PVDF), and analyzed by immunoblotting using primary antibodies to Notch1 (Epitomics), Hes1 (Epitomics), Jagged1 (Abcam), Runx2 (Santa cruz Biotechology), OCN (Abcam), OPN (Epitomics), LaminB1 (Abcam), and β-actin. HRP-conjugated anti-rabbit or anti-mouse secondary antibodies were used as the secondary antibodies. The results were normalized to the loading control β-actin, and an ECL detection system was used for the data analysis.

2.8. Cytotoxicity and Apoptotic Assay. Cell viability of BMSCs with Jagged1 treatment was assessed by a colorimetric method (Cell Counting Kit-8; Beyotime, Beijing, China) using tetrazolium salt according to the manufacturer's procedure after long-term (1-2 weeks) treatment. The number of apoptotic BMSCs cells in short-term (3 days) treatment has also been evaluated by flow cytometry with AnnexinV-FITC Apoptosis Detection Kit (keygentec, Nanjing, china).

2.9. Statistical Analysis. Results are expressed as mean ± standard deviation. Statistical analysis was conducted using GraphPad Prism 6 software. Differences between groups were evaluated for statistical significance using a one-way analysis of variance; P values less than 0.05 were considered statistically significant. All experiments were repeated in triplicate.

3. Results

3.1. ALL Cells Inhibit the Osteogenic Differentiation of BMSCs. The effect of ALL cells on the osteoblast differentiation markers was investigated. We used a coculture system with ALL cells and confluent BMSCs obtained from healthy volunteers. In these coculture systems, the osteogenic differentiation of BMSCs was assessed by ALP activity, the expression of OPN, and OCN and mineralization. Firstly, it was found that ALL cells, but not the normal BMNCs, reduced OPN and OCN mRNA expression in BMSCs after 3-day coculture (Figure 1(a)). OPN and OCN protein expressed by BMSCs were also consistently inhibited (Figure 1(b)). Furthermore, the ALP activity of BMSCs cocultured with ALL cells was significantly lower than that with normal BMNCs after 14

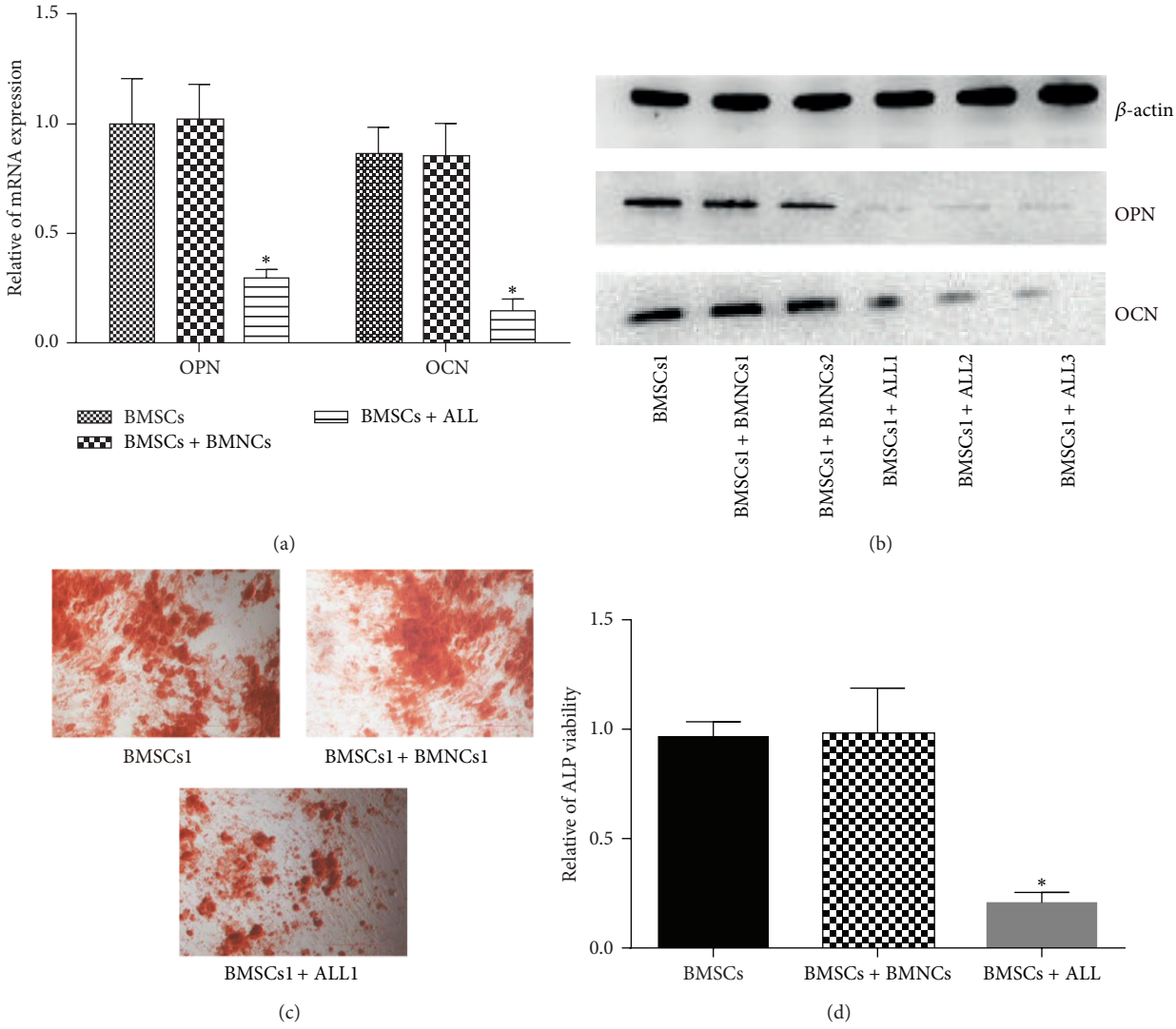

Figure 1: Effect of ALL cells on osteoblast differentiation of bone mesenchymal stem cells (BMSCs). BMSCs obtained from healthy bone marrow mononuclear cells (BMNCs) were cocultured with acute lymphoblastic leukemia (ALL) cells from six ALL patients or BMNCs from three healthy donor in osteogenesis induction medium for 3 days or 14 days. (a) The mRNA expression of Osteopontin (OPN) and Osteocalcin (OCN) were analyzed using real-time RT-PCR. (b) OPN and OCN protein were assessed by western blot analysis after 3-day coculture. (c) Calcium deposits were detected using von Kossa staining. (d) Alkaline phosphatase (ALP) levels were detected using an ALP kit ($^*P < 0.05$ versus BMSCs cultured alone or coculture with BMNCs).

days (Figure 1(d)). Lastly, the mineralization in cocultured BMSCs after 14 days was assessed by Alizarin red S staining. Significant reduction of the mineralization levels was observed in BMSCs cocultured for 14 days with ALL cells but not in the control BMNCs (Figure 1(c)). Taken together, these results indicate that ALL cells significantly inhibit the osteogenic differentiation of BMSCs. No difference in the inhibitory effect of ALL cells was observed in the coculture systems.

3.2. ALL Cells Activate Notch Signaling in Cocultured BMSCs. We further studied the expression and activation levels of Notch signaling in the process of the osteogenic differentiation of BMSCs under coculture conditions. First, the Jagged1 expression levels in ALL cells and normal BMNCs were evaluated by real-time RT-PCR and western blotting. Results showed that the expression of Jagged1 was significantly higher in ALL cells than in BMNCs (Figures 2(a) and 2(c)). Meanwhile, Notch1 expression in the BMSCs cocultured with ALL cells was significantly higher than that in the control BMNCs (Figures 2(b) and 2(d)), suggesting that Notch1 expression is negatively correlated with the osteogenic differentiation of BMSCs. Consistent with the observed Notch1 levels, the expression of Hes1, which is a target gene of Notch pathway, was also markedly increased in cocultured BMSCs. Thus, ALL cells can activate Notch signaling in BMSCs and suggest a negative correlation between Notch signaling and osteogenic differentiation of BMSCs.

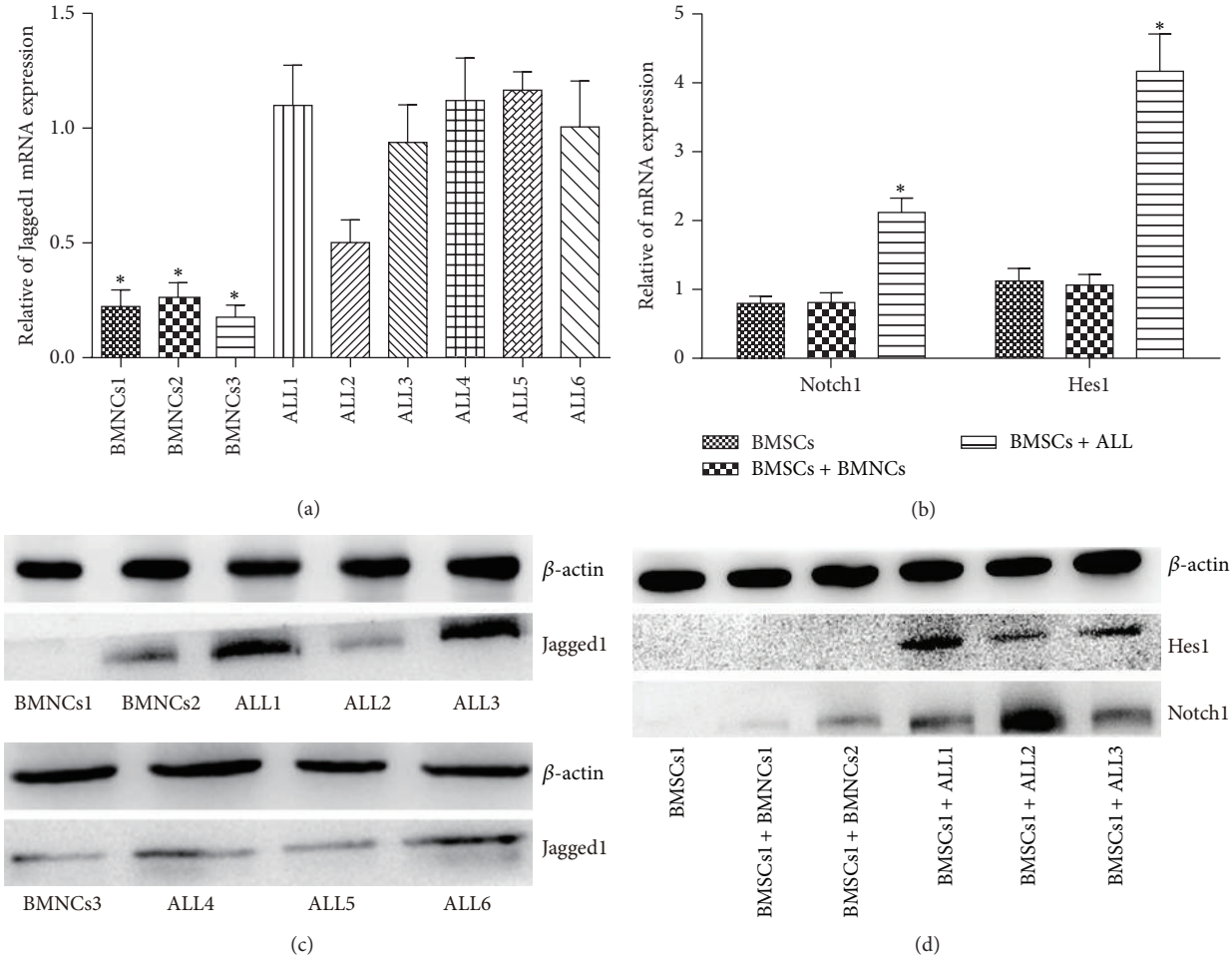

Figure 2: ALL cells activate Notch signaling in BMSCs in coculture. (a), (c) The Jagged1 expression levels in ALL cells and normal BMNCs were evaluated by real-time RT-PCR and western blotting ($^*P < 0.05$ versus BMNCs). (b), (d) The mRNA and protein expression levels of Notch1 and Hes1 in BMSCs were analyzed using real-time RT-PCR and western blot analysis after 3-day cocultured with ALL cells or BMNCs ($^*P < 0.05$ versus BMSCs cultured alone or coculture with BMNCs).

3.3. Jagged1 Overexpressed in ALL Cells from Leukemia Children with Invasion Osteoclasia or Osteoporosis. To assess whether the invasion osteoclasia or osteoporosis is due to the Jagged1 overexpressed in ALL cells, the Jagged1 expression levels was evaluated in ALL cells from 63 leukemia children with or without invasion osteoclasia or osteoporosis by real-time RT-PCR. A significant overexpression of Jagged1 was observed in leukemia children with invasion osteoclasia or osteoporosis compared with those who did not have invasion osteoclasia or osteoporosis (Figure 6(c)).

3.4. Recombinant Notch Ligand Jagged1 Impaired the Osteogenic Differentiation of BMSCs. The cooccurrence of the enhanced Notch expression and impaired osteogenic differentiation by BMSCs cocultured with ALL cells prompts us to further investigate the role of Notch signaling in this process. We cultured BMSCs on immobilized soluble Jagged1 ligand in osteogenic induction medium for 3 days, with IgG-Fc as the control. To determine whether Jagged1 stimulates Notch activation, Notch1 and Hes1 expression were analyzed in BMSCs. The results showed that Notch1 and Hes1 levels were increased by Jagged1 treatment compared with the controls (Figures 3(a)-3(b)). Osteogenic differentiation markers were also assessed, as previously mentioned. The ALP activity was significantly lower in the Jagged1-treated cells (Figure 3(c)). In addition, the mRNA and protein expression levels of OPN and OCN were reduced in the Jagged1 group (Figures 3(a)-3(b)). Consistent with these findings, Jagged1 protein inhibited osteogenic mineralization (Figure 3(d)). These results imply that Notch signaling is critical for the impairment of the osteogenic differentiation of BMSCs.

3.5. Anti-Jagged1 Neutralizing Ab Rescued the Osteogenic Differentiation of Cocultured BMSCs. To further confirm the role of Notch signaling in this process, anti-Jagged1 neutralizing Ab was introduced into coculture systems with BMSCs and ALL cells to inhibit Notch signaling. As shown in Figures 4(a)-4(b), Notch1 and Hes1 expressions were clearly inhibited by anti-Jagged1 neutralizing Ab. After BMSCs and

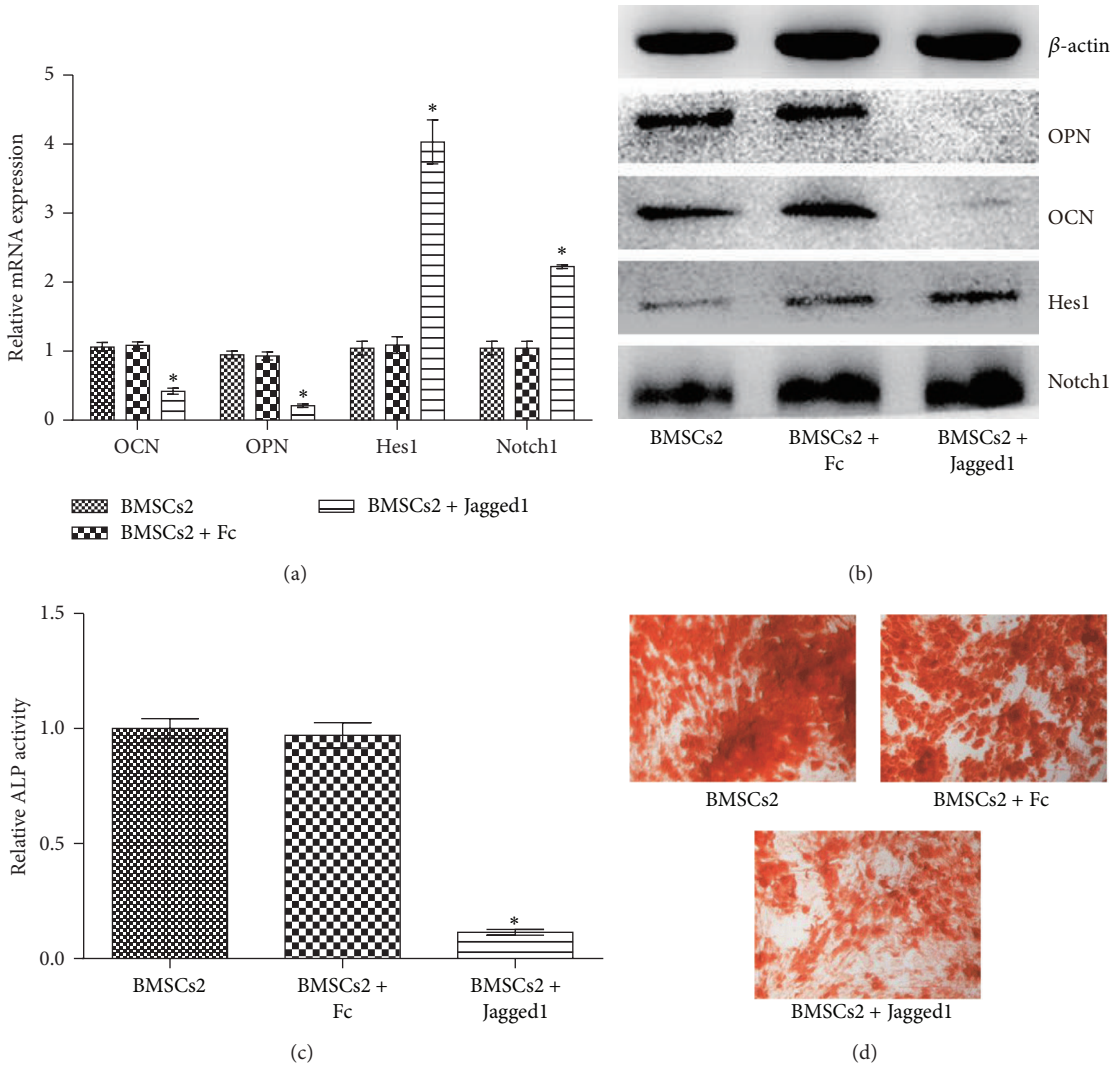

Figure 3: Effect of recombinant protein Jagged1 on osteoblast differentiation of BMSCs. We cultured BMSCs on immobilized soluble Jagged1 ligand in osteogenic induction medium for 3 days or 14 days, with Ig G-Fc as the control. (a-b) The mRNA and protein expression levels of osteogenic differentiation markers OPN and OCN, and Notch1 and Hes1 were analyzed using real-time RT-PCR and western blot analysis after 3-day ($^*P < 0.05$ versus BMSCs2 cultured alone). (c) ALP levels were detected using an ALP kit ($^*P < 0.05$ versus BMSCs2 cultured alone). (d) Calcium deposits were detected using von Kossa staining.

ALL cells were cocultured with anti-Jagged1 neutralizing Ab under osteogenic conditions for 3 days, the mRNA and protein expression levels of OPN and OCN were elevated in the anti-Jagged1 neutralizing Ab group (Figures 4(a)-4(b)). In addition, the ALP activity was significantly higher than that without anti-Jagged1 neutralizing Ab (Figure 4(c)). Consistent with these findings, the inhibitory effect on Notch signaling promoted osteogenic mineralization. These results suggest that inhibition of Notch signaling can rescue the impaired osteogenic differentiation of BMSCs.

To exclude that the inhibitory effect observed on BMSCs in our Jagged1 treatment could be due to toxicity, we tested the viability of BMSCs by flow cytometry and confirmed that no toxic or apoptotic effect was present in BMSCs after 3 days (Figure 5(a)). And then we have evaluated the viability of BMSCs in the presence and absence of recombinant Notch ligand Jagged1 or anti-Jagged1 neutralizing Ab using a cytotoxic assay. The viability of BMSCs was evaluated after 7 and 14 days. No significant reduction of BMSCs viability was observed at any time point. Figure 5(b) shows the percent of cell viability at 2 weeks.

3.6. Effect of ALL Cells on Runx2 Expression in BMSCs. To investigate whether ALL cells could affect the expression of the critical osteoblast transcription factor Runx2, BMSCs with ALL cells were cocultured. First, we found that Runx2 mRNA expressed by BMSCs was not modified after 3 days of coculture (Figure 6(a)). However, Runx2 protein expression in BMSCs nucleus, as evaluated by nuclear extract western blots, was modified in the presence of ALL cells (Figure 6(b)). To further investigate the connection between Notch signaling and Runx2, anti-Jagged1 neutralizing Ab was applied

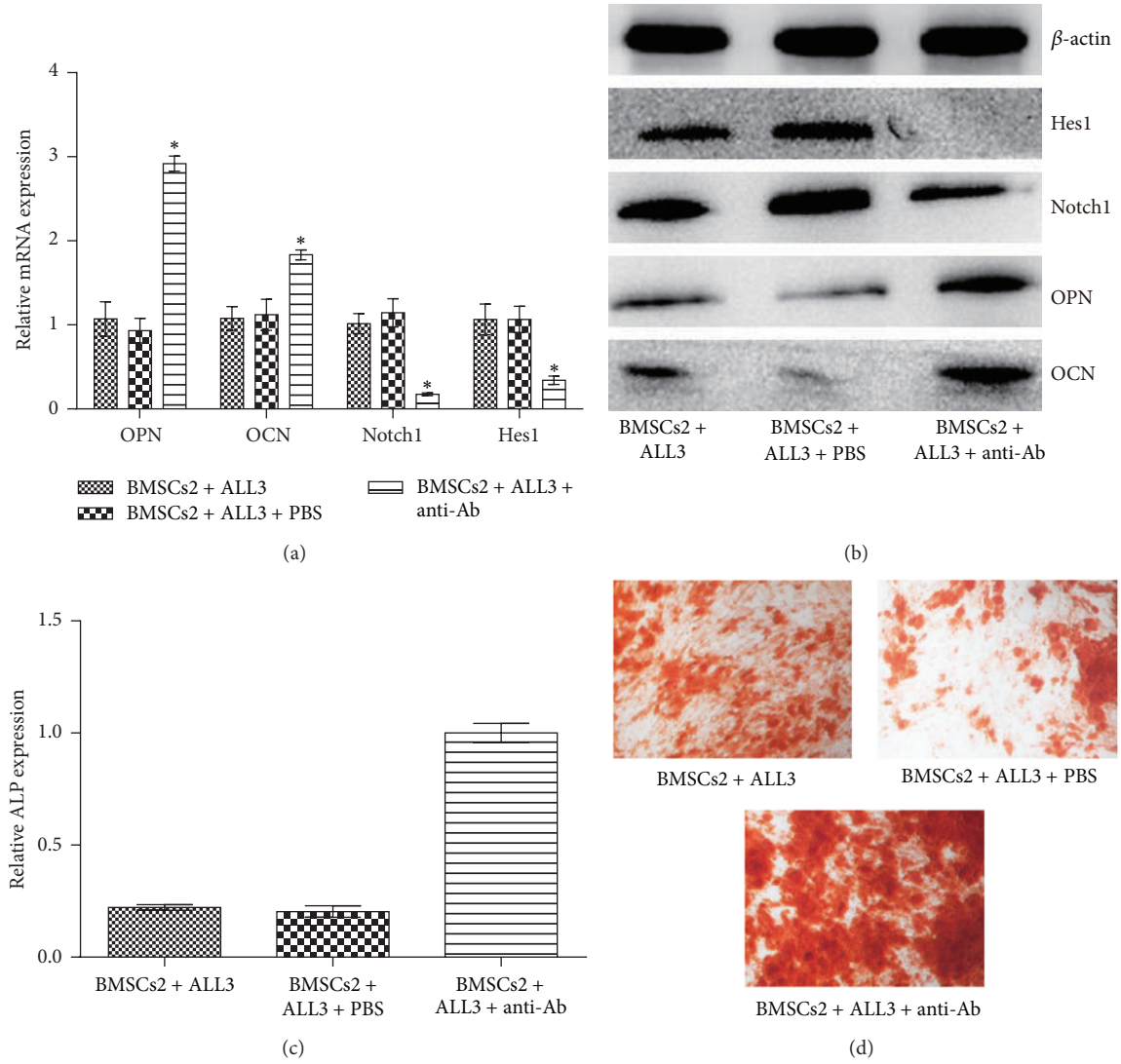

FIGURE 4: Effect of anti-Jagged1 neutralizing Ab on osteoblast differentiation of BMSCs in cocultures. BMSCs2 and ALL cells were cocultured with or without anti-Jagged1 neutralizing Ab under osteogenic conditions for 3 days or 14 days. (a-b) The mRNA and protein expression levels of osteogenic differentiation markers OPN and OCN, and Notch1 and Hes1 were analyzed using real-time RT-PCR and western blot analysis after 3-day coculture. (c) ALP levels were detected using an ALP kit ($^*P < 0.05$ versus BMSCs2 cocultured with ALL cells). (d) Calcium deposits were detected using von Kossa staining.

to inhibit Notch signaling and results showed that Runx2 mRNA expressed by BMSCs was not modified but protein level was elevated (Figures 6(a)-6(b)). In summary, Notch signaling showed an inhibitory effect on Runx2 protein level but not on Runx2 mRNA expression.

4. Discussion

The inhibition of normal hematopoiesis is partially responsible for the impairment of the bone marrow microenvironment in leukemia [15, 16]. Osteoblasts have long been known as important parts of the bone marrow microenvironment and have been known to support HSCs in vitro. Recent data suggest that BMSCs give rise to cells of the osteogenic lineage, and studies indicate that leukemic cells can inhibit osteoblastic cell function and decrease osteoblastic cell numbers [17]. However, how ALL cells implement this process is poorly understood.

Our data demonstrate that the osteogenic differentiation of BMSCs is inhibited by ALL cells, as demonstrated by the decreased expression of osteogenic markers. The inhibitory effect of ALL cells on the osteogenic differentiation of BMSCs might explain the decreasing number of osteoblasts, which leads to the destruction of the bone marrow microenvironment and impairs support of normal hematopoiesis. This conclusion is in agreement with an in vivo study showing that osteoprogenitor numbers are decreased in the long bones of

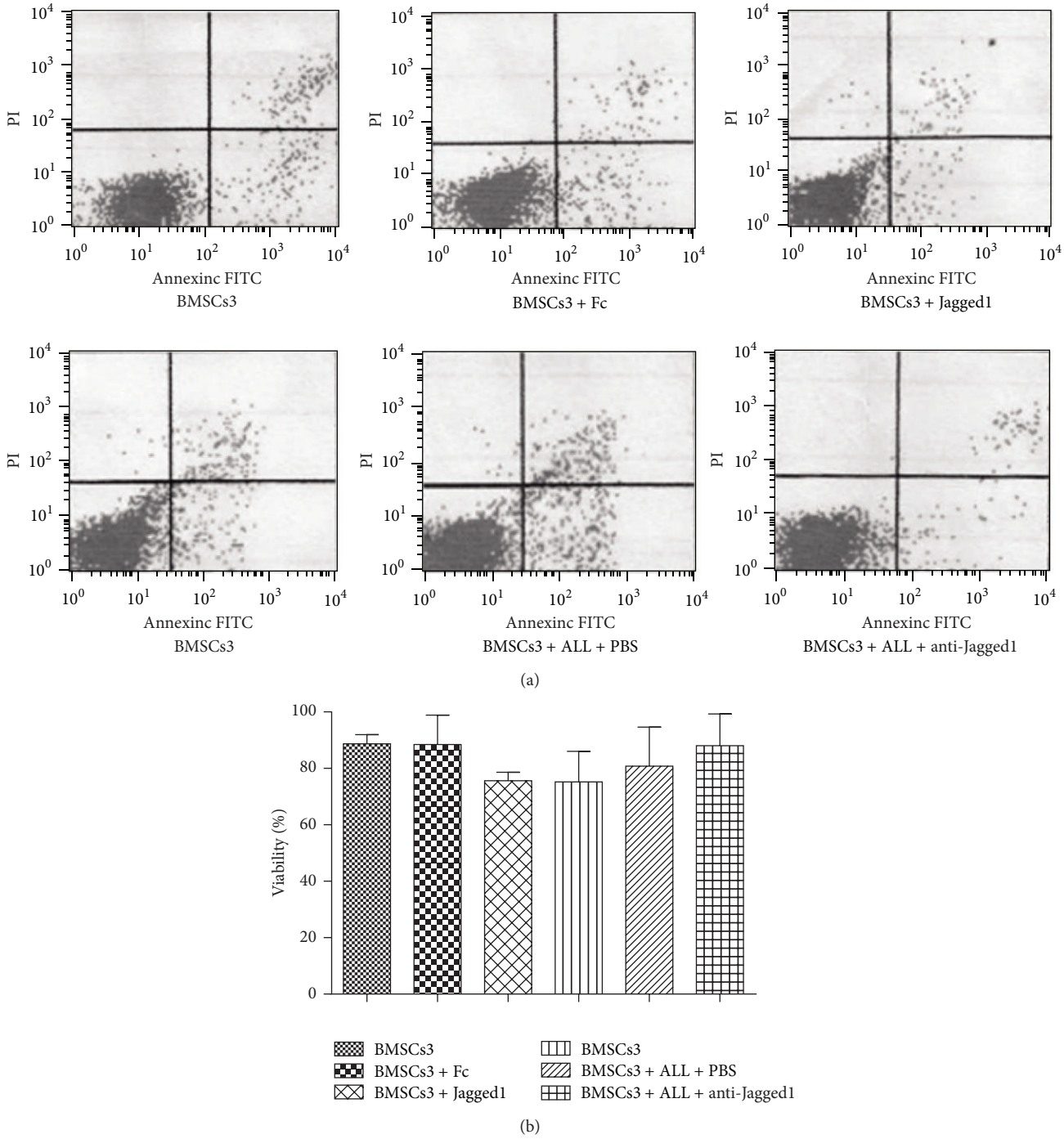

Figure 5: No toxic and apoptotic effect on BMSCs in the Jagged1 treatment. (a) The presence of both death and apoptotic BMSCs has been investigated by flow cytometry after 3 days in the presence and absence of recombinant Notch ligand Jagged1 or anti-Jagged1 neutralizing Ab. (b) The viability of BMSCs in the Jagged1 treatment using a cytotoxic assay after 14 days.

leukemic mice [17, 18] and increased osteoblasts in mouse models of acute leukemia decrease leukemia blasts in the bone marrow and reestablish normal hematopoiesis [18]. Meanwhile, numerous studies have shown a decrease in the markers of bone formation in pediatric acute leukemia cases at diagnosis before corticosteroid treatment [19]. The inhibition of ALL cells in the osteogenic differentiation of BMSCs is further supported by previous studies that maintaining a pool of mesenchymal progenitors led to a deficit in osteoblast production and resulted in precipitous bone loss [10].

Some previously published data have shown that leukemia BMSCs exhibit similar differentiation potential compared with BMSCs from health donors [20]. These contradictory results could be explained by the possibility

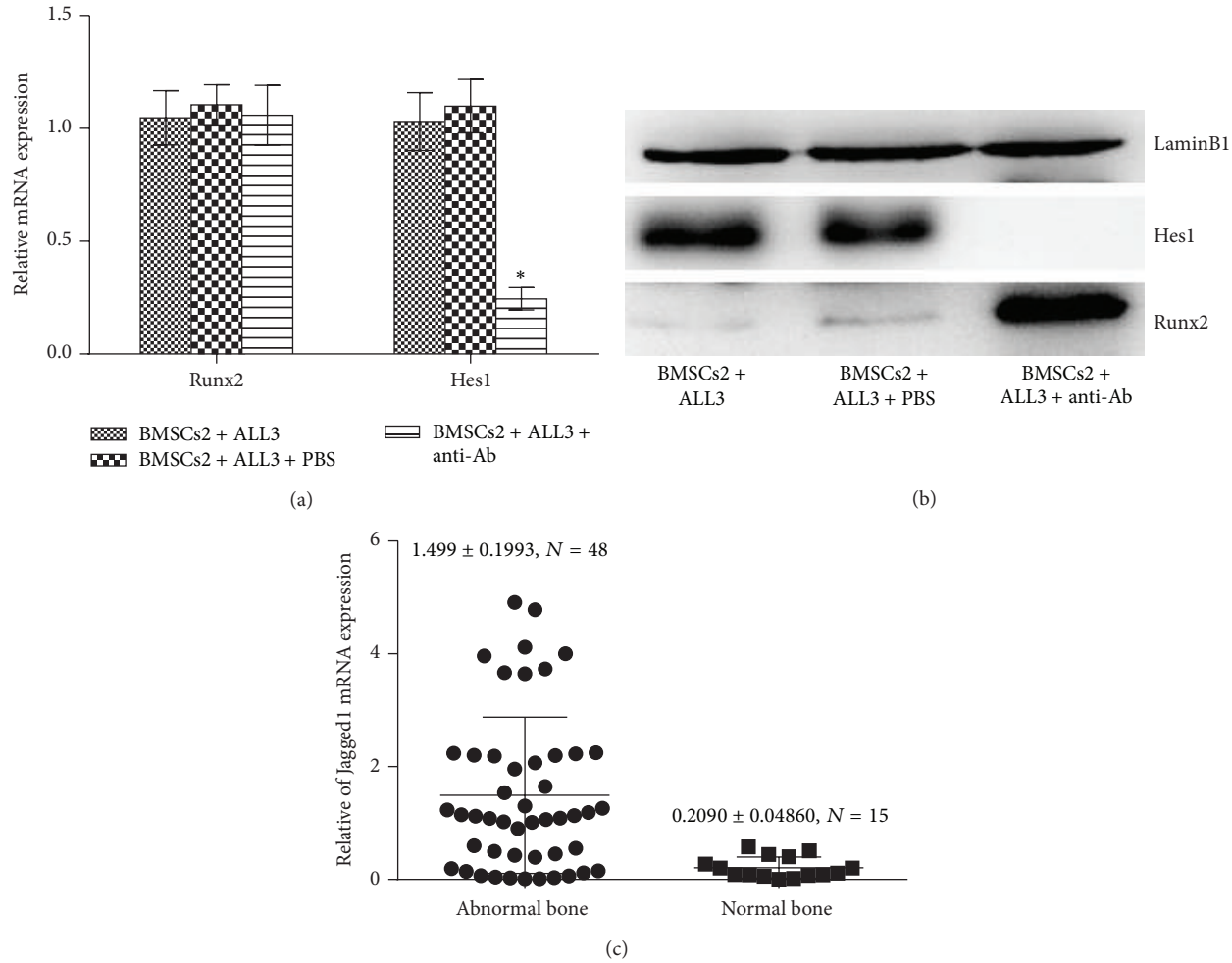

Figure 6: (a) The mRNA expression of Runx2 and Hes1 was evaluated by real-time RT-PCR in BMSCs2 after 3-day coculture with ALL cells with or without anti-Jagged1 neutralizing Ab ($^{*}P < 0.05$ versus BMSCs2 cocultured with ALL cells). (b) The protein expression of Hes1 and Runx2 were assessed. (c) The Jagged1 expression levels were evaluated in ALL cells from 63 leukemia children with or without invasion osteoclasia or osteoporosis by real-time RT-PCR. Abnormal bone represented leukemia children with invasion osteoclasia or osteoporosis.

that the effect of ALL cells on BMSCs is reversible. Once leukemia cells are removed, the BMSCs return to normal, as shown by the increased expression of bone formation markers after the reduction in disease burden by chemotherapy [21]. In addition, a recent study demonstrated that osteoblasts regulated ALL cells dormancy and protected them from cytotoxic chemotherapy [22]. One potential explanation for these dissimilar results is that leukemic cells not only inhibit osteoblastic cell function and decrease osteoblastic cell numbers, but also can change the osteoblast, which further create a favorable niche for ALL cells.

The mechanism by how ALL cells inhibit osteogenic differentiation of BMSCs was also investigated in this study. We focused on Notch signaling since this pathway regulates osteogenic differentiation. Previous studies have demonstrated that Notch signaling maintains a pool of mesenchymal progenitors by suppressing osteoblast differentiation [10]. The osteogenic differentiation potential of mesenchymal stem cells can be promoted by inhibiting Notch1 activity in vitro [23]. In addition, abnormal Notch signaling is associated with cancer, including leukemia. Studies have indicated that Notch1 and Jagged1 are highly expressed in B- and T-cell-derived Hodgkin's lymphoma and anaplastic large cell tumor cells [14]. Notch ligands Jagged1/2 and Delta ligands are expressed in BMSCs and, in the context of leukemia, BMSCs can enhance Notch signaling in human B-ALL cells via Jagged1 and rescue B-ALL cells from drug-induced apoptosis in vitro [24]. However, whether the abnormal Jagged1 in ALL cells affects the osteogenic differentiation of BMSCs is unknown.

In accordance with previous reports [13, 14], we observed that Jagged1 was highly expressed in ALL cells compared with normal BMNCs. Consistent with Notch-ligand interactions, enhanced signaling was observed in BMSCs after coculturing with ALL cells, as demonstrated by the increased expression of Notch1 and Hes1. In addition, we found that the expression of osteogenic markers was decreased and, once anti-Jagged1 neutralizing Ab was added to the coculture system, the osteogenesis potential of BMSCs was regained, suggesting that ALL cells can inhibit the osteogenic differentiation of

BMSCs by activating Notch signaling. The involvement of Notch signaling in the osteogenic differentiation of BMSCs is further supported by the evidence that Notch signaling stimulation by a soluble Jagged1 ligand decreases the expression of osteogenic markers. Moreover, the mRNA expression of Jagged1 in ALL cells supports our in vitro study. Children with invasion osteoclasia or osteoporosis highly expressed Jagged1 in comparison with children without invasion osteoclasia or osteoporosis. This evidence suggests that the overexpressed Jagged1 in ALL cells might activate the Notch signaling in BMSCs and lead to a reduction of the number of osteoblastic cells.

Runx2 is a crucial transcription factor in osteogenic differentiation, regulating the expression of osteoblast markers such as ALP, OCN, and OPN [25]. Hes1, which is downstream of Notch signaling, may mediate the Notch-induced inhibition of osteoblast differentiation by inhibiting Runx2 activity [26]. Similarly, in our coculture system, we found that ALL cells decreased Runx2 protein expression. This finding is in agreement with a previous study showing that the expression of Runx2 in Notch1 knockdown BMSCs was upregulated. In contrast, human myeloma cells only block Runx2 activity, without modifying Runx2 expression in coculture system with a mesenchymal/stromal cell line [27]. This result could be explained by the different experimental system applied by different researchers and the potential involvement of other signaling pathways.

In conclusion, our findings indicate that abnormal Notch signaling not only induces leukemia cell proliferation but also inhibits the osteogenic differentiation of BMSCs, which further disturbs normal hematopoiesis. The prevention of abnormal Notch signaling in BMSCs would be beneficial for the restoration of normal hematopoiesis. Furthermore, an in vivo study on the mechanisms involved in BMSCs differentiation into osteoblast and other lineages in the context of ALL is urgently required.

Abbreviations

BMSCs: Bone mesenchymal stem cells
ALL: Acute lymphoblastic leukemia
OPN: Osteopontin
OCN: Osteocalcin
ALP: Alkaline phosphatase
HSC: Hematopoietic stem cell
CT: Computerized tomography
BMNCs: Bone marrow mononuclear cells
PBS: Phosphate-buffered saline
PVDF: Polyvinylidene difluoride membrane
RT-PCR: Reverse transcription-polymerase chain reaction.

Conflict of Interests

The authors declare that they have no competing interests.

Acknowledgments

The authors thank colleagues for providing technical assistance and insightful discussions during the preparation of the paper. Gui-Cun Yang collected the clinical data and samples and drafted and revised the paper. You-Hua Xu directed the conception and design of the study. Hong-Xia Chen contributed to data analysis. Xiao-Jing Wang conducted studies on cells culture. Xi-Zhou An revised the English writing of the paper. All authors have seen and approved the final paper. This work was supported by translational medicine foundation of Children's Hospital of Chongqing Medical University (no. 7000003).

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Review Article

Stem Cells and Regenerative Medicine: Myth or Reality of the 21th Century

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Since the 1960s and the therapeutic use of hematopoietic stem cells of bone marrow origin, there has been an increasing interest in the study of undifferentiated progenitors that have the ability to proliferate and differentiate into various tissues. Stem cells (SC) with different potency can be isolated and characterised. Despite the promise of embryonic stem cells, in many cases, adult or even fetal stem cells provide a more interesting approach for clinical applications. It is undeniable that mesenchymal stem cells (MSC) from bone marrow, adipose tissue, or Wharton's Jelly are of potential interest for clinical applications in regenerative medicine because they are easily available without ethical problems for their uses. During the last 10 years, these multipotent cells have generated considerable interest and have particularly been shown to escape to allogeneic immune response and be capable of immunomodulatory activity. These properties may be of a great interest for regenerative medicine. Different clinical applications are under study (cardiac insufficiency, atherosclerosis, stroke, bone and cartilage deterioration, diabetes, urology, liver, ophthalmology, and organ's reconstruction). This review focuses mainly on tissue and organ regeneration using SC and in particular MSC.

1. Introduction

Most of human tissues and organs do not regenerate spontaneously, justifying why cell therapy is today a significant tissue and organ repair strategy. The concept of regenerative medicine is an emerging multidisciplinary field to revolutionize the way "*to improve the health and quality of life by restoring, maintaining or enhancing tissue and functions of organs.*"

The history of SC began in the mid nineteenth century with the discovery that some cells could generate other cells. In the beginning of the 20th century, SC were discovered when it was found that the bone marrow contained hematopoietic SC and stromal cells [1, 2]. The first successful transplant was performed by Dr. Thomas in Cooperstown, NY, in the late 1950s. The transplant involved identical twins, one of whom had leukemia, avoiding the problems associated with nontwin transplants, such as graft-versus-host disease [3]. It was not until 1968 that the first successful nontwin (allogeneic) transplant was performed. In this case, the donor was a sibling of the patient [4]. The first successful unrelated donor transplant took place in 1973 in New York when a young boy with a genetic immunodeficiency disorder received multiple marrow transplants from a donor identified as a match through a blood bank in Denmark. The first successful unrelated donor transplant for a patient with leukemia took place in 1979 at the Hutchinson Center. Since then, bone marrow transplantation expanded rapidly during the 1990s [5].

In 1998, cells from the inner cell mass of early embryos were isolated as the first embryonic stem cell lines [6, 7]. Later, in 2006, Takahashi et al. described the IPS (induced pluripotent stem cells) [8–10]. Several categories of stem cells can be used in regenerative medicine including embryonic stem cells (ESC), fetal stem cells (FSC), and adult stem cells (ASC) [11, 12]. Not all stem cells are of equal interest in terms of ability for clinical applications and are able to evolve into different specialized cells. Fetal and adult stem cells are undifferentiated cells, which can be found within fetus or in adult tissues or organs. They are able of limited self-renewal and are multipotent, which means that they can differentiate in several types of tissue cells. Although adult stem cells cannot be expanded in culture indefinitely, the use of these cells does not present ethical problems.

Multipotent SC, self-renewing, and adherent MSC, represent a small fraction of the marrow stroma [13–21]. These nonhematopoietic stromal cells are usually harvested *in vitro* from bone marrow but also from other tissues of mesodermal origin: fetal or neonatal tissues (umbilical cords or placenta), adipose tissue, joint synovium, dental pulp, and so forth [22–30]. MSC are characterized by their capacity of self-renewal and differentiation in different cells types (chondrocytes, endothelial cells,. . .). They were initially identified as progenitors able to produce colonies of fibroblast-like cells (CFU-F for colony forming units-fibroblast), to differentiate into bone or cartilaginous tissues, and to support hematopoiesis. Indeed, MSC cultivated under adapted conditions differentiate into cells of conjunctive tissues: osteoblasts, chondrocytes, tenocytes, adipocytes, and stromal cells supporting the hematopoiesis [31]. They can also differentiate into vascular smooth muscle cells, sarcomere muscular cells (skeletal and cardiac), and endothelial cells [32–36]. Recent publications even state that they can differentiate into nonmesodermal cells such as hepatocytes, neurons, or astrocytes [37–42].

MSC do not have a defined profile of surface antigen expression but there are available markers to identify them. They are mainly characterized by the expression of different antigens, CD105, CD73, CD90, Stro-1, CD49a, CD29, and CD166. On the other hand, MSC do not express antigens CD34 and CD45 (specific of the cells of hematopoietic origin), glycophorin (specific of blood cells), antigens of differentiation of the various leucocyte populations (CD14, CD33, CD3, and CD19), and HLA-DR [43–46]. The International Society for Cellular Therapy suggested a consensual definition: cells must adhere on plastic, express CD75, CD90, and CD105 and not CD34, CD45, HLA-DR, or CD11b, CD19, and are capable of differentiation into chondrocytes, osteoblasts, and adipocytes [26, 47]. Under current conditions of *in vitro* culture [48], the results obtained showed that the proliferation of MSC remained within the limit of Hayflick of 40 *in vitro* population doublings but was affected by the age of the donors [49–54]. Recent studies show that the ability of expansion and differentiation of MSC is donor-dependent. It seems that the number of MSC and their ability of *in vitro* differentiation and tissue regeneration *in vivo* decrease with age and according to the donor pathology [55]. They generally do not circulate in the peripheral blood but are resident in mesenchymal tissues [56]. Bone marrow mesenchymal stem cells (BM-MSC) can provide a support for the growth of the hematopoietic stem cells through the secretion of cytokines and through the creation of cellular interactions either directly (adhesion molecules) or indirectly (production of the extracellular matrix components). Today, nonstandardized protocols exist for their culture, differentiation, and self-renewal ability. In addition, some MSC could be more immature, without any tissue specialization, and their existence has been suspected in human [57–59].

IPS result in the acquisition of a novel state followed by the *in vitro* reprogramming of an adult cell after addition of selected transcription factors. The major advance in this field was performed in 2006 with the possibility of a direct reprogramming of somatic cells into pluripotent cells starting from fibroblasts [8, 9]. Generation of IPS depends on the genes used for the induction (*Oct 3-4* and *Sox* gene family are determinant regulators for the induction process). In the course of the reprogramming, an extinction of the characteristic genes of the fibroblast, a reexpression of embryonic genes (*SSEA 1* and *4*), and activation of telomerase are observed. However, the efficiency of the technique is low. It is likewise necessary to underline that the IPS are exposed to a significant risk of malignant transformation due to the presence of the oncogene c-Myc used in the reprogramming. The present interest of this type of lines and its nonembryonic origin is the possibility of establishing specific lines of deficient patients for clinical research. The IPS are thus a tool for the study of the mechanisms of cell differentiation and genetic diseases and also for pharmacological screening [60].

2. Main Clinical Applications of Stem Cells

The majority of medicine specialities and different applications can benefit in the next decade from the progress in regenerative medicine: most are at experimental stages, with the exception of bone marrow transplantation. Cell therapy covers very large potentials in many clinical fields in cancer and in regenerative medicine [61–63], and more than 3,000 trials with SC are currently in progress (https://www.clinicaltrials.gov/).

Nevertheless, before SC therapeutics can be applied in the clinic, more research is necessary to understand their behaviour upon transplantation as well as the mechanisms of their interaction with the diseased microenvironment. Many authors underlined that regenerative medicine is likely to transform in the future the way we practice medicine, using pharmacological or surgical procedures. The mechanism of action of SC is still being determined. The general consensus today suggests that the most probable mechanism may be through the release of cytokines and other growth-promoting factors.

Before clinical applications, many challenges are to be solved [64].

(i) How to differentiate SC to the desired cell phenotype and which biological and environmental parameters are important during culture for differentiation?

(ii) What are the best suitable cells: which precursors or differentiated cells?

(iii) What are the possible immunological barriers when allogenic cells are used?

(iv) What are the best biomarkers to identify pluripotent/multipotent/precursors cells?

(v) What is the role of the microenvironment (scaffolds, mechanical signals)? [65]

(vi) What are the bioreactive molecules such as cytokines or growth factors that can support the formation of the desired tissue?

(vii) Are there potential karyotype changes during cell culture?

(viii) The translation from laboratory to clinics by using good laboratory practice (GPL) could impact on cell properties?

(ix) Which are the best methods to trace cells *in vivo*?

It is important to note that clinical applications of biotherapies are strongly controlled in Western countries. Harvesting cells or tissues of human origin can only be performed in health centers accredited by Public Authorities (in France, different regulation laws describe the procedure of authorization related to preparation, storage, and clinical use of cells and tissues). The European Regulating Authorities are also very strict about the nature of the clinical trials and about the choice of the patients. Before grafting, different points must be precised.

(i) The severity degree of the pathology has to be considered.

(ii) What type of grafting is planned for the patients?

(iii) The site of grafting should be defined.

(iv) What is the benefit for the patient?

(v) What is the clinical evaluation method to investigate the functionality of the graft?

(vi) Possible side effects.

2.1. Stem Cells and Cancer. Cancer SC has been for long a concept of hematology, particularly in acute myeloid leukaemia. However, more recently, research studies have described the concept of tumor initiating cells in solid tumors [66–72]. The anticancer cell therapy includes bone marrow grafting and in particular the injection of autologous or allogenic hematopoietic stem cells (HSC) CD34+. This population (CD34+) is however heterogeneous regarding its ability to generate the various lines and is the object of many research studies [73]. The graft of HSC has gained an essential place in therapeutic oncohematology [74, 75]. By 1950s, the fundamental role of hematopoietic tissue in protection against radiations was highlighted. The first clinical trials in 1959 showed the feasibility of an engraftment of allogenic marrow [3]. In 1968, the first compatible allogenic grafts HLA were successfully carried out among patients presenting severe combined deficits [4]. Then, the first cryopreserved autografts of bone marrow were reported in lymphomas. Since then, studies were pursued to improve the clinical trials and to decrease, in autologous situations, the relapses linked to the residual disease often present in the graft. Other studies aimed to prevent, in allogenic situations, the graft versus host disease [76].

Using chemical agents or specific monoclonal antibodies, *ex vivo* manipulations of grafts were developed to eliminate tumoral cells or T lymphocytes. By 1984, new sources of HSC have been highlighted in the peripheral and placental blood [77, 78]. That is a major step toward the development of grafts of blood HSC. The first placental blood graft was performed by Gluckman in Paris in 1998 [79]. Since 1993, banks of cryopreserved grafts of placental origin have been developed [80–82].

The use of cytotoxic T cells or NK cells, isolated and amplified *in vitro*, can be proposed for anticancer applications [83–87]. The use of B cells, CD4+ T cells, regulatory T cells [88, 89], and myeloid dendritic or predendritic cells producing interferon is also possible. The injection of dendritic cells for antitumoral immunization, mainly in the residual disease, but also as adjuvant therapy, is the basis of different clinical trials. However, much remains to be understood as the cells nature, their capacity to homing to specific sites (tumor, nodes), and their capacity to stimulate the immune system.

Several clinical trials have been proposed (34 in the beginning of 2014). The main applications are MSC and graft failure, graft versus host disease, and treatment of myelodysplasia [90, 91]. This point will not be developed in this review but a lot of information can be found in the literature [92–94].

2.2. Stem Cells and Tissue Regeneration. Regenerative medicine, based on the graft of tissue native cells (i.e., myocytes, chondrocytes, etc.) or SC able to differentiate into somatic cells, holds great promise if clinical hurdles can be overcome, particularly their possible tumorigenic property. This was highlighted in a case report involving a child who received fetal neural SC as a treatment for a neurodegenerative disease, but who later unfortunately developed multifocal glioneuronal tumor from transplanted neural stem cells [95]. Many studies have been published in this area in the last 20 years [96–100].

The regeneration of damaged tissues or organs implies the existence of cells able to proliferate, differentiate, and give a functional contribution to the regenerative processes. Among the possible middle-term therapeutic applications, cardiac insufficiency, atherosclerosis, osteoarticular diseases, diabetes, and liver diseases can be considered.

In regenerative medicine, four important issues have to be taken into account: (1) the choice of the reparative cells that can form a functional tissue; (2) if necessary, the choice of appropriate scaffolds for transplantation; (3) the role of bioreactive molecules, such as cytokines and growth factors that support the formation of the desired tissue; (4) grafting and safety studies (GMP compliance). More than 3,000 clinical trials are indexed in "https://www.clinicaltrials.gov/" (mainly in USA (25%), Europe (30%), and Asia (40%)), with most of them using MSC.

2.2.1. Heart Disease. Every year in France, 10,000 new cases of serious cardiac insufficiency are detected. Heart transplants

remain the only treatment for the most advanced stages but the shortage of donors and complications of immunosuppression restrict the indications. Surgical remodeling of the left ventricle only deals with the particular anatomical forms and recent negative results have led to a review of the indications. Mechanical ventricular assistance remains a temporary solution for those waiting for a transplant. There is thus a need for new treatment solutions. Xenotransplantation is not progressing since the immunological challenges are considerable and there are major safety considerations. Gene therapy and IPS are still in their infancy [27, 101] and the complexity of the mechanisms involved in heart failure does not lend itself to this therapeutic approach. Finally, cell therapy has a place, but only in patients who retain a sufficient reserve of contractile cells. The numerous trials have not made it possible to reach a conclusion at the present time [102–109].

Today more than 40 clinical trials are listed with a majority of bone marrow, Wharton's jelly and adipose stem cells [110–113]. Histologic observations in autopsy of samples of allogenic cardiac grafts in sex mismatch showed the formation of cardiomyocytes with the receiver genotype in the myocardial tissue coming from the donor [50]. Y genotype cardiomyocytes have been shown in the myocardium of female mice that received an intravenous injection of bone marrow coming from male mice. Isotypic studies showed the homing of progenitor stem cells from bone marrow towards the lesion sites after a coronary ligation. The molecular signals leading to tissue repair are unknown. However some cytokines released during cardiac ischemia could be involved.

The treatment of myocardial infarction (MI), however, is subject to a significant constraint: the immediate availability of cells. The intracoronary injection of stem cells prepared starting from a withdrawal of bone marrow did not lead to significant improvements (3% maximum of the ejection fraction of the left ventricle). In the same manner, the intravenous injection of MSC does not give significant results. In the case of heart failure, the cell therapy turns out to be no efficient and it seems difficult today to envisage a regenerative therapy. At the end of 2007, the US based stem cell company *Osiris Therapeutics* completed a human trial using allogeneic SC for the treatment for heart disease. An intravenous drip was used to deliver of the shelf MSC to patients that had recently suffered a heart attack. No deaths occurred, and the treatment is now widely thought as safe [109].

Today there is no regulatory approved cell treatment for myocardial infarction, but research and clinical studies offer the hope for successful cell therapy in the next decades.

2.2.2. Peripheral Arterial Disease. Lower limb ischemia causes a decreased blood flow in the lower leg with intense pain and swelling [114]. Recently, preliminary results of a Phase I clinical trial using adult SC treatment for severe limb ischemia was presented with endothelial progenitor cells (EPC) and MSC. The cells, obtained by bone marrow aspiration, were mixed and infused into damaged vessels. According to this study, there were no adverse effects as a result of the infusions. More importantly, their patients experienced a progressive and lasting improvement in clinical parameters including walking tests, oxygen pressure, angiography, and quality of life. The use of adult SC therapy in ischemia patients would allow the development of new mature and stable capillaries. These cells have shown the property of differentiation into endothelial or smooth muscle cells but also produce a significant amount of vascular growth factors [115–120].

Harvest Technologies Corp. (MA, USA) presented some positive results from a 30-patient clinical trial of a stem cell-based treatment of critical limb ischemia (CLI). Clinical evaluation of the patients with thromboangiitis obliterans disease conducted for 12 weeks showed that the treatment had significant clinical effect. The most important finding was that more than 85% of patients were able to save their legs. Other major endpoints also showed significant improvement including quality of life assessment and individual perception of pain; there was 100% reduction in the use of pain medications. Limb perfusion, as measured by TcPO2, also showed statistically significant improvement. Thirty-three percent of the patients had serious ulcers and 90% of these showed 90% or better wound closure in 26 weeks. There were no adverse events associated with the treatment.

In 2009 *Pluristem Therapeutics Inc*. (Haifa, Israel) began a Phase I clinical trial with a placenta-derived SC product for the treatment of CLI (end-stage of peripheral artery disease). The different trials (12 patients) generally evaluated the safety of the product in patients with CLI.

2.2.3. Ischemia Stroke. Cerebral infarct is a process, in which brain damage increases with time. Therefore the time when treatment is started is critical. At present, the only effective treatment (tissue plasminogen activator) has to be administrated very soon after the stroke [114]. In animal models, intravenous administration of hUCB cells to rats, after induction of stroke by occlusion of the middle cerebral artery, promoted the improvement of neurological function. The cells were mainly found in the cortex and the striatum of the damaged hemisphere and outside the brain, in bone marrow and spleen, and in very small amounts in muscle, heart, lungs, and liver. These authors found that some of the injected cells showed neuronal markers (NeuN2 and MAP2), astrocytic markers (GFAP), and endothelial cell markers (FVIII) [121].

Actually, IPS cells should be ideally generated without using viral vectors and without teratoma formation for being suitable for clinical use. But today the main clinical trials in topic use autologous bone marrow MSC [122–124].

2.2.4. Nervous System and Neurodegenerative Diseases. The classical notion of a renewal of adult neuronal cells is today questioned, but the therapeutic applications still remain uncertain. The spinal cord repair is currently the purpose of a great deal of work after injury [125, 126] (10 trials) with bone marrow SC. In 2010, a first study from cells derived from embryo SC producing oligodendrocytes was carried out in a volunteer in connection with the *GERON Company* [127, 128]. So far, no information has been given on the result of the test and the study seems to have been stopped. In France, one group is involved in a clinical study, in cooperation with

a German team, using autologous bone marrow SC. Other studies should also begin in USA, Portugal, and China.

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease characterised by a progressive muscle weakness that can result in paralysis and death. The multicausality of neuron death poses a considerable problem to the development of new therapeutic strategies, including cell therapy. Numerous hypotheses have been developed about the origin of ALS, but it seems that the immune system may be involved. Cell transplantation approaches in ALS remain to generate a neuroprotective environment for degenerating motor neurons by transplantation of nonneuronal cells, rather than to replace lost motor neurons. Among the cell therapy approaches tested in motor neuron disease animal models, systemic injection of human cord blood mononuclear cells has proven to reproducibly increase the life span of SOD1G93A mice, a model of familial ALS, even if only few transplanted cells were found in the damaged areas. Bigini et al. showed that human cord blood (mononuclear cells) significantly enhanced symptoms progression and prolonged survival in SOD1G93A mice and were localized in the lateral ventricles, even 4 months after administration [129]. However, hCB-MNCs were not found in the spinal cord. These results strengthen the hypothesis that the beneficial role of transplanted cells is not due to cell replacement but is rather associated with the production and release of circulating protective factors. They observed in this study that hCB-MNCs release a series of cytokines and chemokines with anti-inflammatory properties that could be responsible of the functional improvement of mouse models of motor neuron degenerative disorders. The clinical trials (25 beginning of 2014) are mainly focused on Parkinson [130] and Alzheimer [131] diseases and amyotrophic lateral sclerosis [132] with bone marrow and umbilical cord SC.

2.2.5. Bone and Cartilage [133–135]. The prevalence of osteoarthritis and degenerative joint disease will increase in the near future, driving the market for SC therapies for joints and cartilage [136]. In fact, most of the pioneering work using stem cells for bone and tendon repair was carried out in the veterinary field, mainly injuries in racehorses. Adult MSC are able to differentiate in bone, cartilage, tendon, ligament, and muscle. Today the most studied source for bone and cartilage is the bone marrow with different scaffolds. For bone the problem is more complex, when compared to cartilage, because bone is a vascularized tissue and the formation of mineralized bone matrix is not sufficient to lead to a functional tissue [137–139].

Cartilage is a mesenchymal tissue composed of one cell-type (chondrocytes), extracellular matrix (ECM), and water. Chondrocytes represent only 1-2% of cartilage volume. The cartilage ECM is composed of collagen fibers (mainly type II collagen) supporting glycoproteins and proteoglycans which have a protein core associated with glycosaminoglycan molecules such as hyaluronic acid (HA) and chondroitin sulfate. The tissue fluid, mainly water, contributes to the particular mechanical properties of cartilage and provides nutrition and exchange with synovial fluid and with extracellular fluid or other adjacent tissues. Hyaline cartilage functions with minimal friction. It demonstrates an excellent ability to provide and adaptation to compression and distributes loads on the surface of the joint [140, 141].

Because of the limited self-healing capacity of cartilage, repair of articular defects caused by degenerative joint diseases or traumatic injuries represents an open challenge and current therapies for cartilage repair are inadequate for restoring form and function [142–144]. *In vitro* preparation of functionally developed biocartilage substitutes is an attractive concept for future clinical treatments of cartilage injuries and degeneration. Today a FDA approved cellular-based therapy for cartilage defects uses chondrocytes [145]. In this application, autologous cells are harvested from a biopsy and expanded *ex vivo* to obtain a large number of cells for transplantation. Autologous expanded chondrocytes have a low risk of immune rejection but they have a tendency to dedifferentiate (loss of phenotype) *in vitro*. In other words, the influence of mechanical forces on cell function *in vitro* has been demonstrated for engineering cartilage and bones [146]. In cartilage production, dynamic mechanical stresses on chondrocytes and MSC promote differentiation and increase matrix production [147–149].

Many clinical studies performed have demonstrated the therapeutic potentials of MSC from bone marrow or adipocytes [150–153]. Wharton's jelly MSC offer another source that has proven a chondrogenic differentiation potential and could be used in an allogenic context [46, 154–156].

While MSC therapy is promising, the incomplete understanding of their biological characteristics and function limits today the utilization of MSC in clinical application [157]. Role of growth factors, cytokines, receptors, transmembrane signalling, and adhesive proteins in MSC interactions are still elusive. In a study of the effects of MSC in a caprine model of traumatic osteoarthritis it was showed that intra-articular delivery of autologous cells to meniscectomized joints resulted in significant meniscal and regeneration and chondroprotection. In another study, subcutaneous implantation of hydroxyapatite scaffolds loaded with allogenic MSC allows cartilage obtention. The present clinical trials for cartilage repair are mainly focused on osteoarthritis 29 trials with auto- or allo-SC from bone marrow, umbilical cord, or adipose tissues [158–160].

2.2.6. Dermatology. In mammals, cell renewal on the external surface of the skin is ensured by the keratinocytes of the basement layer which divide actively and are differentiated into cells of the stratum corneum. That activity implies the existence of SC. Unlike the SC of hair follicles confined in a niche, the SC of the *epidermidis* are spread along the basement membrane. The main clinical trials [10] are mainly on limb ischemia in diabetic patients [161, 162].

2.2.7. Pancreas and Diabetes [163]. The cell graft appears to be an alternative to the medical treatments for pancreas diseases. The first attempt of cell therapy by grafting of islets of Langerhans was published more than ten years

ago [164–167]. Other cell sources have also been proposed for pancreas and diabetes cell therapy like adult [168, 169] or fetal [170–172] MSC, embryonic stem cells [173–175], or even IPS [176, 177] because of their differentiation potential into insulin-producing cells and their immunomodulatory properties. Two studies with different therapeutic approaches are currently investigating the influence of cord blood stem cells on improving the function of pancreatic beta cells. In the first approach, children with young-onset diabetes are infused with autologous cord blood without chemotherapy. Initial results have shown that such autologous cord blood transplantation without chemotherapy has not resulted in adverse effects but has not significantly improved the situation either. All children are still dependent on administration of insulin. In the second approach, adult patients with newly diagnosed diabetes mellitus underwent nonmyeloablative chemotherapy after receiving reinfused stem cells from autologous bone marrow. Different trials on diabetes [22] type 1 or 2 are mainly performed with autologous or allogenic bone marrow or Wharton's jelly MSC [178–181].

2.2.8. Liver Diseases. In response to a variety of chronic injuries such as hepatitis, alcohol or drug abuse, metabolic diseases, autoimmune attack of hepatocytes or the bile duct epithelium, and congenital abnormalities, liver fibrosis occurs and finally leads to hepatic cirrhosis and liver failure. Liver transplantation is the accepted treatment option for this end-stage liver diseases and acute liver failure resulting in irreversible liver dysfunction. However, it is limited by the shortage of donor organs. Moreover, it is difficult to accept such a heavy surgical treatment for some patients because of the shortage of donor organs. In fact, correction of hepatocyte functional deficiency is the prime goal of liver transplantation. There is growing evidence in support of cell therapy. As an alternative to liver transplantation, some authors tried to use hepatocytes to treat patients with liver diseases instead of liver transplantation. However, the obstacle against their clinical applications is the requirement of large number of hepatocytes that are not available from patients themselves and as well as from other donors either. Thus, it is necessary to search for a novel source of cells. SC therapy has been accepted as one of the new approaches to recolonize liver. Several studies reported the hepatocyte differentiation potential of embryonic, fetal, or adult MSC but also IPS [182–187].

As the liver contains three different cell types, which are organized in three-dimensional structures, growth and regeneration processes are highly complex. Therefore the idea of using one-type of SC leading to these three types of cells to repair liver is acceptable. Various populations of SC are under investigation in terms of their regenerative capabilities. Recently, studies showed that extrahepatic adult MSC of different origins have demonstrated their ability to express a hepatocyte-like phenotype after being differentiated *in vitro*. These cells which include MSC derived from bone marrow, umbilical cord, adipose tissue, and placenta are used in 32 trials mainly for cirrhosis (after hepatitis, alcohol abuse, and liver transplantation) [188–191].

2.2.9. Urology and Erectile Dysfunction. The group of Atala in USA performed urethra transplant in young patients, prepared *in vitro* from bladder cells cultivated on a collagen and polyglycol acid matrix [192].

Recently, the SC therapy for erectile dysfunction has been investigated. Transplantation of SC (adipose-derived stem cells or bone marrow stem cells...) was performed by intracavernous injection [193, 194]. More recent studies used combinatory therapy by supplementing stem cells with angiogenic proteins. The different studies reported better erectile function after SC mainly by intracavernous injection [195].

The main potential applications are postprostatectomy and postradiotherapy, diabetes associated erectile dysfunction, and Peyronie's disease [196]. Human clinical trial of erectile dysfunction with SC is not yet approved as treatment but one clinical trial in Korea was published in 2010 and two preclinical trials have been approved in USA and France.

2.2.10. Retina. The different ophthalmologic treatments with SC (mainly bone marrow) are related to retina diseases, macular degeneration, glaucoma, and hereditary dystrophy [197–199].

2.2.11. Hematology: Preparation of Red Blood Cells. In vivo production of red blood cells (RBC) can be of a great practical interest. Recently, RBC preparation has been possible *in vitro* based on CD34+ stem cells [200, 201]. The protocol is in three steps: (1) proliferation and induction of the erythroid differentiation, (2) culture on a model reproducing the physiological microenvironment with mesenchymal cells, and (3) culture in the presence of stromal cells alone and without any growth factor. With this protocol, the cells undergo the various phases of differentiation to red cells. The industrial development would require developing suitable bioreactors. Another solution would be to have a permanent and unlimited supply of blood products. A first option is to use embryonic SC whose differentiation gives: first CD34+ stem cells, then erythrocytes [202]. Another approach consists in using induced pluripotent SC or IPS [203]. Different lineages of IPS and/or embryonic SC are currently used experimentally; beyond the difficulties in controlling complete differentiation, one major issue to be solved is that of insufficient yields [204].

2.3. Stem Cells and Whole Organ Engineering. The relevance of research into the creation of reconstructed organs is justified by the lack of organs available for transplantation and the growing needs for an ageing population. On a technical level, the development of these reconstructed organs involves two complementary stages: decellularization of the target organ with a need to maintain the structural integrity of the extracellular matrix and recellularization of the matrix with stem cells or resident cells [205, 206].

Whole organ engineering like liver, kidneys, heart, or lung is particularly difficult because of the structural complexity and heterogeneity of organ and cell types. But new ways of researches are currently focused on the matrix to support recellularization and a promising approach is the direct use of

extracellular matrix of the whole organ. Thus rodent, porcine, and rhesus monkey organs have been decellularized to obtain a scaffold with preserved extracellular matrix and vascular network.

Decellularization can be achieved through an intra-arterial infusion of a solution containing the detergent Triton X-100 and ammonium hydroxide. This method causes all the cellular elements to disappear, leaving elements of the extracellular matrix and the vascular system. Other methods of decellularization have also been used, employing other chemicals, enzymes, or physical ways (ultrasounds) [207, 208].

Several types of cell can be considered for recellularization purposes: SC (embryonic, fetal, and adult SC) or the patient's autologous cells. SC probably represent the ideal source of material due to their ability to proliferate. Their use appears to be limited, nevertheless, by their allogenic nature, which could possibly trigger an immune response and consequent rejection, in addition to the risk with ESC of the formation of teratomas *in vivo*. Fetal cells conserve their ability to proliferate and are easily differentiated without running the risk of induction of teratomas *in vivo*. These obstacles could be removed in future by using nuclear transfer techniques from the patient's somatic cells (IPS). Finally, the stem or progenitor cells present in most organs are another source of cells that could be used for *in vitro* organogenesis. But, they often remain difficult to define, isolate, and grow in culture.

Furthermore, the type and number of cells to be used for recellularization vary depending on the organ to be reconstructed. Apparently, specific cells of the organ to be reconstructed are indispensable. Other types of cell, such as endothelial cells and fibroblasts, are also needed, since they promote the functional cell phenotype and contribute to the structural organization of tissue. The matrix of the vascular system of the organ to be reconstructed needs to be reendothelialized so as to orientate the blood flow and prevent thrombosis.

Currently, growing organs *in vitro* and *ex vivo* can take several weeks until they have completely developed in the matrix. For seeding the use of an extracorporeal pulsating or continuous infusion system (bioreactor) is indispensable for providing the cells with an oxygen supply and keeping the infusate at a constant temperature [209, 210]. The infusion liquids are derived from the culture media used for the cells in question. They need to contain growth factors or other molecules that are more specific to each organ. Finally, there is another hypothetical possibility for recellularization, the transplanting of a decellularized organ into the recipient, in the hope that recellularization will occur directly from the recipient's own cells.

Encouraging work has recently shown the feasibility of creating bioorgans for the reconstruction of heart, lungs, liver, and kidneys. Clinical applications still remain a distant prospect, however.

2.3.1. Heart. Heart construction could be an alternate option for the treatment of cardiac insufficiency in the future. It is based on the use of an extra-cellular matrix coming from an animal's heart and seeded with cells likely to reconstruct a normal cardiac function. Though the decellularization techniques now seem to be under control, the issues posed by the selection of the cells capable of generating the various components of cardiac tissue are not settled yet. In addition, the recolonization of the matrix does not only depend on the phenotype of cells that are used but also impacted by the nature of biochemical signals emitted. The complexity of those problems results in the full replacement of the heart with a biomaterial substitutes to standard transplanting is one prospect [211]. However, it is more realistic to hope, in the medium run, partial replacements of the heart with recellularized matrices reinforcing portions of the failing myocardium or with direct cellular therapy with SC.

The decellularization of animal hearts (rats and, more recently, hearts of large mammals) has been performed by D. Taylor through the infusion of chemical detergents. This study shows that the integrity of the matrix (collagen, fibronectin, laminin, fiber orientation, etc.) can be maintained as well as the permeability of the vascular tree and the competence of the heart valves [212].

Recellularization is more difficult due to the diversity of the cell populations that need to be reconstituted. Three ways to achieve this goal can be considered. (i) Use of a single population of pluripotent cells is capable of giving rise to all types of heart cells through the effect of environmental signals (an approach that appears currently to be rather unrealistic). (ii) Use of adult cells already differentiated for target lineages. The obtaining of fibroblasts and vascular cells can be achieved, especially as they can be taken from a future "recipient" of the reconstituted organ, as has been successfully demonstrated in the creation of implantable blood vessels. (iii) The third intermediate strategy consists of using a single population of progenitor cells at the mesodermic stage that would be liable, depending on the signals produced by the host tissue, to achieve differentiation *in situ* in the three main cell types (cardiomyocytes, endothelial cells, and smooth muscle cells). The problem of obtaining cardiogenic cells is also more complex since they not only need intrinsically contractile properties, but they must also be capable of coupling and modulating their frequency in response to neurohumoral or pharmacological stimuli. The plasticity of adult somatic cells is limited; however, it does not allow them to differentiate into cardiomyocytes. This property is only possessed by pluripotent cells, capable of acquiring a cardiac phenotype under the influence of the appropriate signal inducers. Such pluripotent cells could be human embryonic stem cells (hESC) whose allogenic character poses the problem of rejection (to say nothing of the ethics debate) or autologous, adult somatic SC rendered pluripotent through reprogramming (IPS). Regardless of the origin of such pluripotent cells, however, their clinical use implies an *in vitro* differentiation stage and then a selection process so that only the cardiogenic progenitors would be used. More recently, a direct conversion of the adult cells (fibroblasts) into cardiomyocytes has been proposed, again passing through the pluripotent cell stage. This approach still seems to be remote for clinical applications [213].

An important challenge is the transfer of cells into the matrix to recolonized [214]. While cell infusion destined for

the vascular system appears to be logical for the endothelium, intramural injection of cells for cardiogenic purposes is less obvious.

In summary, by the complete replacement of a human heart by another heart constituted from a matrix of animal origin and seeded by cells capable of providing the organ with effective, mechanical activity remains a remote prospect and is unlikely to become a reality within the next 10 to 20 years.

Another strategy for cardiac repair is the preparation of cardiac patch [215, 216]. The construction of the high biocompatible biomaterials pretreated with SC will offer a promising method to improve the effects of SC therapy for myocardial infarction. Thus the development of this cardiac SC patch has high therapeutic perspectives for the treatment of the disease and prevention of the chronic heart failure. However the materials suitable for the treatment of MI need to have specific quality: biocompatibility, resistant to the mechanical force *in situ*, suitable for the cell amplification, and being with suitable size of pores for the cell communication which is necessary for the formation of the functional tissue. Under microscope, the pore size needs to be at least 50 μm which is necessary for the vascularization of the patch and assure the MSC metabolism. The biological materials have more advantages than artificial materials because the integration of the cells is optimal for the construction of the cardiac SC patch. As the MSC derived from Wharton's jelly are easy to collect, the umbilical artery can be collected at the same time. The natural matrix of the umbilical artery possesses the essential property for the construction of a biocompatible cardiac patch.

2.3.2. Lungs. About fifty million people throughout the world are living with chronic respiratory failure at a terminal stage. The only treatment for this disease that seriously reduces life expectancy is, in selected cases, lung transplantation, but the results still are poor.

A tracheobronchial graft remains a challenge [217–221]. Research has not yet found an ideal cell substitute for the airways of the lung. Failures have been observed with synthetic prostheses, bioprostheses, tracheal allografts, and autografts. In fact, not only epithelial tissue regeneration but also even cartilaginous regeneration has been observed. Research seems to indicate that this regeneration of tracheal tissue might be possible from an aortic matrix and SC taken from bone marrow [217]. Studies have been performed in humans in the context of extended cancer of the trachea and conservation surgery in cases of lung cancer. The research has also contributed to better understand tissue regeneration mechanisms [222].

Pulmonary regeneration using SC is more complex [223]. In fact, several types of local progenitor cells that contribute to cell repair have been described at different levels of the respiratory tract. Moving towards the alveolus, one finds bronchioloalveolar SC as well as epithelial cells and pneumocytes. In the category of "local SC," cells of the subpopulation have been identified that are differentiation markers which *in vitro* mimic stromal mesenchymal cells. The role of these cells in tissue repair has been demonstrated in animal models. Recently it was described that resident, multipotent pulmonary SC are capable of self-renewal as well as clonogenicity. The phenotype and functional characteristics of these new cells have been specified *in vitro* and *in vivo*.

The lung also contains resident specific MSC that have been described and characterised [224–226]. These cells do not play a direct part in epithelial renewal but establish communication with the epithelium, thus ensuring their role as a local cytoprotector [227].

Finally, numerous studies performed on animals have shown a beneficial role played by exogenous MSC produced by bone marrow. The effects observed in lesional pulmonary edema, sepsis, pulmonary hypertension, and even idiopathic pulmonary fibrosis have resulted in clinical applications that are currently being assessed [228–231]. The immunomodulatory, anti-inflammatory, antiapoptotic, and angiogenic properties of MSC today place these cells at the heart of tissue repair. Contrary to past hypotheses, these cells do not seem to differentiate themselves into alveolar epithelial cells and their method of action would involve paracrine mechanisms, not all of which have as yet been explained.

With respect to the creation of a bioartificial lung,recent works on the subject have been realized with decellularized rat lung in order to obtain a supporting matrix [232]. Epithelial and endothelial cells were then injected into a pulmonary matrix followed by a five-day incubation period in a bioreactor. Morphological studies found an aspect closely resembling the animal's own lung with respect to the alveolar cells (volume, number, and size) and *in vitro* physiological studies also showed that the ventilatory capacities and gas exchanges had also been maintained. An *in vivo* implantation of the bioartificial lung produced spontaneous ventilation for six hours. After this, pulmonary edema occurred. Several research routes, such as improvement in differentiation and maturation of the injected cells, a longer incubation period in the bioreactor and optimization of postoperative ventilation have been proposed.

Recently, others authors also decellularized a rat lung using chemical treatment, retaining only the framework matrix of the lung. This decellularized lung was then placed in a bioreactor that was used to mime the physiological conditions (negative pressure and pulsatile vascular perfusion). Epithelial cells from new-born rats were injected through the trachea and endothelial cells were injected into the vascular system. After four to eight days of incubation, this biolung was grafted on to a rat [221]. The compliance measurements were substantially different between the native lung, the decellularized lung, and the lung produced by bioengineering, with greater opening pressures reflecting a less functional surfactant in the bioengineered lung. Yet there was nothing to indicate rigidity of the matrix, thus ruling out the development of fibrosis, and gaseous exchanges were covered, attesting to the functional nature of this lung.

The decellularization of lungs was then reproduced on the lungs of pigs, nonhuman primates, and even humans [233–235]. Embryonic SC or MSC were used to cellularize decellularized lungs [235–237].

This initial research opens up a promising route for developing a functional bioartificial lung, with the prospect

of application to humans within 15 to 20 years [238]. However many questions remain to be answered: is the use of a decellularized pulmonary matrix the only possible solution? Which cells should be chosen for recellularization, MSC or resident pulmonary cells? What is the optimal incubation time in a bioreactor? Would the technique be applicable to the human lung with its very extensive alveolar surface?

2.3.3. Liver. Recent researches have shown that it was possible to use decellularized liver treated by detergents as scaffold, which keeps the entire network of blood vessels and the ECM [239]. The intact blood vessel network will mimic the circulation in organ and provide appropriate oxygen and nutrient supply for bioartificial liver [240]. The ECM is composed of a complex mixture of molecules and arranged in three-dimensional spatial organization that support the cell seeding, growth, and differentiation. Decellularized liver keeps the texture of the original organ. This natural structure can provide a three-dimensional matrix in favor of cell proliferation, differentiation, and function, which promotes the emergence of the idea to use decellularized organ in bioengineering liver. The liver decellularization is carried out by perfusing detergents like Triton X-100 or sodium dodecyl sulfate, *via* the portal vein [241]. This method can destroy cell membrane and take off debris of cells and at the same time keeps the extracellular matric complete with blood and biliary vessels. This matrix maintains the liver-specific proteins proportions for collagens I and IV, fibronectin, and laminin. The intact vascular system is useful for recellularization.

Besides decellularized whole organ scaffold, the choice of cells selected to repopulate a decellularized liver scaffold is critical to the function of bioengineered liver. At present, potential cell sources are hepatocyte and MSC. SC, such as liver stem cells, ESC, IPSCs, and MSC, are a promising alternative for primary hepatocytes. Recent studies have shown that MSC originated from extrahepatic tissues can differentiate into endoderm cell-lines as hepatocytes. Several methods have successfully differentiated MSC into hepatocytes, such as stimulation MSC by cytokines as growth factors direct and indirect coculture of MSC with hepatocytes, or promotion of MSC differentiation in a three-dimensional matrix. In some cases, differentiation of MSC into hepatocytes can also be an alternative approach for whole organ transplantation in treatment of acute and chronic liver diseases [183]. Moreover, it has been shown that decellularized liver scaffold supports hepatic differentiation of MSC, leading to cells with morphological and functional characteristics of mature hepatocytes [242, 243].

2.3.4. Kidney [244–246]. The kidney is certainly one of the most difficult organs to reconstruct due to its tissue complexity and the heterogeneous nature of the cells from which it is constituted. There is relatively few researches on kidney autoconstruction, though experiments performed on rats, pigs, and monkeys [247]. The first demonstration of the feasibility of the technique was provided by Ross in the rat [248]. They seeded the decellularized organ with pluripotent murine embryonic stem cells antegrade through the artery or retrograde through the ureter. The cells introduced were differentiated into glomerular, tubular, and vascular structures. They nevertheless lost their embryonic phenotype as it could be seen from the appearance of immunohistochemical markers. Nakayama et al. [249] decellularized sections of the kidneys taken from macaques at various growth stages from fetus to adult, *via* intermediate ages, with the aim of optimizing decellularization techniques and recellularization *in vitro* [249, 250]. They demonstrated that the appearance of Pax-2 and vimentin markers after the cells had been implanted originated from the kidneys of the fetus.

As with other organs, the research into the construction of a kidney raises numerous questions about the preparation of a matrix and the sources of the cells used for recellularization. Biological matrices have proved their superiority over the synthetic matrices sometimes used in tissue engineering. In the case of the kidney, the most frequently employed matrices are allogenic, even though xenogeneic matrices can be considered, although they might be subject to specific immunological and regulatory issues.

In summary, a large number of questions and problems remain to be solved before a kidney can be prepared or constructed from ECM. Furthermore, none of the "self-constructed" organs in animals has proved to be capable of performing the vital function in the recipient for longer than a few hours. In the case of the kidney, no transplant has yet been reported even though it is the main challenge for research. The objective remains plausible, however, even if clinical applications appear to be very remote, certainly not before 15 to 20 years.

3. MSC Secretome for Tissue Repair: Towards a Cell-Free Therapy

Even if initially MSC were proposed for cell therapy based on their differentiation potential, the lack of correlation between functional improvement and cell engraftment or differentiation at the site of injury has led to the proposal that MSC exert their effects not through their differentiation potential but through their secreted product [251, 252]. The secretion of bioactive factors is then thought to play a predominant role in the mechanisms of action of MSC. Haynesworth et al. [253] were the first to report that MSC synthesize and secrete a broad spectrum of growth factors, chemokines, and cytokines that could exert significant effects on cells in their vicinity. Since that, many researches have been focused on the characterization of the MSC secretome, including both soluble factors and factors released in extracellular vesicles (e.g., exosomes and microvesicles) and their therapeutic potential [254–256].

The results from most investigations show that MSC-conditioned medium or its components mediate some biological functions of MSC. Several studies have reported that MSC-derived exosomes have functions similar to those of MSC, such as repairing tissue damage, suppressing inflammatory responses, modulating the immune system, or even decreasing cancer cells proliferation [257–264].

Together these studies provided pivotal support for the paracrine hypothesis such that MSC therapy is increasingly rationalized on MSC secretion rather than its differentiation potential. However, the mechanisms are still not fully understood and the results remain controversial. Compared with cells, exosomes are more stable and reservable, have no risk of aneuploidy, a lower possibility of immune rejection following *in vivo* allogeneic administration, and may provide an alternative therapy for various diseases.

4. Conclusions

The regeneration of tissues and organs and the use of SC for clinical uses are and will remain a challenge for the development of cell therapy and tissue engineering. Fetal and adult SC and in particular MSC provide exciting therapeutic tools of regenerative medicine. However basic research should be developed to better understand the biological process and molecular mechanism of SC differentiation, as well as the role of the mechanical signals.

Several challenges should be overcome:

(i) increase of the yield of preparation of the differentiated stem cells and study of the heterogeneous character of the preparations;

(ii) possibility to have a standardized and reproducible product (preparation of controlled batches);

(iii) technical problems regarding the definition of scaffolds, cells used, long-term stability, and culture medium. In particular, the impact of the biomaterial used remains to be defined;

(iv) grafting (biotissue can be introduced via direct cell implantation (cell therapy), biotissue transplantation, or gene therapy);

(v) risk of teratogenic effect and of immune reaction (i.e., in the umbilical cord cells the immune risk being weaker);

(vi) religious and legal issues with respect to the different country regulations.

Current knowledge allows optimism for the future but definitive answers can only be given after long-term randomized and controlled clinical trials.

Conflict of Interests

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

Authors' Contribution

J.-F. Stoltz and N. de Isla contributed equally.

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Review Article

Cell Therapy in Patients with Critical Limb Ischemia

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Critical limb ischemia (CLI) represents the most advanced stage of peripheral arterial obstructive disease (PAOD) with a severe obstruction of the arteries which markedly reduces blood flow to the extremities and has progressed to the point of severe rest pain and/or even tissue loss. Recent therapeutic strategies have focused on restoring this balance in favor of tissue survival using exogenous molecular and cellular agents to promote regeneration of the vasculature. These are based on stimulation of angiogenesis by extracellular and cellular components. This review article carries out a systematic analysis of the most recent scientific literature on the application of stem cells in patients with CLI. The results obtained from the detailed analysis of the recent literature data have confirmed the beneficial role of cell therapy in reducing the rate of major amputations in patients with CLI and improving their quality of life.

1. Introduction

Critical limb ischemia (CLI) is an important condition in the general population with a strong social impact [1]; the prevalence of CLI in the population aged 60–90 years is estimated as 1% (0.5–1.2%) [1, 2] with male to female ratio around 3 : 1 and 5–10% of patients with asymptomatic peripheral arterial obstructive disease (PAOD) or claudication will progress to CLI at 5 years from the first diagnosis. Several studies have shown that over 50% of CLI patients do not have any PAOD symptoms 6 months prior to the onset of CLI [3]. The major risk factors for PAOD include smoking, hyperlipidemia, hypertension, and—for development of CLI—diabetes. Diabetic patients are, at least, fivefold more likely to develop CLI than nondiabetic patients.

CLI is the end stage of PAOD and the macrovascular lesions induce a reduction of distal perfusion. Nutrient blood flow to the tissues and microcirculation exchange are severely altered [4].

Strategies to treat CLI and its related symptoms include both pharmacologic therapy and invasive procedures [5]; however, about 25% of patients still progress each year to limb amputations [6]. Pathophysiologically, chronic ischemia exceeds tissue capacity for oxygen diffusion and nutrients from peri-ischemic territories, as well as for endogenous remodeling. Recent therapeutic strategies have focused on restoring this balance in favor of tissue survival using exogenous molecular and cellular agents to promote regeneration of the vasculature: these are based on stimulation of angiogenesis by extracellular and cellular components [7–9]. Several studies have shown that bone marrow-derived endothelial and hematopoietic progenitors may restore tissue vascularization after ischemic events in limbs, retina, and myocardium [10–14]. Dysfunction in the vascular bed in ischemic conditions, attrition of the microvasculature, and the difficulty or impossibility to adapt to the need for increased blood flow are the critical points through which we investigate cellular mediators and tissue-specific

chemokines, which facilitate selective recruitment of bone marrow-derived stem and progenitor cells to specific organs and the factors that promote differentiation of the progenitor cells [15, 16]. The different families of chemokines are determined by the numbers and spacing of cysteine residues adjacent to the amino terminus: CC, CXC, CX3C, and XC. The CC chemokines primarily attract mononuclear cells, including monocytes, eosinophils, basophils, dendritic cells, and T lymphocytes. CXC chemokines primarily attract neutrophils (CXCL1–3 and CXCL5–8) or lymphocytes (CXCL4 and CXCL9–16). Peripheral blood monocytes express CCR1, CCR2, CCR3, CCR5, and CXCR4. Evidences show that a large cohort of chemokines affects monocytes/macrophage recruitment and consequently influences arteriogenesis and response of tissues to ischemia.

The principle that characterizes the therapeutic application of stem cells is the restoration of vascular cellularity, the control and the support of the newly formed vessels which must ensure an adequate supply of oxygen in critical ischemic areas. Thus, oxygen tension plays several roles in the expression of different genes such as the vascular endothelial growth factor (VEGF) family and proangiogenic growth factor.

The aim of this study is to perform a systematic analysis of the most recent scientific literature on the application of stem cells in patients with CLI of different etiologies.

2. Material and Methods

PubMed, Scopus, and ScienceDirect databases were searched for articles using the terms: Peripheral Arterial Obstructive Disease, Critical Limb Ischemia, Stem Cells Therapy, Angiogenesis and Limb Loss.

Only publications in English were included. Titles and abstracts were screened by 1 author (B. L.) to identify potentially relevant studies. All potentially eligible studies were subsequently evaluated in detail by 1 reviewer (B. L.) through consideration of the full text. Reference lists of retrieved articles were also searched for relevant publications.

Inclusion required clinical trials in which therapy with stem cells in CLI patients was performed. Studies were excluded if not performed in English language, if performed in animals or in vitro, if the cohort was defined by the presence of CLI and an additional confounding disease process (e.g., chronic renal failure or cerebrovascular diseases), or if CLI specific results could not be distinguished from those of a larger population consisting of individuals without CLI. Studies were excluded when the primary focus was carotid artery disease, aortic aneurysmal disease, intracranial vascular disease, inflammatory diseases, cancer, nonvascular diseases, and treatment with chemotherapy.

3. Results

Study Selection. The initial database searches yielded 68587 studies from PubMed, 526 from ScienceDirect, and 1 from Scopus in the last 5 years. We evaluated 1031 eligible full text articles (Figure 1).

The biology and physiology of stem cells and their differentiation in vascular cells, the current methods of sampling of stem cells found in literature, the relationship with clinical and adverse effects in treated patients, and the description of the indications to the stem cell therapy in patients with CLI are given below.

3.1. Biology of Vascular Stem Cells. Embryonic stem cells (ESCs) have the competency to self-renew indefinitely while maintaining the potential to give rise to all cell types in the human body; the first human cell line was generated in 1998 by Thompson et al. [17]. Many studies were made to clarify the physiology of stem cells, the stage specific embryonic antigens, and the several factors which maintain "stemness" [18–25]. During embryogenesis, the inner cell mass (ICM), the internal cell component of the blastocyst, gives rise to the primitive endoderm and epiblast, which consists of three primary germ layers: ectoderm, mesoderm, and endoderm. Vascular cells including endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) are predominantly descendants of mesodermal cells; however, an ectoderm origin for VSMCs was detected [26, 27]. The differentiation of mesoderm in vascular cells is regulated by important factors in a complex process with a fine regulation: Brachyury, a transcription factor required for posterior mesoderm formation and differentiation and then downregulated when cells undergo specific development into mesoderm-derived tissues, including cardiac muscle, endothelium, and blood cells [28, 29], bone morphogenetic protein (BMP), a member of the transforming growth factor- (TGF-) β superfamily [30, 31], MIXL1, a homeobox gene involved in hematopoietic specification [32, 33], Nodal [34, 35], CD31 [34], [36], CD34 [37, 38], Sca-1 [39, 40], N-cadherin [41, 42], platelet-derived growth factor receptor- (PDGFR-) α [43, 44], and vascular endothelial growth factor receptor- (VEGFR-) 2 [45, 46].

Blood vessels arise from endothelial precursors through a process known as developmental vasculogenesis [47, 48]: resulting capillaries are small and cannot sufficiently compensate for a large occluded transport artery due to Hagen-Poiseuille law [49, 50]. Arteriogenesis, also called collateral growth, is the transformation of preexistent collateral arterioles into functional collateral arteries: evidences have shown that human bone marrow-derived stromal cells promote arteriogenesis through paracrine mechanisms [51, 52].

Studies showed that ischemia induces plasma elevation of stem and progenitor cell-active cytokines, including soluble kit-ligand (sKitL) and thrombopoietin, progenitor-active cytokines such as granulocyte-macrophage colony-stimulating factor (GM-CSF), and erythropoietin. Thrombopoietin and sKitL may release stromal-derived factor-1 (SDF-1) from platelets accelerating revascularization of the ischemic limbs through mobilization of hemangiocytes [50, 53, 54]. Hemangiocytes induce neovascularization by releasing angiogenic factors and by physically supporting the assembly of endothelial cells. The risk factors due to insufficient collateralization (diabetes, smoking, hyperlipidemia, and advanced age) are the same for a lower number of circulating, monocytic progenitor cells (MPCs) [55–57].

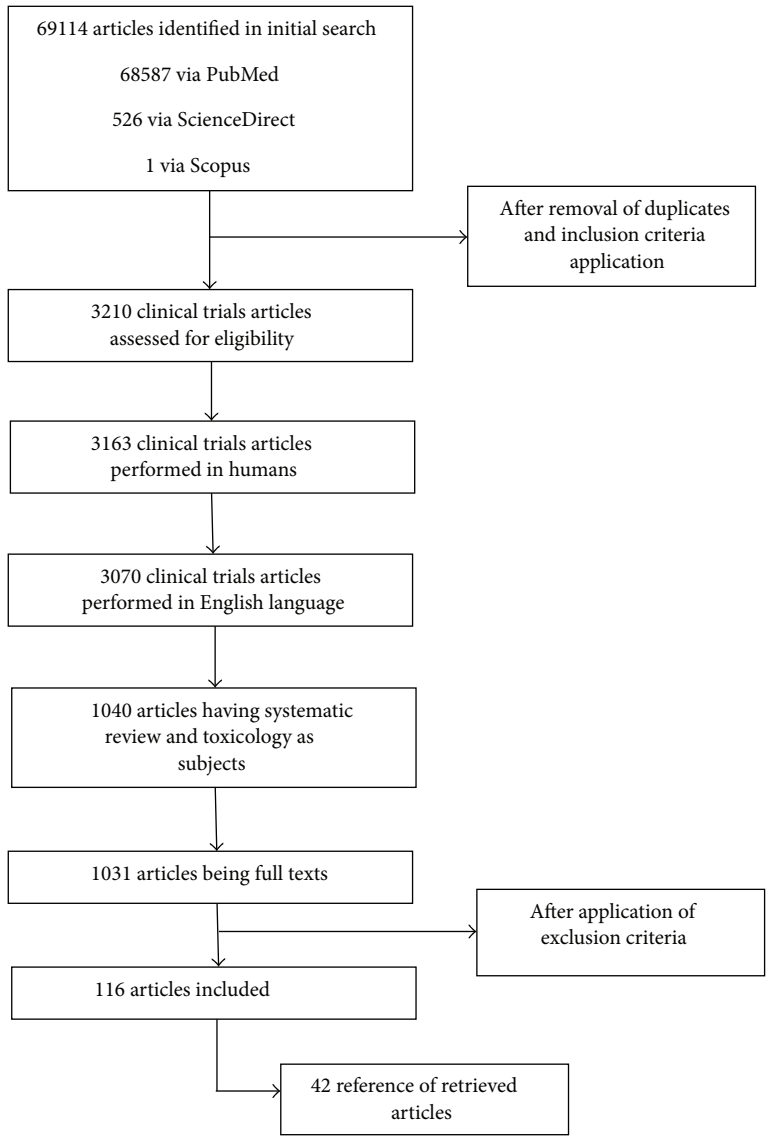

Figure 1: Flow of papers identified from search strategy.

Also immature VSMCs play a central role in blood vessel morphogenesis; they proliferate and migrate and produce extracellular matrix (ECM) components of the blood vessel wall such as collagen, elastin, and proteoglycans. Vascular growth stimuli, such as ischemic injury, in large and small vessels can trigger a process of differentiation of VSMC in which the matrix proteases, known as matrix metalloproteinases (MMPs), may play several roles: MMPs, thus, are not only involved in many vascular [58–72] and nonvascular diseases [73].

3.2. Mobilization of Stem Cells. The hematopoietic stem cell (HSC) resides in the bone marrow (BM) but several chemokines and cytokines have been shown to enhance trafficking of HSC into the peripheral blood. This process, known as stem cell mobilization, results in HSC microenvironmental interactions with the critical ligands, receptors and cellular proteases. Peripheral blood progenitor cells (PBPCs) advantages are avoidance of general anesthesia and pain and other adverse effects related to BM collection. Studies have shown that peripheral blood after cytokines stimulation contained a major number of CD34+ cells and T and NK cells than those in BM collection [74].

Granulocyte colony stimulating factor (G-CSF) and GM-CSF represent the two major cytokines used to mobilize the stem cells. These two factors can stimulate the stem cells that are released from the BM niches into peripheral blood. The BM niche is a structured microenvironment composed of supporting cells that anchor, through cell interaction, stem cells and regulates the self-regeneration, proliferation, and release into the circulation. Supporting cells also provide stem cells survivor with chemical signals: neurotransmitters induce membrane type-1 metalloproteinase (MT1-MMP) expression and MMP-2 activity [58–72, 75], which mediate

Table 1: Comparison of advantages and limitations of different types of stem cells.

Stem cell type	Limitations	Advantages
Embryonic stem cells	Ethical dilemmas, possible immune rejection after implantation, a small number of differentiated cardiomyocytes being generated, leading to teratocarcinomas; genetic instability	Differentiating into cells of all three germ layers
Pluripotent stem cells	Genetic instability, more research needed before using for cardiovascular repair/regeneration	Avoiding ethical concerns
Adult stem cells	Natural regeneration capacity of CSCs being too limited, acquisition and isolation difficulties, more research needed	Avoiding ethical concerns, lower risk of immune rejection
Mesenchymal stem cells	More research needed	Allowing for allogeneic grafting without the use of immunosuppressive agents, self-renewal, proliferating, and differentiating, promoting growth of adjacent cells, less susceptible to mutations, easy to collect
Hematopoietic stem cells	High maintenance, low frequencies, unknown signaling pathways	Proliferating and migrating to injury site in response to physiological/pathological stimuli, capable of myogenesis and angiogenesis
Endothelial progenitor cells	Extremely low numbers in peripheral blood and bone marrow making ex vivo expansion difficult	Increasing its numbers in response to ischemia/cytokine stimuli and migrating to injury site and differentiating into new myocytes

the cleavage of ties (CXCR4, VLA4, VCAM-1, and SCF) holding the stem cells in the BM niche and supporting their blood release.

Several studies have shown that administration of G-CSF and GM-CSF leads to a dose-dependent increase of endothelial progenitor cells (EPCs) in peripheral blood [76–78]. G-CSF promotes not only granulocyte expansion but also reduction of adhesion molecules and disruption of the SDF-1/CXCR4 axis: proteolytic enzymes, neutrophil elastase (NE), and cathepsin G (CG) cleave adhesion molecules as VCAM-1, SDF-1 and CXCR4, and c-kit [79, 80]. CXC chemokine receptor-4 antagonists can mobilize EPCs increasing MMP-9 signaling in the BM [58–72, 81, 82].

GM-CSF, instead, is rarely used because it mobilizes a reduced number of cells compared to G-CSF [83]. Generally, VEGF, fibroblastic growth factors, and stromal cell-derived factors have the ability to recruit EPCs; parathyroid hormone, statins,and other ligands may be used to mobilize stem cells, alone or in combination with G-CSF [84, 85].

Stem cell treatments with BM-derived cells (BMDCs) show safety outcomes but also adverse events related to cell collection and mobilization. Porat et al. postulated that alternatively activated dendritic cells (DCs) can promote the generation of EPC-enriched stem cells within a one-day culture [86].

Another source of stem cells can be satellite cells of skeletal muscle: these cells are one of the well-studied adult tissue-specific stem cells and have served as an excellent model for investigating adult stem cells. Myogenic precursor cells of postnatal muscle are responsible for the repair and regeneration of muscle fibers in adult tissue, either by fusing together and forming new fibers or incorporating themselves into damaged muscle cells and their myonuclei [87]. Satellite cells are mitotically quiescent or slow-cycling, committed to myogenesis, but undifferentiated. Satellite cells are the only source of new myoblasts in the adult tissue but they decrease with the age. In ischemic conditions these cells can be activated and their behavior is similar to those of bone marrow stem cells [88]. Satellite cells are activated by Myf-5 [89], a transcription factor, and CD34 is required for maintaining the quiescent state of myogenic stem cells [88]. After 6 hours from injury, satellite cells are activated and migrate, after disruption of basal lamina, from adjacent myofibers by projecting across tissue bridges initiated from an outpouching process of the satellite cell itself [90, 91]. Currently, the limit of this method is represented by the low number of in vivo studies which show the effectiveness of neoangiogenesis in patients with CLI.

Mesenchymal stem cells (MSCs) are multipotent cells showing adaptability and secretory capacity: thus, they can mediate reparative processes from the through release of soluble molecules, MSC-derived growth factors, and extracellular matrix components with paracrine mechanisms. It is likely that lower secretion of these important factors is the cause of failed tissue reparation. Studies have shown that cells obtained from older patients with multiple risk factors have impaired functions [92, 93]: for this reason, therapeutic success in CLI patients could be increased by using MSCs from young donors.

Therapeutic administration of stem cells does not have to be derived only from bone marrow but also from adipose tissue [93–95] and umbilical cord [96, 97] and other sources and released cytokines are main driving molecules in reparatory processes in CLI patients [98–100] with different results (Table 1).

3.3. Intramuscular versus Intra-Arterial Administration of Stem Cells. Intramuscular and intra-arterial injection or

a combination of both may be proposed in the treatment of human PAD. The principle of intramuscular injection is the creation of a cell depot with paracrine activity in the ischemic area. Experimental animal studies indicate that BM-derived cells contribute to vascular and muscle regeneration by physically integrating into the tissue and/or by secreting growth factors [101, 102]. The principle of intramuscular injection is the creation of a cell depot with paracrine activity in the ischemic area. Injection of bone marrow mononuclear cells has been reported to promote neovascularization of ischemic tissues effectively. This angiogenic effect may be related to their ability to induce vascular and muscle regeneration by direct de novo vascular and muscle differentiation or paracrine mechanisms through vascular endothelial growth factor secretion. Bone marrow mononuclear cells (BM-MNCs) contained the cell fractions that include EPCs and released various angiogenic factors: incorporation of EPCs in newly formed vessels as well as angiogenesis/arteriogenesis by angiogenic factors released from injected cells likely contributes to the increase in blood. Studies indicate that BMDCs contribute to vascular and muscle regeneration by physically integrating into the tissue and/or by secreting growth factors [103–105].

Intramuscular injection was performed into the gastrocnemius muscle; furthermore, injections were also placed along the occluded native arteries, because the density of preformed collaterals is highest in parallel orientation to the axial arteries: this is the preferred location for collateral growth [106, 107].

The effects of intra-arterial or intra-arterial plus intramuscular cell administration were compared to the effects of intramuscular cell administration. Ankle-brachial index (ABI) and transcutaneous partial pressure of oxygen ($TcPO_2$) were found to be significantly improved only after intramuscular or combined therapy and not after intra-arterial cell therapy only [50, 108, 109]. On the other hand, significantly improved pain and pain-free walking distance were detected and there was no difference between the two. Intramuscular cell therapy significantly improved ulcer healing, while this could not be assessed in detail in trials of intra-arterial cell therapy. Pilot studies by the small number of patients reported the improvement of clinical signs and symptoms of intractable patients with CLI by injection of G-CSF [110, 111] or intramuscular injection of G-CSF-mobilized peripheral blood mononuclear cells [112–114]. Tateno et al. [115] postulated that the implanted peripheral blood mononuclear cells stimulate ischemic skeletal muscle cells to produce muscle-derived angiogenic factors, thereby promoting neovascularization. In all studies, there were no significant differences in the clinical characteristics between patients treated with intra-arterial or intra-arterial plus intramuscular cells [50, 116, 117].

3.4. Adverse Effects in Stem Cells Therapy. Injection of BM-MNCs significantly improved pain-free walking time, rest pain, and tissue oxygen pressure on average 6 months after treatment, whereas injection of peripheral blood mononuclear cells did not exert significant effects [118, 119]. Several studies reported very low mortality rate (<15%) in patients treated with autologous stem cells implantation for CLI [108, 120–122]. These findings suggest that the angiogenic cell therapy using intramuscular implantation of BM-MNCs is valid therapeutic choice and not inferior to the conventional revascularization therapies in patients with CLI. Because of the increased risk and the reduced potential of the treatment, peripheral blood stem cell treatment is less appropriate in the older age [123]. In some studies, deaths have been reported: these were mostly due to acute myocardial infarction, congestive heart failure, and stroke while perforation peritonitis and sepsis have been reported as exceptional [124, 125]. In 2012, Jonsson et al. reported a high incidence of serious adverse events in patients treated with peripheral blood mononuclear cells, causing the investigators to terminate the study [126]. Out of 9 patients, 2 had a myocardial infarction that was believed to be related to the bone marrow stimulation and 1 of the 2 patients died. Another patient had a minor stroke 1 week after stem-cell implantation.

As previously showed, hemodialysis [127], diabetes mellitus [128, 129], and complication with coronary artery disease (CAD) [130–132] are factors that negatively impaired angiogenesis or limb salvage in animal experiments and clinical settings.

3.5. Patients in Whom Endovascular and Surgical Revascularisation Are Not Believed to Be Possible. Patients with poor outflow vessels and extensive comorbidities resulting in unacceptable risk of a revascularization procedure, as well as patients who had previously failed revascularization attempts, are not candidate to surgical and/or endovascular procedures [133–136]. This subgroup of patients without revascularization options, known as NO-CLI, is a population with a high rate of limb loss and death.

One primary endpoint for evaluating the outcomes of NO-CLI therapy is major amputation (AMP), which is usually combined with mortality for AMP-free survival (AFS) [137–140]. AFS captures two hard endpoints, mortality and amputation, that are obviously important to patients and clinicians. Despite the fact that the AFS and mortality are the focal points used in the majority of RCTs in the literature for NO-CLI, they necessarily require periodic review and updating.

In the absence of arterial reconstruction options, novel approaches, such as pharmacologic, gene, or stem cell therapy, are proposed; in particular, regenerative medicine has recently emerged as a new speciality that has created great expectations in the scientific community [141]. Improvement of neovascularization is a therapeutic option to rescue tissue from critical ischemia.

4. Discussion

The gold-standard treatment of severe PAOD and CLI is surgical or endovascular revascularization. However, up to 30% of patients are not candidate for such interventions, due to excessive operative risk or unfavorable vascular involvement. Despite the progress in medical and surgical therapy of

Table 2: Clinical trials using stem cells for treatment of critical limb ischemia.

Authors	Type of cells	Clinical outcomes
Nizankowski et al. [143]	BMCs	Improvement of symptoms (pain, cold sensation), increase in ABI and $TcPO_2$, new collateral vessels
Napoli et al. [144]	BMCs	Increase in ABI and walking distance, ulcer healing, reduction of amputation rates
Procházka et al. [145]	BMCs	Improvement in toe pressure, TBI, LDI, and $TcPO_2$
Matoba et al. [146]	BMMNCs	Long-term improvement in pain scale, ulcer size, and walking distance, reduced amputation rates
Amann et al. [8]	BMMNCs	Limb salvage, increase in ABI and $TcPO_2$
Kawamura et al. [147]	PBMNCs with GcSF	Reduced amputations, mostly in nondiabetic nondialysis patients
Huang et al. [148]	PBMNCs versus BMMNCs	PBMNC administration: higher overall efficacy, improvement in ABI, skin temperature, rest pain, walking distance, $TcPO_2$, ulcers, and amputation rates (both treatments)
Tateishi-Yuyama et al. [149]	Bone marrow MNCs	Increased ABPI and $TcPO_2$ pressure, decreased rest pain
Motukuru et al. [150]	Bone marrow MNCs	Increased ABPI and improvement in ulcer healing
Lara-Hernandez et al. [108]	Peripheral blood CD34+ CD133+ cells after G-CSF mobilization	Increased ABPI and improvement in ulcer healing
Benetti et al. [151]	Human fetal-derived stem cells	
Burt et al. [152]	EPCs (CD34/CD133)	Improvement in amputation-free survival, exercise capacity, pain relief, collateral formation, perfusion, and QoL
MESENDO (II) Clinicaltrials.gov # NCT00721006 [153]	Stem cell mixture	Completed; pending publication
Lasala et al. [154]	BM-MNC EPC + BM-MSC	↑ABI, ↑angiogenesis (MRA), ↑AFS, ↑$TcPO_2$, ↑WH, ↑WT, ↓pain
Dash et al. [155]	BM-MSC	↑angiogenesis (biopsy), ↑WH, ↑WD, ↓pain
NCT01257776 [156]	Adipose-MSC	ABI, AFS, DSA improved
NCT01216865 [156]	Cord-MSC	ABI, AFS, pain, WT, WH improved

patients with CLI, the prognosis of patients with no option for revascularization remains poor: the amputation rate is high as well as mortality rate (20%) within six months.

During the last two decades, a novel therapeutic strategy has been proposed: the stem cells therapy. BMDCs include autologous BMCs, BMMNCs, and EPCs; peripheral blood-derived cells include PBMNCs, PMNCs, ECFCs, CPCs, and EPC, while other cells mainly include MSCs and ADSCs. In 1997, Asahara et al. discovered that bone marrow-derived circulating cells are able to differentiate into endothelium and promote new vessel growth. These cells, known as EPCs, were able to improve tissue perfusion in myocardial and peripheral ischemia through the stimulation of vasculogenesis [142]. As showed in our review, the studies which have applied the stem cell therapy in NO-CLI patients are very numerous. Despite some failures associated with factors that invalidated the functionality of the different stem cells (i.e., diabetes), the results obtained from the detailed analysis of the recent literature data have confirmed the beneficial role of cell therapy in reducing the rate of major amputations, improving distal perfusion, increasing walking distance, reducing pain, improving ABI and $TcPO_2$, and improving overall ischemic symptoms in patients with CLI and their quality of life (Table 2).

Bone marrow aspiration was well tolerated, the most frequent adverse reaction being local pain, responsive to non-steroidal anti-inflammatory drugs; common adverse event was mild anemia. G-CSF stimulation was generally well tolerated, with prevalently minor side effects, including flu-like symptoms, myalgia, fever, and bone pain. Intramuscular or intra-arterial delivery associated with intramuscular injections of BMMNCs cells had positive results in the majority of clinical studies: the procedure appeared to be generally safe and well tolerated and most adverse reactions were expected given the severe underlying disease and could not be directly attributed to cell therapy. The intramuscular administration

seems preferable maybe because cells could hardly reach the target tissue when infused intra-arterially in severely compromised arterial beds.

Thus, bone marrow cells or peripheral blood cells administration? The latter seems to be easier to perform and it might be repeated but it does not appear to be inferior in efficacy: BMMCs seemed to be more effective than mobilized peripheral blood cells in inducing reparative processes because these cells are transiently dysfunctional due to cleavage of the chemokine receptor CXCR4, which is directly involved in stem cell homing. PB-MNCs show comparable or even superior efficacy in comparison to BM-MNCs. In conclusion, BMCs, BM-MNCs, and PB-MNCs are the main cell types used and there is no clear superiority of one cell type over the others. Current literature supports that intramuscular BM cell administration is a relatively safe, feasible, and possibly effective therapy for patients with CLI not susceptible to conventional revascularization.

Based on the recent literature data, treatment-induced improvements are sustainable at 2-3 years: if long-term efficacy becomes definitively established, the stem cell therapy for severe inoperable PAOD will be strongly enhanced. For this reason, multicenter, large-scale and randomized controlled clinical trials may be fundamental and mandatory to prove the safety and efficacy of promoting angiogenesis by the administration of stem cells and for this therapy to become a standard treatment strategy for the patients suffering with CLI.

Another key aspect is that stem cell therapy is an expensive treatment and its cost-effectiveness has not been determined. Thus, a detailed cost-benefit analysis is desirable.

Abbreviations

ABI: Ankle-brachial index
ADSCs: Adipose tissue-derived stem cells
AFS: AMP-free survival
AMP: Amputation
BM: Bone marrow
BM-MNCs: Bone marrow mononuclear cells
BMDCs: BM-derived cells
BMP: Bone morphogenetic protein
CAD: Coronary artery disease
CG: Cathepsin G
CLI: Critical limb ischemia
CPCs: Circulating progenitor cells
DCs: Dendritic cells
ECM: Extracellular matrix
ECs: Endothelial cells
ECFCs: Endothelial colony forming cells
EPCs: Endothelial progenitor cells
ESCs: Embryonic stem cells
G-CSF: Granulocyte colony stimulating factor
GM-CSF: Granulocyte-macrophage colony-stimulating factor
HSC: Hematopoietic stem cell
ICM: Inner cell mass
MMPs: Matrix metalloproteinases
MPCs: Monocytic progenitor cells
MSCs: Mesenchymal stem cells
MT1-MMP: Membrane type 1 matrix metalloproteinase
NE: Neutrophil elastase (NE)
NO-CLI: Patients with CLI and without revascularization options
PAOD: Peripheral arterial obstructive disease
PBMNCs: Peripheral blood mononuclear cells
PBPCs: Peripheral blood progenitor cells
PDGFR-α: Platelet-derived growth factor receptor-α
PMNCs: Peripheral mononuclear Cells
SDF-1: Stromal-derived factor-1
sKitL: Soluble kit-ligand
$TcPO_2$: Partial pressure of oxygen
TGF-β: Transforming growth factor-β
VCAM-1: Vascular cell adhesion protein-1
VEGF: Vascular endothelial growth factor
VEGFR-2: Vascular endothelial growth factor receptor-2
VSMCs: Vascular smooth muscle cells.

Conflict of Interests

The authors declare no conflict of interests.

Authors' Contribution

Rita Compagna and Bruno Amato participated substantially in conception, design, and execution of the study and in the analysis and interpretation of data and also participated substantially in the drafting and editing of the paper. Salvatore Massa and Maurizio Amato participated substantially in data collection and in the analysis and interpretation of data. Raffaele Grande and Lucia Butrico participated substantially in data collection and execution of the study and in the analysis and interpretation of data and also participated substantially in the drafting and editing of the paper. Stefano de Franciscis and Raffaele Serra participated substantially in conception, design, and execution of the study and in the analysis and interpretation of data and also participated substantially in the drafting, editing, and critical revision of the paper. Rita Compagna and Bruno Amato contributed equally to this work and share the first authorship. Stefano de Franciscis and Raffaele Serra contributed equally to this work and share the senior authorship.

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Research Article

Analysis of the Pro- and Anti-Inflammatory Cytokines Secreted by Adult Stem Cells during Differentiation

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Adipose-derived stromal/stem cells (ASCs) are adult stem cells that have the potential to differentiate into mesenchymal lineage cells. The abundance of ASCs in adipose tissue and easy accessibility with relatively little donor site morbidity make them attractive candidate cells for tissue engineering and regenerative medicine. However, the underlying inflammatory process that occurs during ASC differentiation into adipocytes and osteoblast has not been extensively investigated. ASCs cultured in osteogenic and adipogenic differentiation medium were characterized by oil red o staining and alizarin red staining, respectively. ASCs undergoing osteogenic and adipogenic differentiation were isolated on days 7, 14, and 21 and assessed by qRT-PCR for the expression of pro- and anti-inflammatory cytokines. ASCs undergoing osteogenic differentiation expressed a distinct panel of cytokines that differed from the cytokine profile of ASCs undergoing adipogenic differentiation at each of the time points analyzed. Mapping the cytokine expression profile during ASC differentiation will provide insight into the role of inflammation in this process and identify potential targets that may aid in enhancing osteogenic or adipogenic differentiation for the purposes of tissue engineering and regenerative medicine.

1. Introduction

Adipose-derived stromal/stem cells (ASCs) are adult stem cells with multipotential differentiation capacity. The ability for ASCs to differentiate along osteogenic and adipogenic lineage cells makes them ideal candidates for regenerative medicine [1, 2]. Furthermore, the abundance and easy accessibility in harvesting large volumes of adipose tissues allow for large-scale expansion of ASCs for therapeutic purposes [3, 4].

The osteogenic and adipogenic differentiation of ASCs has been shown to require the activation of key transcriptional factors that govern cell fate. RUNX2 has previously been shown to be a master regulator of osteoblast differentiation, as RUNX2 activates and regulates many osteogenic signaling pathways, including but not limited to transforming growth factor beta (TGF-β), bone morphogenetic protein (BMP), Wingless type Wnt, and Hedgehog [5, 6]. ASCs cultured in osteogenic differentiation medium have also been shown to upregulate a key osteogenic factor dickkopf Wnt signaling pathway inhibitor 1 (DKK-1), as early as one day. Additional osteogenic transcriptional factors (connective tissue growth factor (CTGF), platelet-derived growth factor receptor beta (PDGFR-β), TGF-β, insulin-like growth factor binding protein 3 (IGFBP3), and tenascin C (TNC)) were induced after 7 days in osteogenic differentiation medium [7]. In contrast, peroxisome proliferator-activated receptor gamma (PPARγ) is principally regarded as the master regulator of adipogenesis, since no factor can rescue adipocyte formation when PPARγ is knocked out [8]. Induction of CCAAT-enhancer-binding proteins (C/EBPβ, C/EBPδ) and peroxisome proliferator-activated receptor delta (PPARδ) expression occurs during early adipogenic differentiation, while fatty acid binding protein 4 (FABP4), C/EBPα, lipoprotein lipase (LPL), leptin, and glucose transporter 4 (GLUT4)

expression is upregulated during late adipogenic differentiation [9, 10].

While many studies have explored the mechanism(s) governing ASC differentiation, few studies have investigated the associated expression of inflammatory gene expression that occurs during the differentiation of these cells. The expression profile of mRNA encoding these inflammatory cytokines may provide information regarding the mechanism governing ASC differentiation. Herein, ASCs were induced to differentiate into osteogenic and adipogenic lineage cells and assessed by qRT-PCR for the expression of pro- and anti-inflammatory cytokines. These studies demonstrated a systemic and robust upregulation of pro- and anti-inflammatory cytokines that was time-dependent. These studies demonstrated the plasticity of ASCs and identified inflammatory cytokines secreted at different stages of differentiation that may govern the ultimate cell fate of ASCs.

2. Materials and Methods

2.1. Materials. Anti-CD45-PeCy7, anti-CD11b-PeCy5, anti-CD166-phycoerythrin (PE), anti-CD105-PE, anti-CD90-PeCy5, anti-CD34-PE, isotype control fluorescein isothiocyanate (FITC) human IgG1, and isotype-control PE human IgG2a were purchased from Beckman Coulter (Indianapolis, IN). Anti-CD44-allophycocyanin (APC) was purchased from BD Biosciences (San Jose, CA). Type 1 collagenase, bovine serum albumin (BSA, fraction V), calcium chloride, cetylpyridinum chloride (CPC) dexamethasone, isobuytlmethylxanthine, indomethacin, ascorbate 2-phosphate, β-glycerol phosphate, alizarin red s, and oil red o were purchased from Sigma (St. Louis, MO).

2.2. Human Subjects. Primary human ASCs were obtained from subcutaneous abdominal adipose tissue of 3 Caucasian females (mean age 34.6 ± 8.4 and mean body mass index 22.2 ± 1.1) undergoing elective liposuction. Tissues were obtained with written informed consent under a protocol reviewed and approved by the Pennington Biomedical (Baton Rouge, LA) Institutional Review Board. Lipoaspirates were processed by incubating tissue in 0.1% type I collagenase and 1% BSA dissolved in 100 mL of phosphate buffered saline (PBS) supplemented with 2 mM calcium chloride. The mixture was placed in a 37°C shaking water bath at 75 rpm for 60 min and then centrifuged to remove oil, fat, primary adipocytes, and collagenase solution, leaving behind a pellet of cells. Cells were resuspended in medium, which consisted of Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12; Life Technologies, Grand Island, NY) and 10% fetal bovine serum (FBS; HyClone; Logan, UT), plated on 150 cm^2 culture dishes (NUNC, Rochester, NY) and maintained in a humidified 5% CO_2 incubator. Fresh medium was added every 2-3 days until cells achieved 80–90% confluence and were harvested with 0.25% trypsin/1 mM EDTA (Life Technologies) and cryopreserved prior to experimental use.

2.3. Cell Culture. Frozen vials of approximately 10^6 ASCs were thawed, plated onto 150 cm^2 culture dishes in 20 mL complete culture medium (CCM), which consisted of α-MEM (Life Technologies), 20% FBS (Atlanta Biologicals, Duluth, GA), 1% L-glutamine (Life Technologies), and 1% penicillin/streptomycin (Life Technologies), and incubated at 37°C with 5% humidified CO_2. After 24 hours, medium was removed and adherent viable cells were washed with PBS, harvested with 0.25% trypsin/1 mM EDTA, and replated at 100 cells per cm^2 in CCM. Medium was replaced every 3-4 days. For all experiments, cells between passages 2 and 6 were used.

2.4. Flow Cytometry. ASCs were harvested with 0.25% trypsin/1 mM EDTA for 3-4 minutes at 37°C. A total of 3×10^5 cells were suspended in 50 μL PBS and incubated with fluorescence-labeled antibodies. The samples were incubated for 30 minutes at room temperature and washed with PBS. The samples were then analyzed with Gallios Flow Cytometer (Beckman Coulter, Brea, CA) running Kaluza software (Beckman Coulter). To assay cells by forward and side scatter, FACScan was standardized with microbeads (Dynosphere uniform microspheres; Bangs Laboratories Inc.; Thermo Scientific; Waltham, MA). At least 10,000 events were analyzed and compared with isotype controls.

2.5. Colony Forming Unit Assay. ASCs were plated at a density of 100 cells on a 10 cm^2 plate (NUNC) in CCM and incubated for 14 days. Plates were then rinsed with PBS and stained with 3% crystal violet (Sigma) for 30 minutes at room temperature. Plates were washed with PBS and once with tap water. Colonies that were larger than 2 mm in diameter were counted.

2.6. Differentiation Protocols

Osteogenic Differentiation. ASCs were cultured in six-well plates (NUNC) in CCM until 70% confluence. Medium was replaced with fresh osteogenic differentiation medium (ODM) consisting of 50 μM ascorbate 2-phosphate, 10 mM β-glycerol phosphate, and 10 nM dexamethasone. After 14 days, cells were fixed in 10% formalin for 1 hour, washed with distilled water, and stained with 1% alizarin red (pH 4.1) to visualize calcium deposition in the extracellular matrix. Images were acquired at 4x magnification on an Eclipse TE200 (Nikon, Melville, NY) with Digital Camera DXM1200F (Nikon) using ACT-1 software (Nikon). For quantification, alizarin red was extracted from each well with 10% CPC and read at 584 nm (FLUOstar optima). Protein extraction with RIPA buffer (Pierce; Thermo Scientific; Waltham, MA) and protein quantification with the BCA assay (Thermo Scientific) were performed according to manufacturer's instructions. Samples were normalized to the amount of protein in each sample.

Adipogenic Differentiation. ASCs were cultured in six-well plates in CCM until cells achieved 70% confluence. Medium was replaced with fresh adipogenic differentiation medium (ADM) consisting of CCM supplemented with 0.5 μM dexamethasone, 0.5 mM isobuytlmethylxanthine, and 50 μM

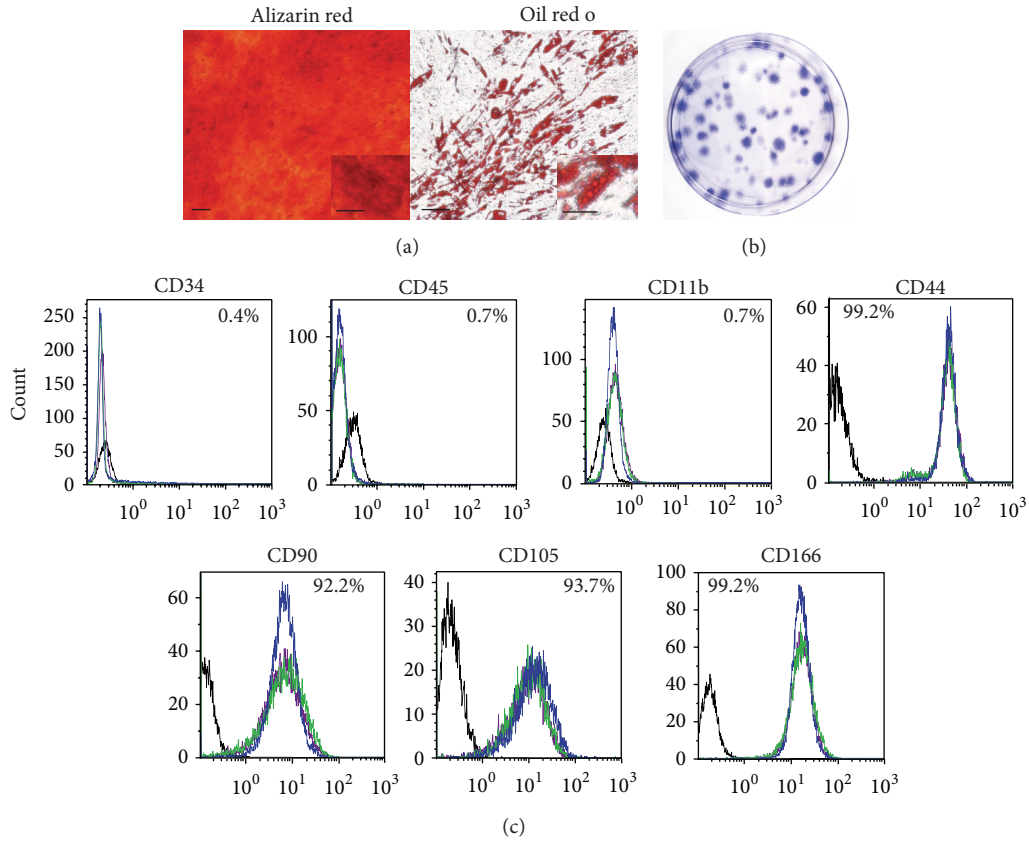


Figure 1: Characterization of ASCs. (a) ASCs were cultured in ODM or ADM for 21 days and stained with alizarin red for osteogenesis or oil red o for adipogenesis. Representative images are shown. Original magnification for osteogenesis is 4x and that of adipogenesis is 10x. Scale bar represents 100 μm. Scale bar for insets represents 25 μm. (b) Cells were seeded at low density and incubated in CCM. After 14 days, colony-forming units were stained with crystal violet. A representative image is shown. (c) ASCs were stained with antibodies against the indicated antigens and analyzed by flow cytometry. Each colored line represents a specific donor ($N = 3$ donors), and respective isotype controls are shown as black lines.

indomethacin. After 14 days, cells in ADM were fixed in 10% formalin for 1 hour and stained with oil red o, composed of 2 parts PBS and 3 parts 0.5% oil red o stock solution to visualize neutral lipids. Images were acquired at 10x magnification. For quantification, oil red o was extracted from each well with isopropanol and read at 544 nm (FLUOstar optima). Protein was isolated with RIPA buffer (Pierce; Thermo Scientific; Waltham, MA) and quantified with the BCA assay (Thermo Scientific) for normalization.

2.7. RNA Isolation, cDNA Synthesis, and Quantitative RT-PCR (qRT-PCR) Analysis. Cells were cultured in CCM, ODM, or ADM and collected after 7, 14, and 21 days. Total RNA was extracted from ASCs using the RNeasy Mini Kit (Qiagen, Valencia, CA), purified with DNase I digestion (Invitrogen) according to manufacturer's instructions, and reverse transcribed using the SuperScript VILO cDNA synthesis kit (Invitrogen) containing random primers. Quantitative real-time PCR was performed using the EXPRESS SYBR GreenER qPCR SuperMix Kit (Invitrogen) according to the manufacturer's instructions. Forward and reverse primer sequences can be found in Table 1. All qRT-PCR primers were designed using Primer3 (Boston, MA) and purchased from Integrated DNA Technologies (Coralville, IA). The expression of human β-actin was used to normalize mRNA content. Samples were tested in triplicate. No-template controls and no-reverse transcription controls were included in each PCR run.

3. Results

3.1. Characterization of ASCs. ASCs were isolated from processed lipoaspirates harvested from subcutaneous adipose tissue and characterized based on differentiation potential, self-renewal capacity, and cell surface marker profile. ASCs were able to differentiate into osteoblast and adipocytes when induced with ODM and ADM, respectively (Figure 1(a)). ASCs seeded at low density were able to generate colony-forming units (Figure 1(b)). Flow cytometric analysis demonstrated that ASCs were negative for CD34, CD45, and CD11b expression and positive for CD44, CD90, CD105, and CD166 expression (Figure 1(c)).

3.2. Expression of Proinflammatory and Anti-Inflammatory Cytokines during Osteogenic Differentiation of ASCs Is Time

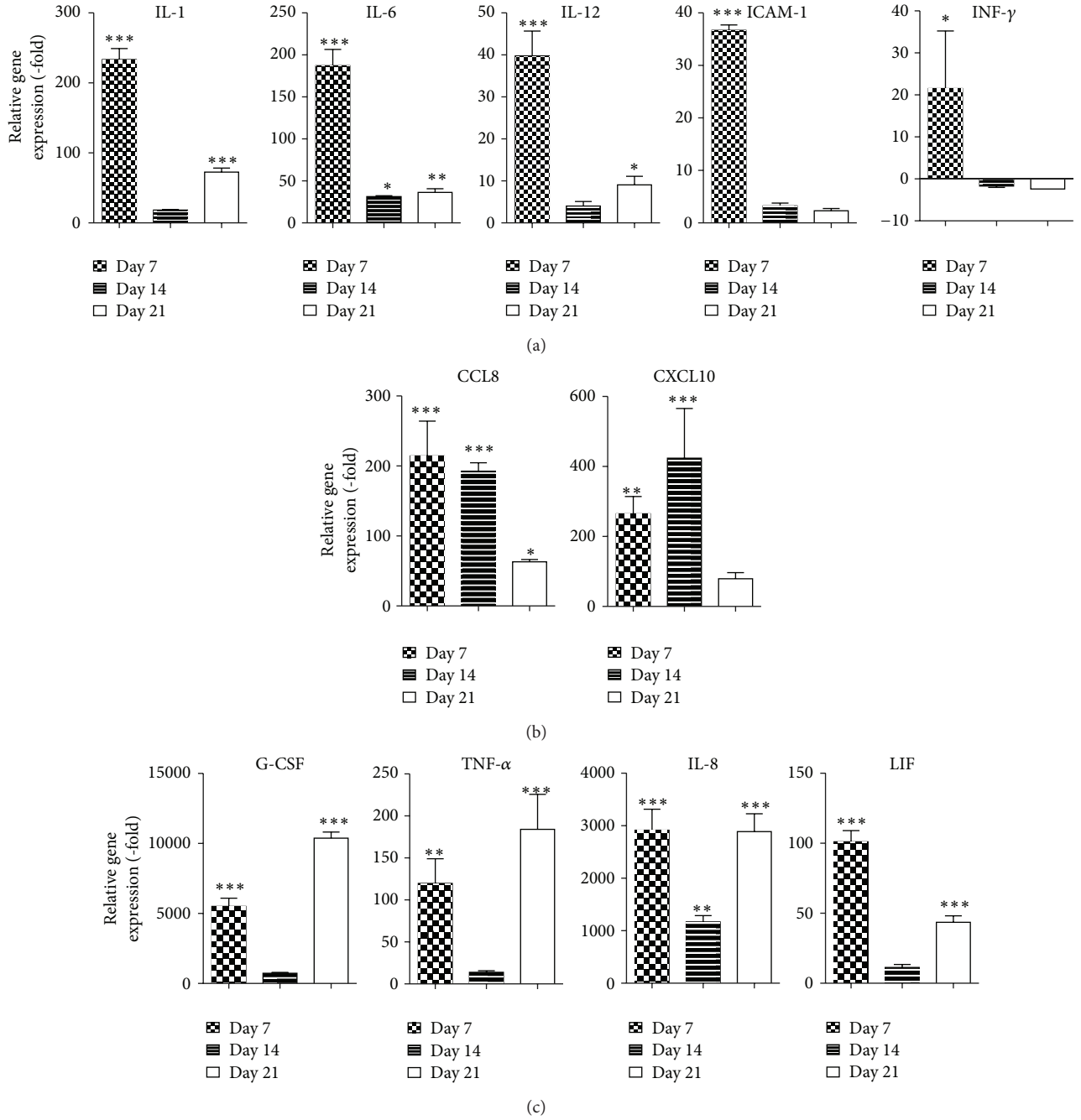


Figure 2: Proinflammatory cytokines are upregulated during osteogenic differentiation of ASCs. ASCs were cultured in CCM and changed to ODM. Cells were harvested on days 7, 14, or 21 and analyzed by qRT-PCR. Data is normalized to undifferentiated cells. Mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ relative to undifferentiated cells.

Dependent. ASCs at passage 2 were expanded in CCM until confluent and cultured in ODM. At 7, 14, and 21 days of culture, cells were harvested and the mRNA expression of pro- and anti-inflammatory cytokines was assessed in comparison to undifferentiated ASCs. ASCs induced with ODM demonstrated an increase in the expression of proinflammatory cytokines interleukin-1 (IL-1; 223.3-fold, $P < 0.001$), interleukin-6 (IL-6; 187.3-fold, $P < 0.001$), interleukin-12 (IL-12; 39.8-fold, $P < 0.001$), intercellular adhesion molecule 1 (ICAM-1; 36.7-fold, $P < 0.001$), and interferon gamma (IFN-γ; 21.5-fold, $P < 0.05$) during the early stages of osteogenic differentiation (day 7), and their expression diminished during the mid and late stages of differentiation (Figure 2(a)). Early and mid stages of osteogenic differentiation of ASCs demonstrated an increase in chemokine (C-C motif) ligand 8 (CCL8; 214.4-fold on day 7 and 192.5-fold on day 14, $P < 0.001$) and C-X-C motif chemokine 10 (CXCL10; 264.5-fold on day 7 and 423.9-fold on day 14, $P < 0.001$) expression,

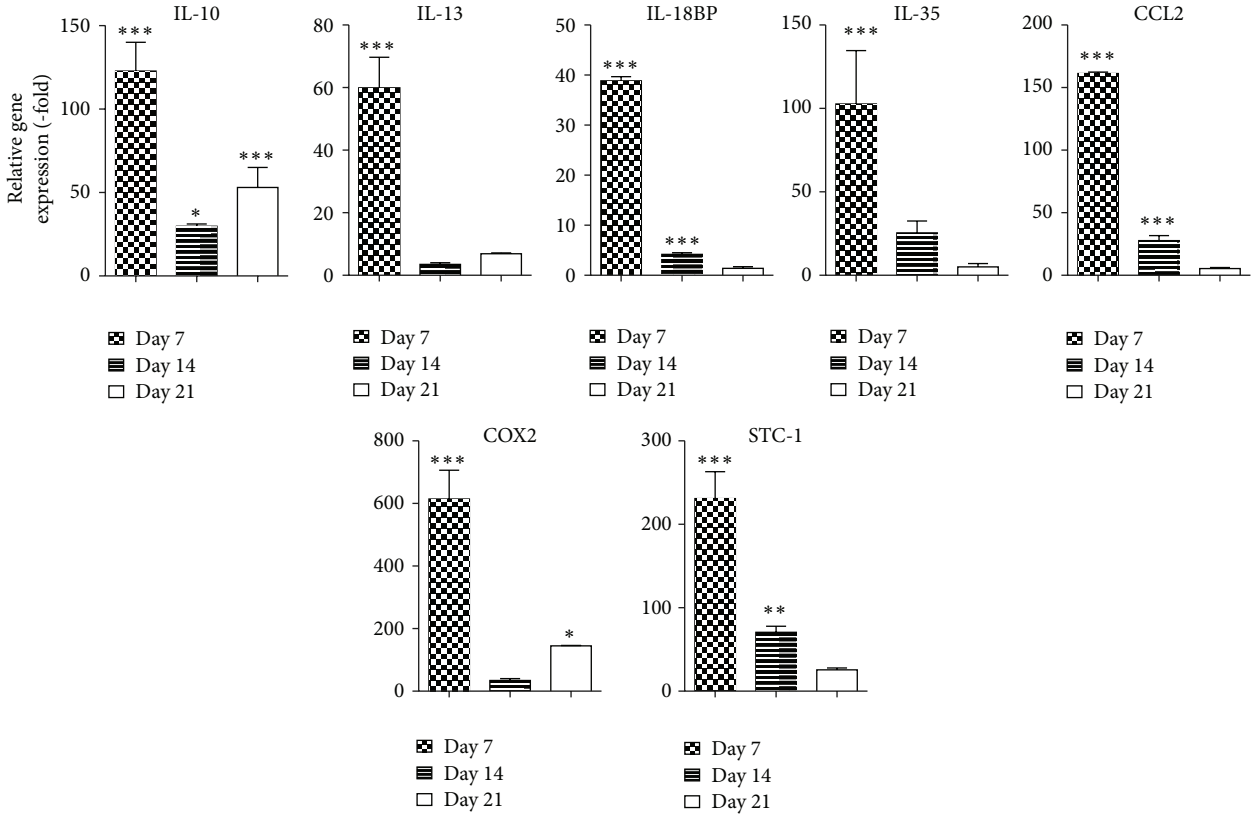
Table 1: qRT-PCR primer sequences for proinflammatory and anti-inflammatory cytokines.

Gene	Forward ($5' \rightarrow 3'$)	Reverse ($5' \rightarrow 3'$)
	Proinflammatory cytokines	
IL-1	CGCCAATGACTCAGAGGAAG	AGGGCGTCATTCAGGATCAA
IL-6	GTAGCCGCCCCACACAGACAGCC	GCCATCTTTGGAAGGTTC
IL-8	GAACTGAGAGTGATTGAGAGT	CTTCTCCACAACCCTCTG
IL-12	TGGAGTGCCAGGAGGACAGT	TCTTGGGTGGGTCAGGTTTG
IL-17	TGAAGGCAGGAATCACAAT	GGTGGATCGGTTGTAGTAAT
CCL8	CACAAGAATCACCAACATCC	TGGTCCAGATGCTTCATG
CXCL10	TCTGACTCTAAGTGGCATTC	ATTGTAGCAATGATCTCAACAC
G-CSF	AGCTTCCTGCTCAAGTGC	TTCTTCCATCTGCTGCCAGATGGT
ICAM-1	CACAGTCACCTATGGCAA	CTGGCTTCGTCAGAATCA
IFN-γ	TCAGCTCTGCATCGTTTTGG	GTTCCATTATCCGCTACATCTGAA
LIF	CCTGGACAAGCTATGTGG	GGTTGAGGATCTTCTGGTC
TNF-α	TCTTCTCGAACCCCGAGTGA	CCTCTGATGGCACCACCAG
	Anti-inflammatory cytokines	
IL-1RA	GTTCCATTCAGAGACGATCT	GTTGTTCCTCAGATAGAAGGT
IL-10	GTGATGCCCCAAGCTGAGA	CACGGCCTTGCTCTTGTTTT
IL-11	GGACCACAACCTGGATTC	GCAGGTAGGACAGTAGGT
IL-13	ATTGCTCTCACTTGCCTT	GTCAGGTTGATGCTCCAT
IL-18BP	ACCATGAGACACAACTGG	ATGCTGGACACTGCTTAG
IL-35	CACGTCCTTCATCCTCAG	GACTCCAGTCACTCAGTTC
CCL2	AGTCACCTGCTGTTATAACTT	CACAATGGTCTTGAAGATCAC
COX2	ACAGTCCACCAACTTACAAT	CAATCATCAGGCACAGGA
HGF	TTATCCTGACGTAAACACCTTTGATATAAC	CTGGGCAGTATTCGGGTTTGA
PTGES2	CCTGGAAGAGATCATCACC	CCTTCTCGTTGAGCATGA
STC-1	AGGATGATTGCTGAGGTG	TGTTATAGTATCTGTTGGAGAAGT
TGF-β	CAGCAACAATTCCTGGCGATA	AAGGCGAAAGCCCTCAATTT
TNFRSF1A	CAGGAAGAACCAGTACCG	TTCTTACAGTTACTACAGGAGAC
TSG-6	CATCTCGCAACTTACAAGC	AGACGGATTCCATAATCAATAATG

which decreased by day 21 (day 21; Figure 2(b)). In contrast, mRNA expression of several proinflammatory cytokines demonstrated a biphasic increase on day 7 and day 21, with minimal induction on day 14: granulocyte-colony stimulating factor (G-CSF) mRNA expression was increased by 5507.2-fold and 10381.3-fold, tumor necrosis factor alpha (TNF-α) was increased by 119.8-fold and 184.1-fold, interleukin-8 (IL-8) was increased by 2915.0-fold and 2888.3-fold, and leukemia inhibitory factor (LIF) was increased by 101.1-fold and 43.6-fold on day 7 and day 21, respectively (Figure 2(c)).

The expression of anti-inflammatory cytokines was also assessed during osteogenic differentiation. Of the 14 anti-inflammatory cytokines assessed, seven genes were significantly upregulated in ASCs following culture in ODM for 7 days, which diminished by days 14 and 21 after induction. Increased expression of interleukin-10 (IL-10; 122.8-fold, $P < 0.001$), interleukin-13 (IL-13; 59.8-fold, $P < 0.001$), interleukin-18 binding protein (IL-18BP; 38.9-fold, $P < 0.001$), interleukin-35 (IL-35; 102.7-fold, $P < 0.001$), chemokine (C-C motif) ligand 2 (CCL2; 161.2-fold, $P < 0.001$), cyclooxygenase 2 (COX2; 614.4-fold, $P < 0.001$), and stanniocalcin 1 (STC-1; 230.8-fold, $P < 0.001$, Figure 3(a)) was observed. In contrast, ASCs cultured in ODM for 7 and 14 days demonstrated a sustained increase in the expression of interleukin-1 receptor antagonist (IL-1RA; 2617.7-fold on day 7 and 3024.7-fold on day 14, $P < 0.001$) and tumor necrosis factor-stimulated gene 6 (TSG-6; 51.2-fold on day 7 and 65.2-fold on day 14, $P < 0.001$; Figure 3(b)). Cells cultured in ODM demonstrated a biphasic induction in mRNA expression of anti-inflammatory cytokine interleukin-11 (IL-11; 4.5-fold on day 7 and 22.9-fold on day 21, $P < 0.001$), tumor necrosis factor receptor superfamily member (TNFRSF1A; 4.5-fold on day 7 and 3.5-fold on day 21, $P < 0.001$), prostaglandin E synthase 2 (PTGES2; 5.7-fold on day 7 and 5.5-fold on day 21, $P < 0.001$), and TGF-β (17.0-fold on day 7 and 28.0-fold on day 21, $P < 0.001$; Figure 3(c)). In contrast, mRNA expression of hepatocyte growth factor (HGF) was reduced by −2.9-fold, −50.0-fold, and −7.1-fold on days 7, 14, and 21, respectively ($P < 0.001$; Figure 3(d)).

3.3. Proinflammatory and Anti-Inflammatory Cytokine Expression during Adipogenic Differentiation of ASCs Vary. ASCs were expanded in CCM until confluent at which time ADM was added to the cells. Again, at 7, 14, and 21



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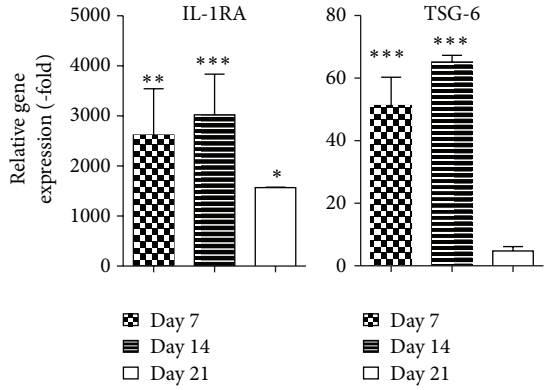

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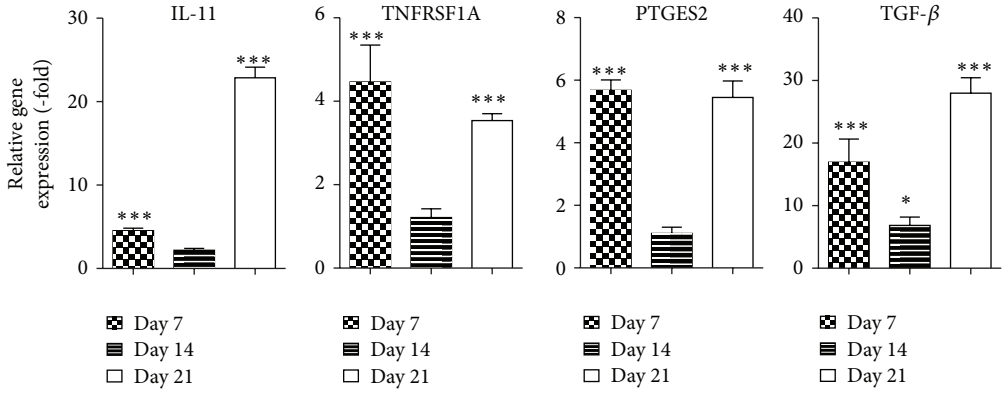

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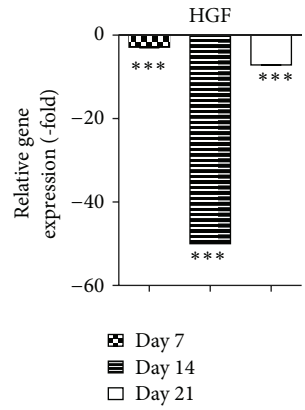


Figure 3: Osteogenic differentiation of ASCs increases expression of anti-inflammatory cytokines. ASCs were grown in CCM and then changed to ODM. After 7, 14, and 21 days, cells were harvested and analyzed by qRT-PCR. Data is normalized to undifferentiated cells. Mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ relative to undifferentiated cells.

days, cells were harvested and the mRNA expression of proinflammatory and anti-inflammatory cytokines was assessed relative to naive ASCs. ASCs induced with ADM for 7 days and 14 days demonstrated an increase in the expression of proinflammatory cytokines IL-12 (223.3-fold on day 7 and 4.3-fold on day 14, $P < 0.05$), interleukin-17 (IL-17; 69.8-fold on day 7 and 11.9-fold on day 14, $P < 0.001$), and ICAM-1 (8.4-fold on day 7 and 2.6-fold on day 14, $P < 0.05$, Figure 4(a)). In contrast, several proinflammatory cytokines demonstrated the highest levels of mRNA expression on day 21: mRNA levels for IL-6 were increased by 9.4-fold, IL-8 was increased by 929.5-fold, G-CSF was increased by 559.4-fold, CCL8 was increased by 163.8-fold, CXCL10 was increased by 43.9-fold, and TNF-α was increased by 139.5-fold (Figure 4(b), $P < 0.001$). In contrast, the mRNA expression of several proinflammatory cytokines was significantly reduced during adipogenic differentiation of ASCs: IL-1 (−4.2-fold on day 14 and −2.4-fold on day 21, $P < 0.001$), LIF (−5.8-fold on day 14, $P < 0.001$), and IFN-γ (−5.0-fold on day 14 and −3.6-fold on day 21, $P < 0.001$).

The analysis of anti-inflammatory cytokines expressed during adipogenic differentiation demonstrated significant differences in induction level that was time dependent. ADM increased gene expression of IL-1RA (1082.6-fold, $P < 0.001$), IL-13 (14.9-fold, $P < 0.001$), IL-18BP (10.2-fold, $P < 0.001$), CCL2 (20.8-fold, $P < 0.001$), and COX2 (4.8-fold, $P < 0.001$) on day 7 (Figure 5(a)). In contrast, the mRNA expression of STC-1 and TSG6 was most significantly increased by 123.6-fold and 8.4-fold, respectively, on day 14 ($P < 0.001$, Figure 5(b)). Adipogenic differentiation of ASCs resulted in the most robust induction of IL-10 (8.8-fold, $P < 0.001$), IL-35 (40.1-fold, $P < 0.001$), TNFRSF1A (5.0-fold, $P < 0.001$), PTGES2 (4.6-fold, $P < 0.001$), and TGF-β (16.7-fold, $P < 0.001$, Figure 5(c)) on day 21. IL-11 and HGF mRNA expression were most significantly reduced on day 14 by −33.3-fold and −14.3-fold, respectively ($P < 0.001$, Figure 5(d)).

4. Discussion

The interest in ASCs for tissue engineering purposes and regenerative medicine has grown significantly due to their accessibility, abundance, and capacity to differentiate into mesenchymal lineage cells. While studies have begun to investigate the mechanism by which ASCs differentiate into adipogenic or osteogenic lineage cells, the precise role of inflammatory cytokines has not been explored extensively. The mRNA levels of many pro- and anti-inflammatory cytokines expressed by ASCs during osteogenic and adipogenic differentiations were assessed. ASCs undergoing osteogenic differentiation expressed a distinct panel of cytokines that differed from the cytokine profile of ASCs undergoing adipogenic differentiation at each of the time intervals analyzed.

A quantitative comparison of the proinflammatory cytokines and anti-inflammatory cytokines expressed during osteogenic differentiation of ASCs demonstrates three distinct groups of cytokines. These groups of cytokines are categorized based on their induction at early, mid, and late stages of osteogenic differentiation (Figure 6). While the current study investigated the inflammatory cytokines secreted during the osteogenic differentiation of these cells in a cocktail of growth factors, others have taken the approach of treating progenitor cells with a similar cocktail of growth factors and supplemented the medium with additional inflammatory cytokines, such as TNF-α and IL-1 [11–14]. Most of these studies were conducted in bone marrow-derived mesenchymal stem cells (BMSCs), which are derived from the mesodermal lineage and have a similar differentiation potential as ASCs. Human BMSCs treated with TNF-α resulted in the activation of NF-κB, leading to increased mineralization and enhanced expression of osteogenic proteins, such as BMP2 and alkaline phosphatase, and transcription factors such as RUNX2 and Osterix [11, 12]. Human BMSCs treated with IL-1, likewise, enhanced differentiation into osteoblasts through the Wnt-5a/receptor tyrosine kinase-like

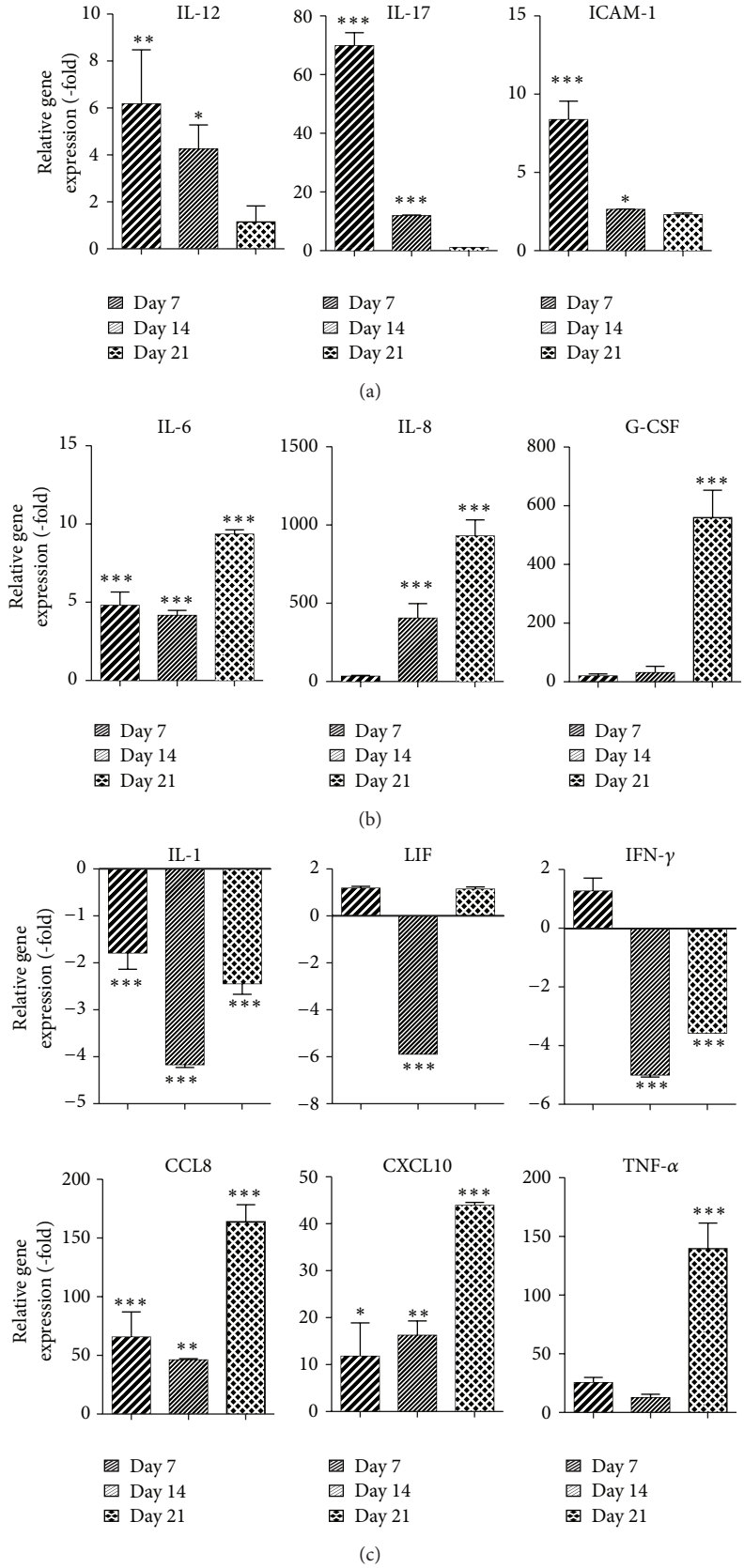
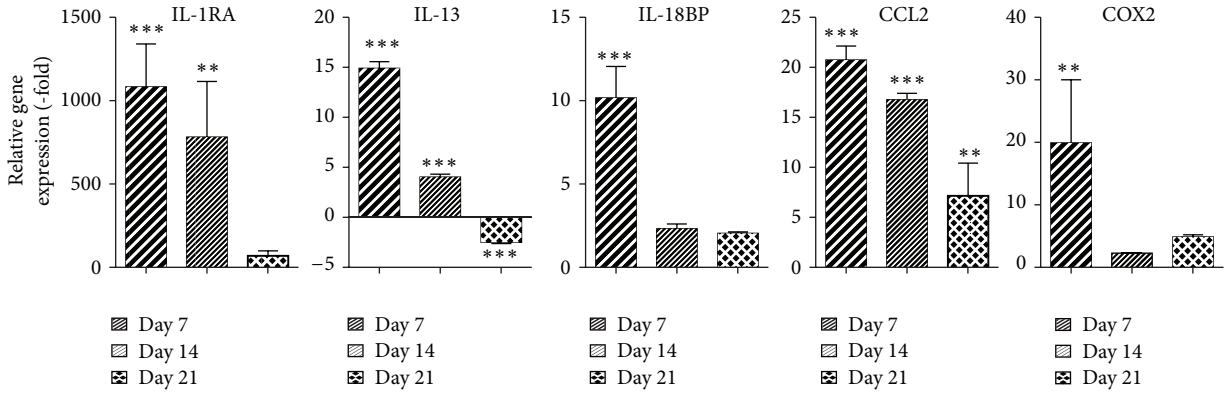


Figure 4: Levels of mRNA expression of proinflammatory cytokines are upregulated during adipogenic differentiation of ASCs. ASCs were grown in CCM and changed to ADM. Cells were harvested on days 7, 14, and 21 and analyzed by qRT-PCR. Data is normalized to undifferentiated cells. Mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ relative to undifferentiated cells.



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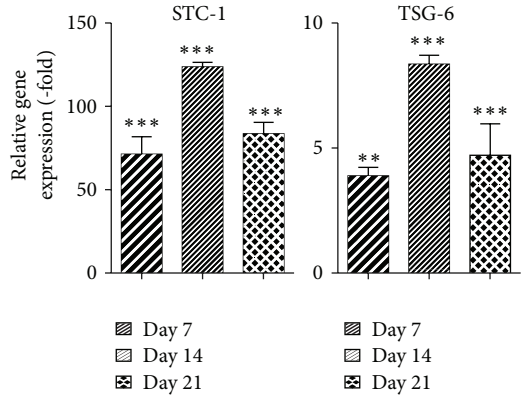

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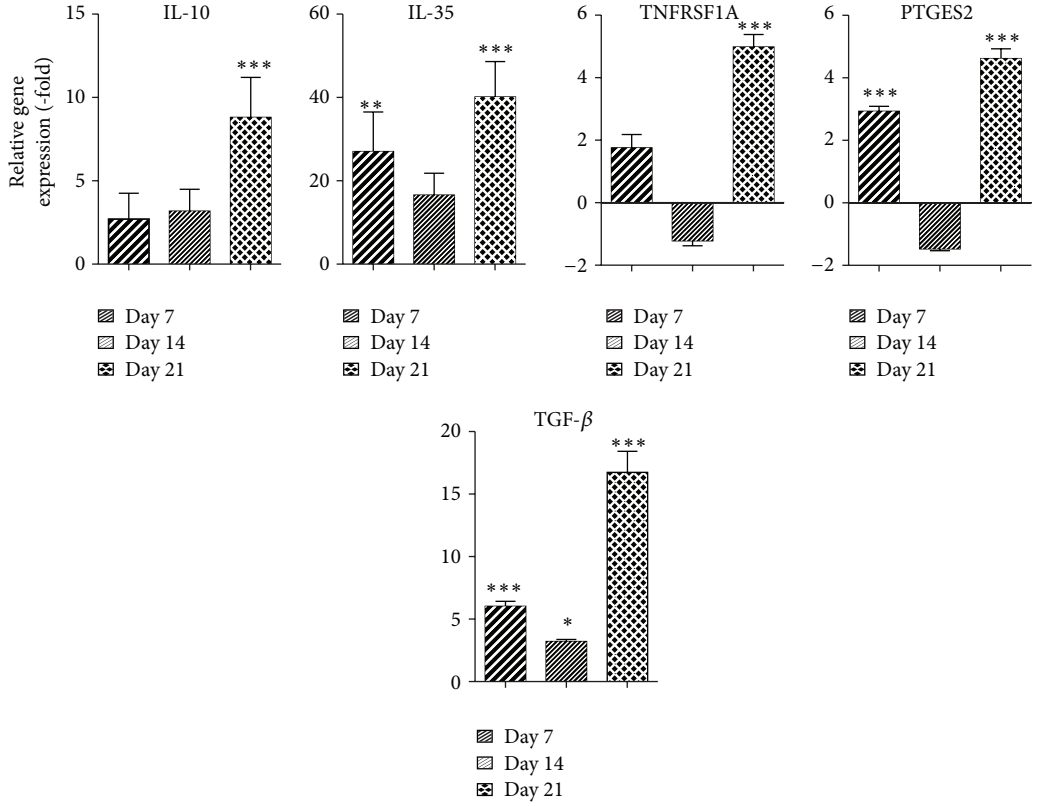

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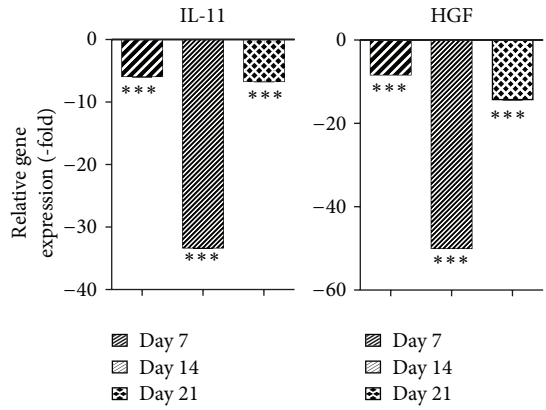

Figure 5: Levels of anti-inflammatory cytokines are elevated during adipogenic differentiation of ASCs. ASCs were grown in CCM and then changed to ADM. After 7, 14, and 21 days, cells were harvested and analyzed by qRT-PCR. Data is normalized to undifferentiated cells. Mean ± SEM. $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$ relative to undifferentiated cells.

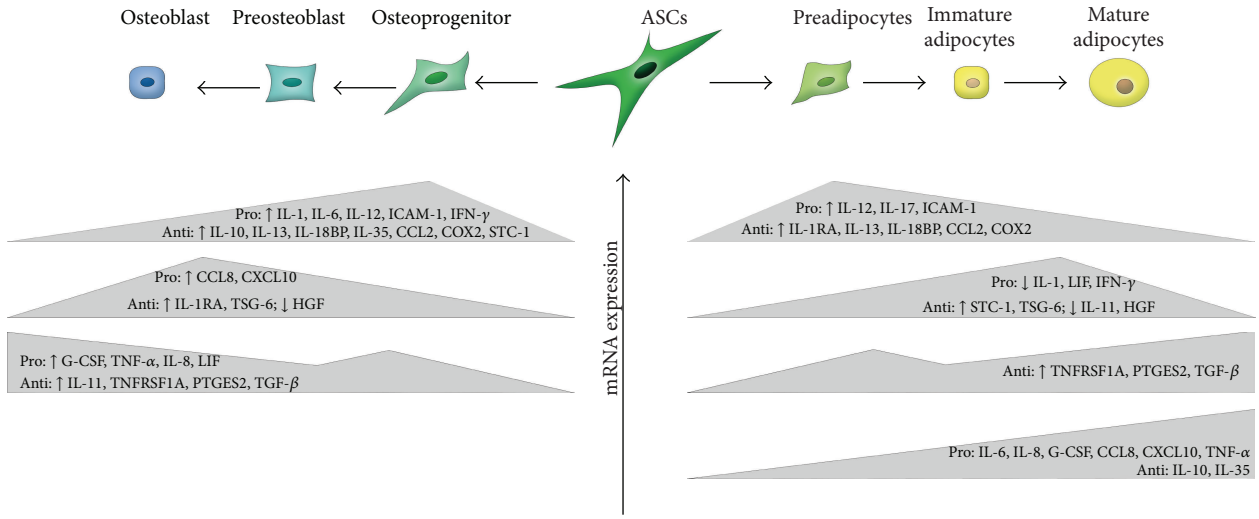

Figure 6: Schematic of ASCs undergoing osteogenic or adipogenic differentiation. Expression of proinflammatory and anti-inflammatory cytokines is dependent on the stage of differentiation.

orphan receptor 2 pathway [13]. Ferreira et al. found that IL-1 also enhanced mineralization through both nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and mitogen-activated protein kinase (MAPK) pathways [14]. The results presented in the current study are consistent with previously published reports showing an upregulation of TNF-α and IL-1 during differentiation. The present study supplements the current body of literature and highlights specific inflammatory factors that should be investigated further based on their induction during osteogenic differentiation. Additional studies, however, are necessary to elucidate the precise mechanism by which these cytokines effect osteogenic differentiation.

Furthermore, it should be noted that the effects of pro- and anti-inflammatory cytokines on osteogenesis might be determined by the physical location. For instance, cytokines have been shown to contribute to a decrease in bone mineral density by inhibiting osteoblast proliferation and differentiation and enhancing the rate of osteoclast differentiation in patients with severe inflammatory disease [15, 16]. Thus, patients diagnosed with such diseases as rheumatoid arthritis and osteoarthritis have a higher incidence of osteoporosis [15, 16]. In contrast, inflammatory cytokines are strongly suspected to induce ectopic bone formation, for instance, in arteries during atherosclerosis or in postburn heterotopic ossification [17, 18]. Consistent with these observations, anti-inflammatory drugs have been shown to reduce the incidence and severity of ectopic bone formation [19].

With respect to adipogenesis, the current study demonstrated a time-dependent expression of cytokines in ASCs during adipogenic differentiation (Figure 6). While limited studies have been conducted on the cytokine profile of

ASCs undergoing adipogenic differentiation, it has been previously shown that cytokines such as IL-1, IL-6, and TNF-α have the ability to inhibit adipogenic differentiation of BMSCs. PPARγ is suppressed by IL-1 and TNF-α, and this suppression is mediated through NF-κB [20]. In the context of obesity, increased proinflammatory cytokines are secreted by the adipose tissue due to tissue hypoxia that results from hypertrophy and hyperplasia of adipocytes [21]. Based on previous reports, this increase in proinflammatory cytokines, such as IL-1 and TNF-α, should result in decreased adipogenesis. However, due to the obesity-associated dysregulation of adipocytes and ASCs, these cells no longer respond to these cytokines properly [22]. Thus, the increase in IL-1 and TNF-α does not inhibit adipogenesis. Additional studies focusing on the impact of other pro- and anti-inflammatory cytokines on adipogenesis will shed light on ASC differentiation for the purposes of soft tissue reconstruction. Furthermore, they may provide insight into what other factors mediate adipogenesis in the context of obesity.

5. Conclusion

The osteogenic and adipogenic differentiation of ASCs alter the expression of an array of cytokines. The levels of induction of these pro- and anti-inflammatory cytokines are dependent on the stage (early, middle, or late) and type (osteogenic or adipogenic) of differentiation. The data presented here provides a framework for understanding the role that cytokine expression may play in tissue engineering projects using ASCs. By understanding which cytokines are upregulated during osteogenic or adipogenic differentiation, it will be possible to specifically target these molecules to enhance osteogenic or adipogenic differentiation for soft tissue defects or bone defects. Furthermore, paracrine expression of these cytokines by differentiating ASCs may change the local environment. By understanding the local environment produced by differentiation ASCs, we will also be better equipped to assess the engraftment and survival of tissue engineered constructs prepared with ASCs.

Abbreviations

ADM: Adipogenic differentiation medium
APC: Allophycocyanin
ASCs: Adipose-derived stromal/stem cells
BSA: Bovine serum albumin
BMP: Bone morphogenic protein
C/EBP: CCAAT-enhancer-binding proteins
C/EBPα: CCAAT-enhancer-binding proteins alpha
C/EBPβ: CCAAT-enhancer-binding proteins beta
C/EBPδ: CCAAT-enhancer-binding proteins delta
CCL2: Chemokine (C-C motif) ligand 2
CCL8: Chemokine (C-C motif) ligand 8
CCM: Complete culture medium
COX2: Cyclooxygenase 2
CPC: Cetylpyridinium chloride
CTGF: Connective tissue growth factor
CXCL10: C-X-C motif chemokine 10
DKK-1: Dickkopf WNT signaling pathway inhibitor 1
DMEM/F-12: Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
FABP4: Fatty acid binding protein 4
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate
G-CSF: Granulocyte-colony stimulating factor
GLUT4: Glucose transporter 4
HGF: Hepatocyte growth factor
IGFBP3: Insulin-like growth factor binding protein 3
ICAM-1: Intercellular adhesion molecule 1
IFN-γ: Interferon gamma
IL-1: Interleukin-1
IL-1RA: Interleukin-1 receptor antagonist
IL-6: Interleukin-6
IL-8: Interleukin-8
IL-10: Interleukin-10
IL-11: Interleukin-11
IL-12: Interleukin-12
IL-13: Interleukin-13
IL-17: Interleukin-17
IL-18BP: Interleukin-18 binding protein
IL-35: Interleukin-35
LIF: Leukemia inhibitory factor
LPL: Lipoprotein lipase
MAPK: Mitogen-activated protein kinase
NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells
ODM: Osteogenic differentiation medium
PBS: Phosphate buffered saline
PDGFR-β: Platelet-derived growth factor receptor beta
PE: Phycoerythrin
PPARδ: Peroxisome proliferator-activated receptor delta
PPARγ: Peroxisome proliferator-activated receptor gamma
PTGES2: Prostaglandin E synthase 2
RUNX2: Runt-related transcription factor 2
STC-1: Stanniocalcin 1
TGF-β: Transforming growth factor beta
TNF-α: Tumor necrosis factor alpha
TNFRSF1A: Tumor necrosis factor receptor superfamily member 1A
TSG-6: Tumor necrosis factor-stimulated gene 6
Wnt: Wingless type.

Conflict of Interests

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

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Research Article

SDF-1/CXCR4 Axis Promotes MSCs to Repair Liver Injury Partially through Trans-Differentiation and Fusion with Hepatocytes

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MSCs have become a popular target for developing end-stage liver therapies. In this study, two models of bone marrow chimeric mice were used to construct the liver failure models. Then it was found that MSCs can transdifferentiate into hepatocyte-like cells and these hepatocyte-like cells can significantly express albumin. Furthermore it was also found that MSCs can fuse with the hepatocytes and these cells had the proliferation activity. However, the percentage of transdifferentiation was significantly higher than fusion. So it was considered that MSCs which transdifferentiated into hepatocyte-likes cells played important roles for repairing the injuring liver function.

1. Introduction

End-stage liver disease (ELSD) is an irreversible condition that leads to the imminent complete failure of the liver [1]. It is often a consequence of chronic liver diseases and is one of the most common causes of death in China. ESLD may be the final stage of many liver diseases. Cirrhosis, viral hepatitis, genetic disorders, autoimmune disorder, toxins, and drugs are all factors that cause ESLD and liver failure [2]. Studies have shown that patients with ESLD are at high mortality risk because of a high incidence of concomitant infection and renal and respiratory failure [2]. The effective therapy for patients with ESLD is liver transplantation [3]. However, many drawbacks such as the relative shortage of donors, operative risk, posttransplant rejection, recidivism of the preexisting liver disease, and high cost limit this technology [4]. Therefore, the exploration of new therapeutic approaches is necessary.

In recent years, bone marrow-derived mesenchymal stem cells (MSCs) have been a popular topic in regenerative medicine and have generated a great amount of enthusiasm as a therapeutic paradigm for a series of diseases. MSCs are a subset of plastic adherent nonhematopoietic stem cells and are characterized by their ability for self-renewal and differentiation into multiple cell types, such as osteoblasts, adipocytes, and chondrocytes [5]. As early as in 1968, it was first discovered that MSCs in bone marrow can differentiate into bone [6]. Subsequently, several studies have demonstrated that the intravenous delivery of MSCs results in their migration to the injury site, such as bone or cartilage fracture, myocardial infarction, and ischemic brain damage [7–11].

Moreover, certain studies have also reported that MSCs can be used to treat a series of liver diseases including cirrhosis, liver fibrosis, and hepatic ischemia reperfusion [12–15]. For example, Peng and colleagues found that the levels of albulin (ALB), total bilirubin (TBIL), and prothrombin time (PT) and the model for end-stage liver disease (MELD) score of patients in a MSC transplantation group markedly improved 2-3 weeks after transplantation compared to the control group [12]. However, the mechanism of repair

remains unclear. How the MSCs transformed into hepatocytes, by differentiation into or fusion with hepatocytes, or neither, is unknown. The results are still controversial [16–20]. In this study, we will demonstrate that MSCs can both differentiate into and fuse with hepatocytes to repair liver damage. In addition, we verified that the SDF-1/CXCR4 axis plays an important role in promoting MSC migration.

2. Materials and Methods

2.1. MSC Transplantation to Generate a Chimeric Mouse Model. Eight-week-old female wild type C57BL/6 mice were obtained from the Third Military Medical University. The animals were housed in a temperature- and humidity-controlled environment with a 12 h light/12 h dark cycle with food (standard laboratory chow) and water available ad libitum. All animal experiments were approved by the Animal Care and Use Committee of the Third Military Medical University and were performed in compliance with the "Guide for the Care and Use of Laboratory Animals" published by the National Institutes of Health.

To determine if MSCs can differentiate into hepatocyte-like cells, we constructed a chimeric mouse model as previously reported [21]. In brief, MSCs were first dissociated from wild type male C57BL/6 mice. Then, the MSCs were injected into the tail veins of female recipient mice, which had been exposed to a 10 Gy whole-body irradiation using a Co^{60} source (Theratron-780 model; MDS Nordion, Ottawa, ON, Canada).

To determine if MSCs can fuse with hepatocytes, we constructed another chimeric mouse model. In brief, the MSCs were dissociated from GFP^+ transgenic female C57BL/6 mice and then injected into the irradiated male recipient mice, which had been exposed to a 10 Gy whole-body irradiation. After 20 days, qRT-PCR with peripheral blood cells was used to confirm the chimeric mouse. The process was performed as previously described [22, 23].

2.2. Construction of the Acute Liver Damage Model. An acute liver damage model was established 20 days after MSC transplantation as previously described [24]. The mice were injected with CCl_4 (Sigma, USA) in the abdominal cavity at a dose of 15 μL/g body weight of 0.3%, 0.6%, 0.8%, or 1% CCl_4 dissolved in peanut oil (Shandong Luhua Group, China). The degree of liver damage was confirmed by histology. On days 2, 3, 4, 7, 14, 21, and 28, damaged liver tissues were collected, and a portion was used for fluorescence in situ hybridization (FISH) and immunofluorescence (IF). Another portion was used for Enzyme-Linked Immune Sorbent Assay (ELISA) to detect the changes in cytokines.

2.3. Double Staining by FISH and IF. To study whether exogenous MSCs can differentiate into hepatocyte-like cells or fuse with hepatocytes, we used double staining by FISH and IF analysis as previously described by Luo et al. [25]. In brief, the tissue sections were incubated with the primary anti-ALB monoclonal antibody (1 : 50 Santa Cruz, USA) or anti-GFP monoclonal antibody (1 : 50 Santa Cruz, USA) at 4°C overnight. On the second day, DNA of the Y chromosome was denatured, and the hybridization process was performed following the manufacturer's protocol (StarFISH Cambio, England). The break-apart probe set included DNA fragments against the Y chromosome probes labeled with Cy3 (Cambio, Dry Drayton, UK). On the third day, the slides were washed to remove unbound DNA sequences. Then, the sections were incubated with the secondary antibody of FITC-labeled goat anti-rat IgG (1 : 50 Invitrogen, USA). After washing, the slides were mounted in 40, 6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA), which specifically stains nuclei. Digital images were captured using a confocal laser-scanning microscope with appropriate filters (Leica Biosystems, Wetzlar, Germany) in ten random fields. Experiments were performed in triplicate.

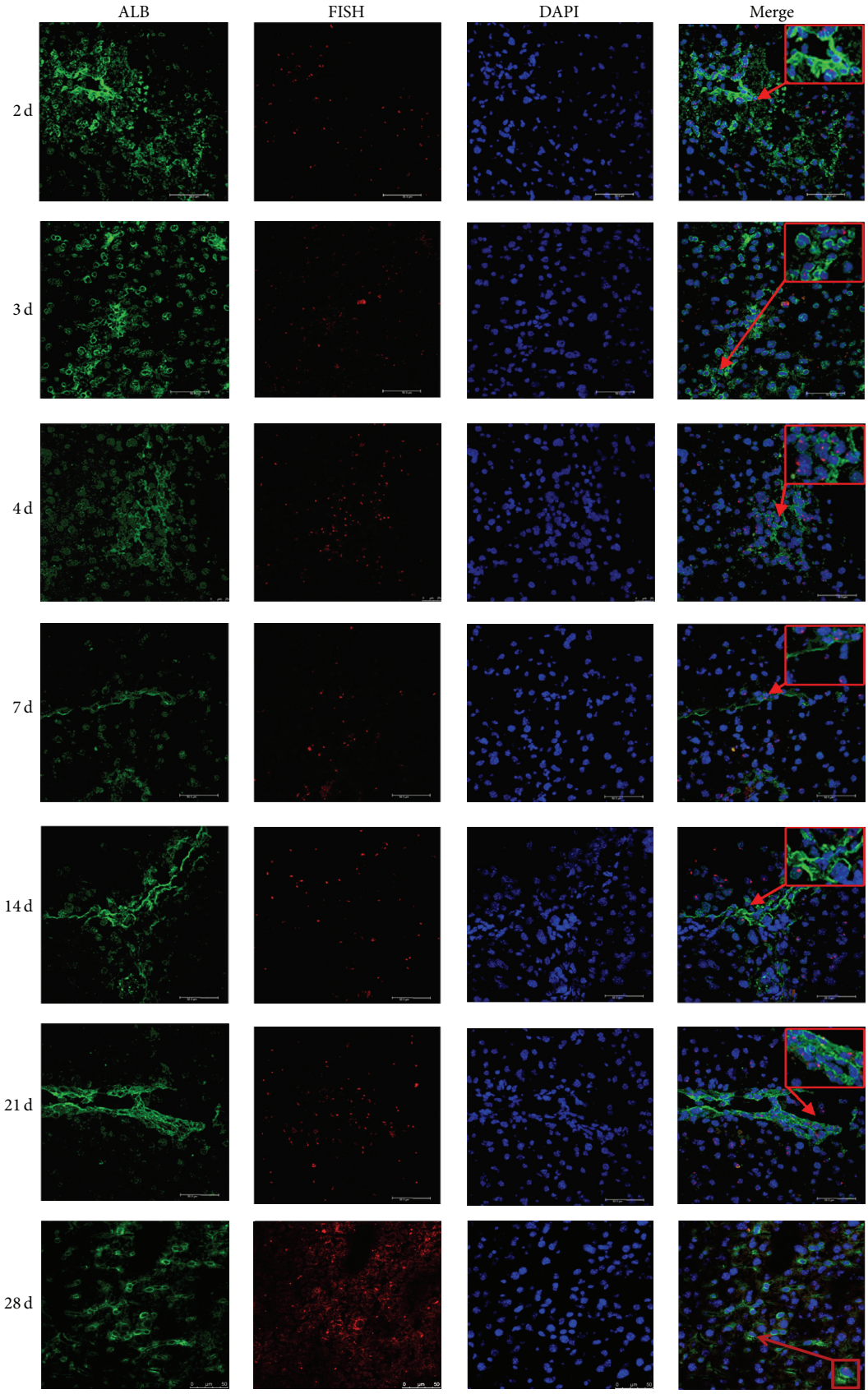
2.4. ELISA. The liver tissues collected on days 0, 2, 3, 4, 7, 14, 21, and 28 after injury were lysed as preciously described [26]. ELISA analysis was used to assess the expression of SDF-1 and CXCR4 in the lysates. Analysis was performed following the manufacturer's protocol for the Duoset ELISA Development kit (R&D Systems, Minneapolis, MN). Optical density (OD) values were read on the Synergy microplate reader (Biotek, Winooski, VT) at 450 nm, and a standard curve was constructed using the provided standards for the quantification of ODs for individual samples.

2.5. In Vivo Chemotaxis Analysis. To analyze the role of SDF-1 in the migration of MSC, 1×10^6 MSCs were preincubated with the SDF-1 inhibitor 17-AAG (Cayman Chemical, Ann Arbor, MI; 20 ng/mL) or PBS for 30 min and injected into the tail veins of bone marrow-destroyed female C57BL/6 mice [27]. The ALI model was created using previously reported methods [24]. FISH and IF were conducted at day 21 after injury.

2.6. Statistical Analysis. 50 mice were included into these experiments; each group contains at least 3 mice. 5 slices were randomly selected for all the samples and 5 pictures were randomly taken for every slice. Three authors counted the number of both positive cells and total cells. The data are expressed as the mean ± SEM. Student's paired t-test was performed for the comparison of data of paired samples, analysis of variance was used for multiple group comparisons, and a Bonferroni posttest was used to determine differences between groups. For all analyses, differences were considered significant at $P < 0.05$. All statistical analyses were performed using the Statistical Program for Social Sciences 13.0 software program (SPSS Inc., Chicago, IL).

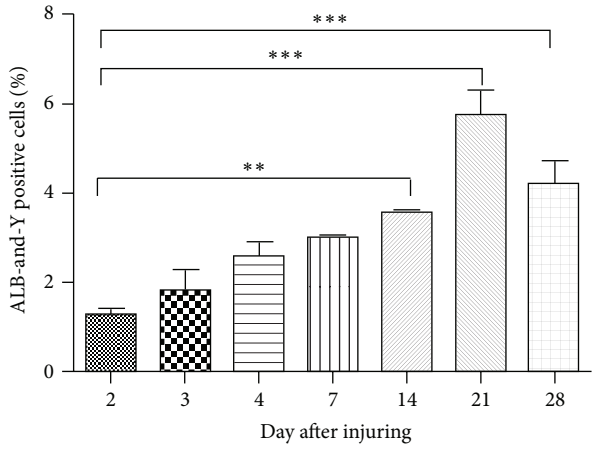
3. Results

3.1. MSCs Were Recruited into Injured Tissue and Transdifferentiated into Hepatocyte-Like Cells. MSCs isolated from male C57BL/6 mice were generously provided by Dr. Guangping Liang (Institute of Burn Research, Third Military Medical University, China). These MSCs were identified as CD44, CD29, and SCA-1 positive but CD117 negative by flow



(a)

Figure 1: Continued.



(b)

Figure 1: MSCs recruited to injured tissue and transdifferentiated into hepatocyte-like cells. (a) The liver specimens were collected on days 2, 3, 4, 7, 14, 21, and 28 after injury. FISH was used to detect Y-chromosome-positive cells, and IF was used to detect ALB-positive cells. The nuclei were stained blue (DAPI). The images were then merged. Bar: 75 μm. Arrow was directed to the double positive cells. All the experiments were repeated three times, at least 3 samples were included in each group. (b) Quantitative data of the ALB and Y-chromosome-positive cells in IHC of different days after injuring ($^{**}P < 0.01$; $^{***}P < 0.001$).

cytometry analyses and were capable of differentiating into adipocytes, chondrocytes, and osteoblasts in vitro [21, 28]. Twenty days after transplantation with these MSCs, the mice were injected with CCl_4 in the abdominal cavity. We determined that 0.3% CCl_4 induced the most damage with a large area of cell degeneration and necrosis. Thus, in subsequent experiments we injected 0.3% CCl_4 into the abdominal cavity to construct the ALI model.

To determine if MSCs can transdifferentiate into hepatocyte-like cells, on days 2, 3, 4, 7, 14, 21, and 28 after CCl_4 injection into chimeric mice, liver specimens were harvested. Because Y-chromosome-positive cells were from the male mice, which represent the MSCs, and ALB is a specific marker for hepatocytes, we used FISH to detect the Y-chromosome-positive cells and IF to detect the ALB-positive cells. As shown in Figure 1(a), expression of the Y chromosome and ALB were detected in the same cells of the damaged liver using laser confocal microscopy. The population of Y-chromosome-positive cells ranged from 13.96% to 18.13%, while the population of both Y-chromosome- and ALB-positive cells ranged from 3.37% to 5.85% (Figure 1(b)). These results show that MSCs can differentiate to hepatocytes.

3.2. MSCs Facilitated Liver Repair Partly through Fusion with Primary Hepatocytes. To determine if MSCs facilitated liver repair through fusion with primary hepatocytes, we constructed a male chimeric mouse model as previously described. In brief, the MSCs were extracted from GFP transgenic female mice, which resulted in the GFP labeling of MSCs in the chimeric mouse model.

On days 14 and 21 after CCl_4 injection, FISH and IF were used to detect Y-chromosome- and GFP-positive cells. As shown in Figure 2(a), expression of the Y chromosome and GFP could be detected in the same cells of the damaged liver using laser confocal microscopy, which suggests that MSCs can fuse with primary hepatocytes. The population of both Y-chromosome- and GFP-positive cells ranged from 0.32% to 0.87% (Figure 2(b)). In addition, we also detected GFP- and Ki67-positive cells with FISH and IF on days 3, 14, and 21. GFP and Ki67 were expressed in the same cells of the damaged liver, which suggests that the MSCs that migrated to the liver have proliferative activity (Figures 3(a) and 3(b)). Together, these results show that MSCs can fuse with hepatocytes and maintain proliferative activity.

3.3. SDF-1/CXCR4 Axis Plays an Important Role in MSC Migration to the Injured Liver. Recent studies have reported that the chemokine SDF-1 and its receptor CXCR4 play a pivotal role in the migration, chemotaxis, homing, and transdifferentiation of MSCs [20]. Therefore, we determined the concentration of SDF-1 and CXCR4 on days 0, 2, 3, 4, 7, 14, 21, and 28 by ELLISA. As shown in Figure 4(a), the concentration of SDF-1 gradually increased and reached its peak on day 21. Consistent with SDF-1, the expression of CXCR4 was also significantly elevated on day 21 (Figure 4(b)).

To further study the role of SDF-1/CXCR4 axis in the migration of MSCs, the female chimeric mice were divided into two groups. One group was injected with MSCs treated with the SDF-1 inhibitor 17-AAG, and the other group was injected with MSCs treated with PBS. On day 21, liver specimens were harvested and analyzed using FISH and IF. As shown in Figures 4(c) and 4(d), Y-chromosome- and ALB-positive cells were notably reduced in the 17-AAG group compared to the PBS group, which suggests that 17-AAG inhibited the migration of MSCs to the damaged liver.

4. Discussion

In 1999, Petersen and colleagues found that BM-derived cells may act as the progenitor of several types of liver cells under

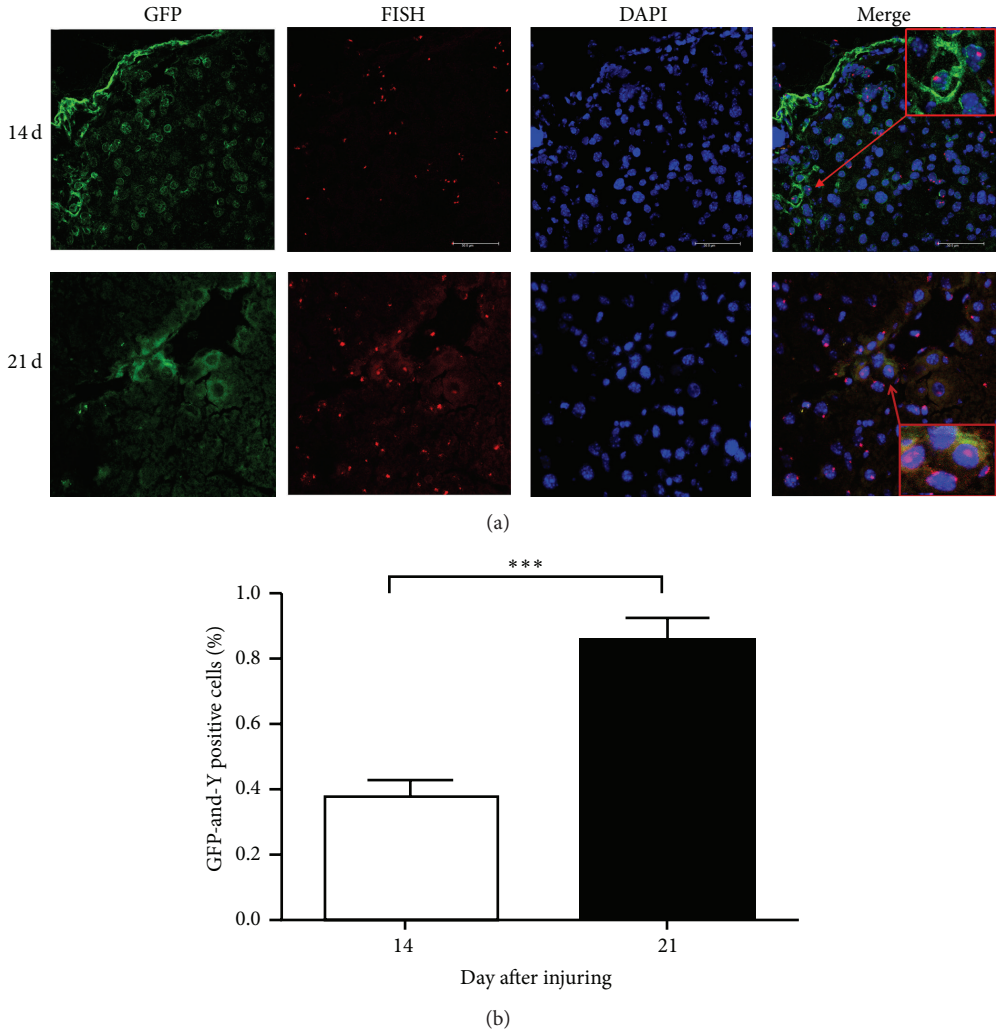


Figure 2: MSCs recruited to injured tissue and fused with hepatocytes. (a) The liver specimens were collected on days 14 and 21 after injury. FISH was used to detect Y-chromosome-positive cells, and IF was used to detect GFP-positive cells. The nuclei were stained blue (DAPI). The images were then merged. Bar: 75 μm. Arrow was directed to the double positive cells. All the experiments were repeated three times, at least 3 samples were included in each group. (b) Quantitative data of the GFP and Y-chromosome-positive cells in IHC of different days after injuring ($^{***}P < 0.001$).

certain physiopathological conditions [29]. Shortly after that, Theise et al. also reported similar results in humans [30]. Liver specimens were obtained from 2 female recipients of therapeutic bone marrow transplants with male donors and from 4 male recipients of orthotopic liver transplants from female donors. Using FISH and diaminobenzidine (DAB) stain, it was found that Y-positive hepatocytes and cholangiocytes could be identified in all study specimens, which suggested that human hepatocytes and cholangiocytes can be derived from extrahepatic circulating stem cells, most likely of bone marrow origin [30]. However, it was still unclear how MSCs changed into hepatocytes. Recently, certain studies have demonstrated that MSCs have the ability to differentiate into cells with hepatocyte-like phenotypes [31–33]. For example, Sato and colleagues had found that MSCs were more potent than $CD34^+$ cells and non-MSCs/$CD34^-$ cells in differentiating into hepatocytes [31]. These results were consistent with our findings. In the female chimeric mouse model, MSCs could differentiate into hepatocytes and were able to secrete albumin (Figure 1). In addition, using a male chimeric mouse model, it was found that MSCs could also fuse with hepatocytes to repair liver damage (Figure 2). However, these results apparently contradicted those by Sato, who showed that no evidence was found for MSCs fusing with hepatocytes [31]. This contradiction may be a result of Sato using human MSCs in rat model, which may lead to interspecies hybrids during cell division. Interspecies hybrids will "kick out" or loosen chromosomes of one or the other species because the chromosome complement of interspecies hybrids is not stable [34]. In addition, we also found that the differentiation rate was significantly higher than that of fusion rate, which suggests that differentiation rather than fusion is the main pathway for repairing the damaged liver.

Chemokines play important roles in controlling cell migration. It has been reported that the SDF-1/CXCR4 axis is constitutively expressed in a wide range of tissues such

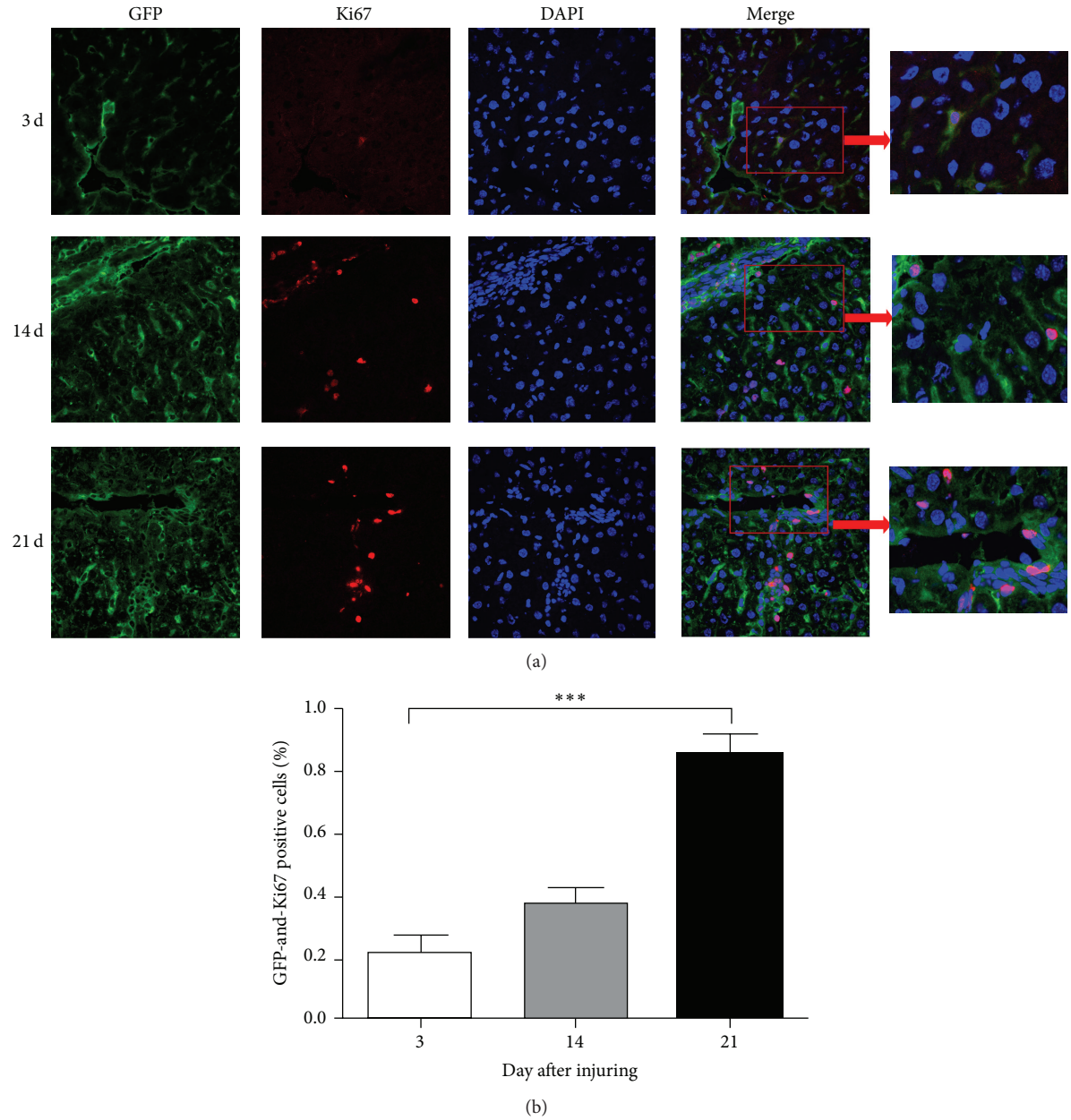


Figure 3: MSCs migrated to the damaged liver and had the proliferative activity. (a) IF analysis of Ki-67 expression (red) to visualize primary hepatocyte cells indicated that some of the donor GFP$^+$ MSCs (green) are overlapped. Nuclear DAPI (blue) staining was used as a counterstain. The images were then merged. Bar: 75 μm. Arrow was directed to the double positive cells. All the experiments were repeated three times, at least 3 samples were included in each group. (b) Quantitative data of the GFP and Ki67 positive cells in IHC of different days after injuring ($^{***}P < 0.001$).

as the brain, heart, kidney, liver, lung, and spleen and also involved in several diseases such as rheumatoid arthritis, ischemic cardiomyopathy, and several brain diseases [35–37]. Moreover, studies have demonstrated that the interaction of SDF-1 with its receptor CXCR4 plays a role in mediating MSC migration to the site of injury [38, 39]. Although it is considered that CXCR4 is expressed primarily in the cell rather than on the surface, it has been assumed that the majority of intracellular CXCR4 in MSCs is mobilized to the cell surface during cytokine stimulation [40, 41]. Furthermore, various organs increase the expression of SDF-1 when responding to tissue damage, such as irradiation, hypoxia, or toxic agent exposure [42, 43]. Therefore, we assume that CCl_4-induced liver injury is a strong promoter of SDF-1 expression and CXCR4 mobilization. Figures 4(a) and 4(b) show an increase in SDF-1 and CXCR4 concentrations at day 7, which peaked at day 21. Notably, as shown in Figures 4(c) and 4(d), the migration of MSCs significantly decreased when treated with the SDF-1 inhibitor 17-AAG. These data demonstrate that the SDF-1/CXCR4 axis plays an important role in MSC migration

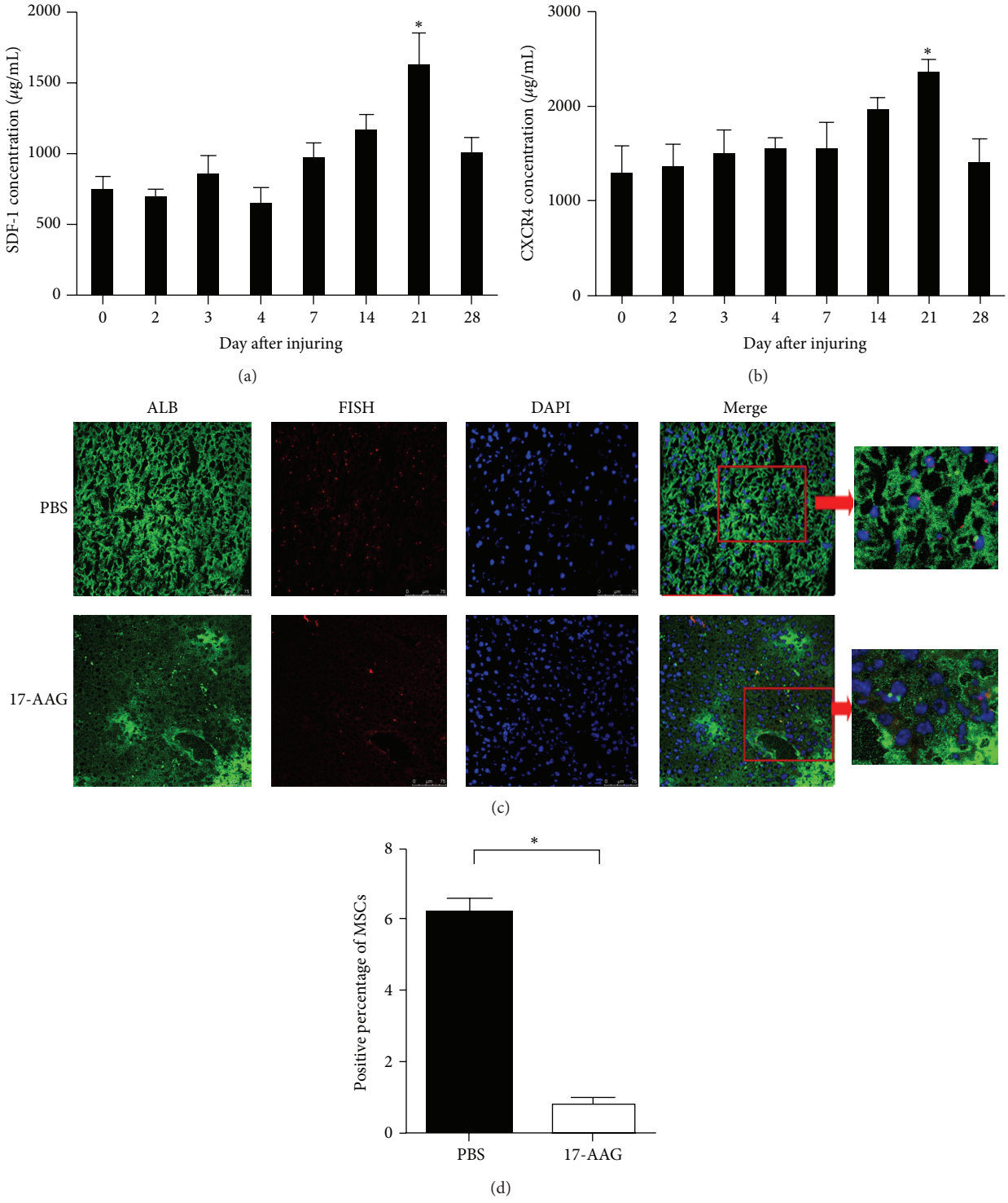


Figure 4: The CXCL12-CXCR4 axis plays an important role in the chemotaxis of MSCs. (a-b) ELISA was used to detect SDF-1 and CXCR4 protein levels in the damaged liver tissue at different times after injury. The data are the mean ± SEM from three independent experiments ($^*P < 0.05$ compared with day 0). (c-d) 1×10^6 MSCs were preincubated with 17-AAG (SDF-1 inhibitor, 20 ng/mL) or PBS for 30 min and injected into the tail veins of bone marrow-destroyed female C57BL/6 mice. Then, liver damage was induced in the mice, and tissue sections were collected 21 days after injury. (c) IF was used to detect ALB-positive cells, and FISH was used to identify the Y chromosome-positive cells. Bar: 75 μm. Arrow was directed to the double positive cells. (d) Both ALB- and FISH-positive cells were counted. All the experiments were repeated three times, at least 3 samples were included in each group.

to the injured liver for repair. Furthermore, recent study also found that MSCs secreted scrapie responsive gene 1 (SCRG1) and its receptor bone marrow stromal cell antigen 1 (BST1) played important roles for cell self-renew and migration [44]. However, it still needs further study to find its role in MSCs treatment.

However, our results cannot completely explain the curative effects of MSCs for ELSD in the clinical context. A recent study reported that despite the role of transdifferentiation and fusion, MSCs also possess another attractive ability for cell-based therapies. An increasing number of studies have shown that MSCs have potent immunosuppressive qualities [45]. English et al. found that MSCs cocultured with purified $CD4^+$ T cells result in a significant increase in $FoxP3^+CD25^+CD4^+$ T cells, while MSC-derived factors such as TGFβ and PEG2 play important roles during induction [46]. In addition, recent study also found that MSCs secreted VCAM-1 also played important roles to induce immunosuppression environment [47]. So our future studies will focus on how MSCs induce an immunosuppressing microenvironment to repair the damaged liver.

In conclusion, we report that MSCs can repair the damaged liver by differentiating into and fusing with hepatocytes. Among these two, MSCs differentiating into hepatocytes is the main pathway in repairing the damaged liver. Furthermore, we also found that the SDF-1/CXCR4 axis plays an important role in the chemotaxis, homing, and differentiation of MSCs to the injury site.

Abbreviations

MSCs: Mesenchymal stem cells
ESLD: End-stage liver disease
CCl_4: Carbon tetrachloride
ALB: Albumin
TBIL: Total bilirubin
PT: Prothrombin time
MELD: Model for end-stage liver disease
SDF-1: Stroma-derived factor-1
CXCR4: Chemokine (C-X-C motif) receptor 4
GFP: Green fluorescent protein
ELISA: Enzyme-linked immunosorbent assay
FISH: Fluorescence in situ hybridization
IF: Immunofluorescence.

Conflict of Interests

The authors had no conflict of interests.

Authors' Contribution

Ning-Bo Hao and Chang-Zhu Li contributed equally to this study.

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Research Article

Adipose-Derived Mesenchymal Stem Cell Exosomes Suppress Hepatocellular Carcinoma Growth in a Rat Model: Apparent Diffusion Coefficient, Natural Killer T-Cell Responses, and Histopathological Features

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We sought to evaluate the effects of adipose-derived mesenchymal stem cells (ADMSCs) exosomes on hepatocellular carcinoma (HCC) in rats using apparent diffusion coefficient (ADC), natural killer T-cell (NKT-cell) responses, and histopathological features. ADMSC-derived exosomes appeared as nanoparticles (30–90 nm) on electron microscopy and were positive for CD63, tumor susceptibility gene-101, and β-catenin on western blotting. The control ($n = 8$) and exosome-treated ($n = 8$) rats with N1S1-induced HCC underwent baseline and posttreatment day 10 and day 20 magnetic resonance imaging and measurement of ADC. Magnetic resonance imaging showed rapidly enlarged HCCs with low ADCs in the controls. The exosome-treated rats showed partial but nonsignificant tumor reduction, and significant ADC and ADC ratio increases on day 10. On day 20, the exosome-treated rats harbored significantly smaller tumors and volume ratios, higher ADC and ADC ratios, more circulating and intratumoral NKT-cells, and low-grade HCC ($P < 0.05$ for all comparisons) compared to the controls. The ADC and volume ratios exhibited significant inverse correlations ($P < 0.001$, $R^2 = 0.679$). ADMSC-derived exosomes promoted NKT-cell antitumor responses in rats, thereby facilitating HCC suppression, early ADC increase, and low-grade tumor differentiation. ADC may be an early biomarker of treatment response.

1. Introduction

Hepatocellular carcinoma (HCC) is the sixth most common cancer and the third most frequent cause of cancer-related death [1]. HCC treatment has greatly changed during the past decade. Surgery or ablation is effective for treating early HCC [1, 2]. Liver transplantation is beneficial for markedly cirrhotic liver with HCC [1, 3]. Transarterial chemoembolization (TACE), radioembolization, and targeted therapy may improve survival in individuals with advanced HCC [1, 4–6]. Unfortunately, the outcome of patients with advanced HCC remains far from being satisfactory [1, 4–6], and studies of more effective therapeutic strategies are essential.

Exosomes are nanoparticles (30–100 nm) produced by reverse budding of multivesicular bodies, fusion with plasma membranes, and secretion from the surfaces of cells into the extracellular space where they enter the vascular system or various biological fluids [7]. Exosomes from tumor cells

may affect the immune system via the suppression of T-lymphocytes, natural killer cells, and mature dendritic cells. Exosomes from normal immune cells may trigger antitumor responses resulting in the immunosuppression of cancer [7, 8]. Liver is an organ of innate immunity with abundant lymphocytes and is rich in natural killer T-cells (NKT-cells) [9, 10]. Although the effect of stem cells on tumor growth is controversial, recent studies demonstrated the inhibitory effects of mesenchymal stem cells on HCC [11, 12]. However, the effects of stem cell-derived exosomes on liver immunity and suppression of HCC have not been highly investigated.

In patients with advanced HCC, modified Response Evaluation Criteria in Solid Tumors (mRECIST) and the European Association for the Study of the Liver (EASL) criteria are commonly used to assess the treatment response after TACE by measuring the dimensions of the enhanced components [13, 14]. Diffusion-weighted (DW) imaging allows for the assessment of water molecule motion to monitor treatment-associated alterations in the tumor microenvironment. Quantification of the changes in water diffusion, the apparent diffusion coefficient (ADC), has been advocated as a better cellular biomarker than MR morphological criteria for assessing advanced HCC [15–17]. The correlation of ADC values with histologic grades of HCC differentiation has also been reported [18, 19]. However, application of ADC as a biomarker for the assessment of cell-based therapies of cancer has not been described. We hypothesized that exosomes purified from the culture medium of adipose-derived mesenchymal stem cells (ADMSC) may promote NKT-cell antitumor immunity. In our study, we used a rat model of HCC to determine the ADC changes during ADMSC-derived exosomes treatment, NKT-cell responses, and the correlated histopathological features observed during the suppression of tumorigenesis.

2. Materials and Methods

2.1. Animals. The Institutional Committee of Kaohsiung Chang Gung Memorial Hospital and Chang Gung University College of Medicine on Animal Care, Use, and Research approved all experimental procedures (Approval number 2011070502). Thirty male Fischer-344 (F344) rats (National Laboratory Animal Center, Taipei, Taiwan) weighing 150–200 g at 4 weeks of age were maintained in pathogen-free animal facilities (24°C ± 1, 55% ± 10 humidity) with water and commercial rat food provided ad libitum.

2.2. ADMSC Preparations and Cultures, Exosome Isolation, Electron Microscopy, and Exosome Protein Quantification and Characterization. The rats were anesthetized with inhalational isoflurane, and the adipose tissues surrounding the epididymis were dissected. The procedures for the ADMSC cultures and the isolation of exosomes from the culture medium were performed as previously described [10, 20] and are summarized in Figure 1. The exosomes isolated from all F344 rats were pooled for electron microscopic assessment, protein separation and characterization, and western blot analysis. For transmission electron microscopy (JEM2100, JOEL Inc., Peabody, MA), the isolated exosomes were pelleted, fixed in 2.5% glutaraldehyde in cacodylate buffer at 20°C for 1 hour, and stained with 2% uranyl acetate after 3 washes with phosphate buffered saline (PBS). The proteins in Dulbecco's modified Eagle medium (DMEM) (Gibco) supplemented with 10% serum before and after cell culture were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The exosomes produced by ADMSC in DMEM were purified and the proteins in different exosome fractions (1 μg, 2 μg, 10 μg, and 50 μg) were also separated by SDS-PAGE. The gel was stained with Coomassie blue for analysis. For western blot analysis of the culture medium, conditioned medium, and exosome fractions, the following primary antibodies were used: mouse monoclonal anti-CD63 (Santa Cruz Biotechnology), rabbit polyclonal antitumor susceptibility gene-101 (TSG101) (Abcam), and anti-β-catenin (Abcam).

2.3. Tumor Cell Culture and Cell Inoculation. N1S1 rat HCC cells (CRL-1603; ATCC, Manassas, VA) were cultured in Iscove's modified Dulbecco medium (IMDM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco) and 0.1% streptomycin (Gibco) and passaged three times per week. Intravenous cyclosporine (20 mg/kg/day) was administered for four days prior to tumor induction. After anesthesia, the rat was restrained on a warm-pad at 37°C. After minilaparotomy, the left hepatic lobe was exposed and 2×10^6 N1S1-cells, with >97% cell viability as determined by trypan-blue exclusion, in 300 μL complete media were inoculated using a 22-gauge needle into the subcapsular site of the left lobe leading to pale-whitish discoloration around the point of injection. After sufficient hemostasis via gentle compression with a cotton-swab, the abdominal incision was closed followed by topical application of antibiotic ointment.

2.4. Blood Samplings, Rationale of Exosome Dosage, Exosome Treatment, and MR Imaging. The time points for blood sampling (0.5 mL of blood sampled via tail vein before HCC induction, 10 days after induction, and on posttreatment day 5 and day 15), exosome treatments (after baseline and on posttreatment day 10 MR imaging), and liver MR and DW imaging (baseline, posttreatment day 10 and day 20) are shown in Figure 2. The exosome dosage (100 μL exosomes with protein concentration 20 μg/μL) was based on a preliminary trial in 6 rats in which exosome was administered via penile vein at three different dosages (40 μg/μL; 20 μg/μL; 10 μg/μL; each in two rats). Two rats receiving the highest concentration (40 μg/μL) had penile phlebitis. Although no complications were noted in the rats treated with the other two concentrations, the time of injection was shorter while the degrees of tumor reduction were better in animals receiving 20 μg/μL as revealed in the explanted liver after the animals were sacrificed. Therefore, this dosage was utilized in the current study whereas an equal amount of culture medium was injected via penile vein in the control group. Longitudinal changes of the NKT-cells in the circulating blood were assessed using a FC500 flow cytometer (Beckman Coulter), immunocytochemical staining with purified

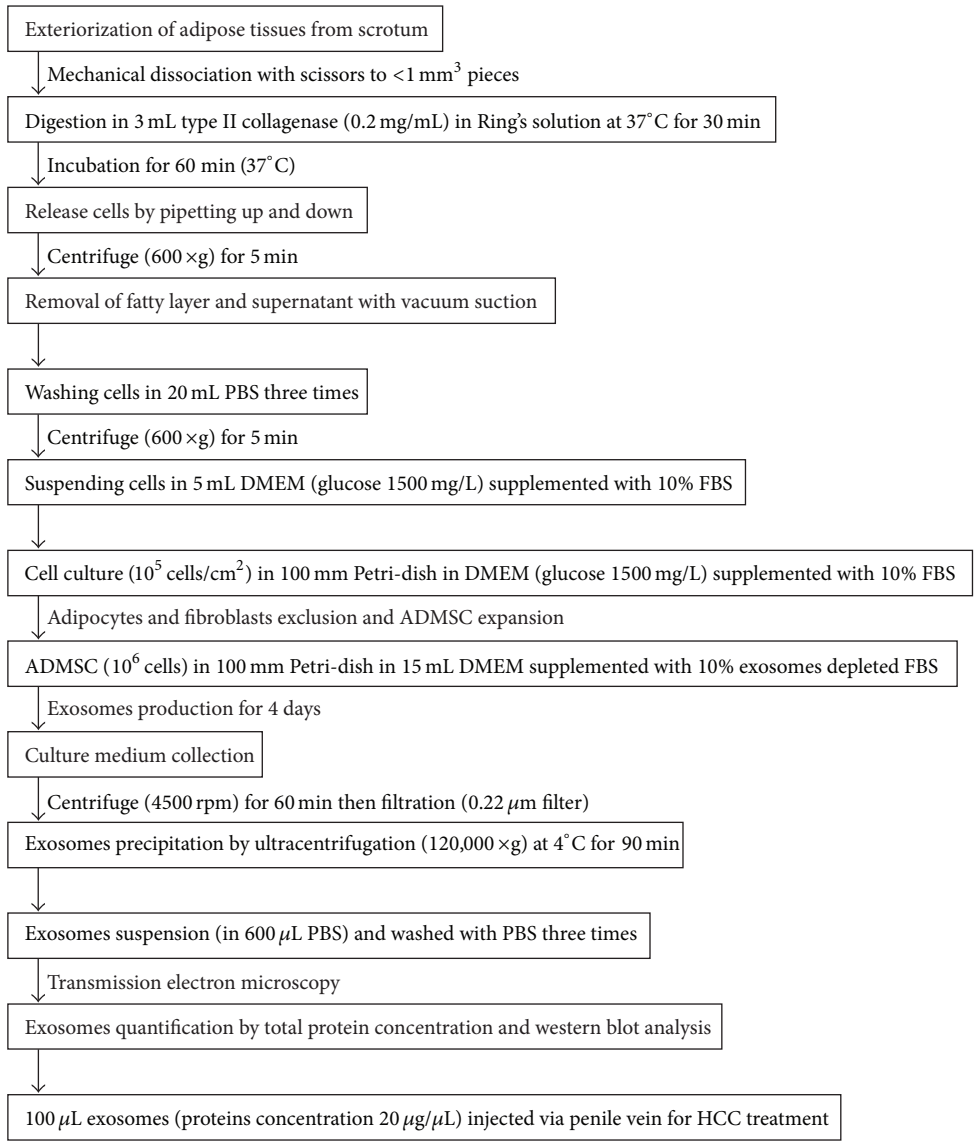


Figure 1: The flowchart shows the preparation and cultures of adipose-derived mesenchymal stem cells (ADMSCs), isolation of exosomes from culture medium, protein quantification and characterization of exosomes, and the final injection of exosomes via the penile vein for HCC treatment (min: minutes, g: gravity, PBS: phosphate buffered saline, DMEM: Dulbecco's modified Eagle's medium, FBS: fetal bovine serum, rpm: rotation per min).

anti-mouse CD3 antibody (1 : 500, BioLegend), and purified mouse anti-rat CD161a antibody (1 : 500, BD Pharmingen) for cellular positivity of CD3 (T-cells marker) and CD161 (NKT-cells marker) and CXP analysis software.

2.5. Liver MR Imaging. The liver MR imaging was performed using a 3.0 T MR imager (Signa VH3, GE HealthCare) and a Mayo Clinic BC-10 MRI coil. After anesthesia, the rat was placed in a supine position in a plastic holder. The imaging parameters are described in Table 1. The axial liver MR imaging included free-breathing precontrast T1- and T2-weighted, DW imaging (b = 0 and b = 800 sec/mm^2, with motion-sensitive gradients applied in three orthogonal directions to minimize the effects of diffusion anisotropy), and contrast-enhanced T1-weighted imaging (0.1 mmol/kg, Magnevist, Bayer-Schering). The HCC assessments were performed on a workstation (AW4.2; GE Healthcare) by the consensus of two experienced radiologists. The contours of the entire tumor on the enhanced T1-weighted images were manually drawn as regions of interest (ROIs), and the whole-tumor volume was determined. The ADC maps were generated using built-in software (Functool; GE Healthcare). The ROIs for whole-tumor volume measurement were also used for the ADC measurements. The day 10/baseline (D_{10}/baseline) and day 20/baseline (D_{20}/baseline) tumor volume ratios and the D_{10}/baseline and D_{20}/baseline ADC ratios were calculated.

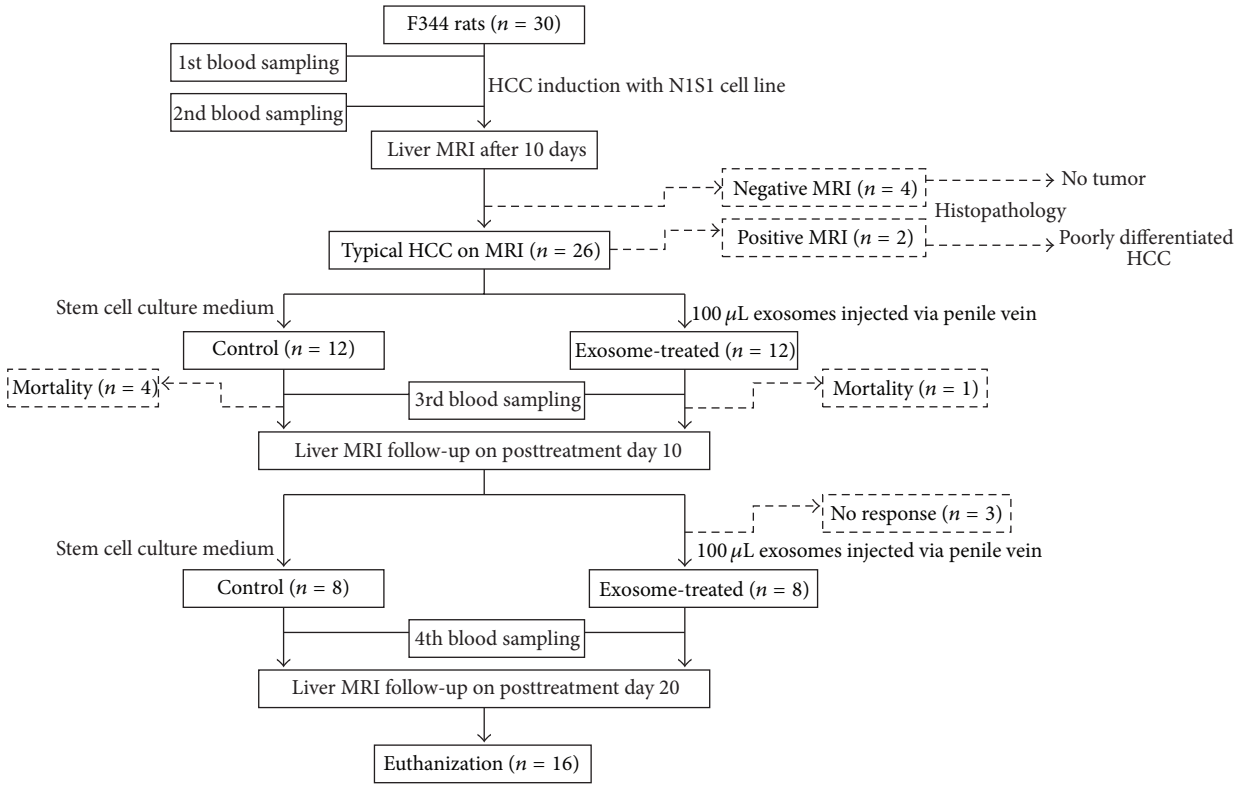


Figure 2: The flowchart shows the timetable for blood sampling (before and 10 days after HCC induction, posttreatment day 5 and day 15), exosome treatment (after baseline and posttreatment day 10 MR), liver MR and DW imaging (baseline, posttreatment day 10 and day 20), and final killing. Please note that two rats with typical HCC features and four rats with no HCC revealed on baseline MR imaging were killed for histopathological confirmation of MR findings.

2.6. Histopathological and Immunohistochemical Staining. Hematoxylin-eosin stained sections were blindly graded by a pathologist (20 years of experience) as grade I (well differentiated), grade II (moderately differentiated), grade III (poorly differentiated), or grade IV (undifferentiated) according to the Edmondson-Steiner (E-S) criteria [21]. The major grade within the tumor was used for correlation. Immunohistochemical staining with CD8α (type I or invariant NKT-cells marker) was performed to assess the number of intratumoral NKT-cells. For quantification, 3 sections of the central part of the tumor were selected for each rat, and 3 randomly selected high power fields (×400) were analyzed for each section. The mean number of CD8α+ NKT-cells for each animal was then determined by adding all numbers and dividing by 9.

2.7. Statistical Analysis. Within-group comparisons of whole-tumor volume and ADC measured at baseline and on posttreatment day 10 and day 20 were made using a one-way analysis of variance followed by post hoc multiple comparisons with the Tukey-Kramer test, whereas the D_{10}/baseline and D_{20}/baseline volumes and ADC ratios were analyzed by Wilcoxon signed rank test. The relationship between the tumor volume ratio and ADC ratio was assessed with a simple linear regression analysis. The mean tumor volume, volume ratio, ADC, ADC ratio, percentage of circulating NKT-cells, and number of intratumoral NKT-cells between the two groups were compared by Mann-Whitney test. The frequencies of low-grade (E-S grades I-II) versus high-grade HCC (E-S grades III-IV) in the two groups were compared with Fisher exact test. Statistical analysis was performed using SYSTAT software (SPSS for Windows, version 13; IL, USA), and $P < 0.05$ was considered statistically significant.

3. Results

3.1. Animals. Twenty-six of 30 rats showed typical HCC features (T1 hypointensity, T2 hyperintensity, hyperintense on arterial phases enhanced images and hypointense on venous phases enhanced images, DW hyperintensity, and ADC maps hypointensity), on the baseline MR imaging, and two rats were killed with histopathological confirmation of poorly differentiated HCC. The other four rats with negative MR imaging showed no tumor on subsequent histopathological examination. The tumor induction rate in the F344 rats with the N1S1 cells was 90% (26/30). Four rats in the control group and one rat in the exosome-treated group died before the second MR follow-up. Three exosome-treated rats showed >30% tumor enlargement (nonresponder) on posttreatment day 10 MR imaging were excluded. The response rate to the intravenous ADMSC-derived exosomes treatment was 72.7%

Table 1: Sequence parameters for liver 3.0-T MR imaging in rats with HCC.

	Precontrast T1-weighted	T2-weighted	Diffusion weighted	Postcontrast T1-weighted (3 phases)
Sequence	FSPGR	SSFSE	SE/EPI	FSPGR
Repetition time (msec)	200	5000	6000	200
Echo time (msec)	2.1	83.6	Minimal	2.1
Flip angle (degree)	70	NA	NA	70
Matrix	192 × 256	192 × 256	64 × 64	192 × 256
Field of view (cm^2)	10 × 7	10 × 7	10 × 7	10 × 7
Section thickness (mm)	3	3	3	3
Intersection gap (mm)	0.3	0.3	0.3	0.3
Number of excitations	6	1	4	6
Number of slices	13	13	13	13
b-value used (sec/mm^2)	NA	NA	0, 800	NA

FSPGR: fast spoiled gradient-recalled echo, SSFSE: single shot fast spin-echo, Se/EPI: spin-echo/echo-planar, TR: repetition time, *E*: echo time, NA: not applicable.

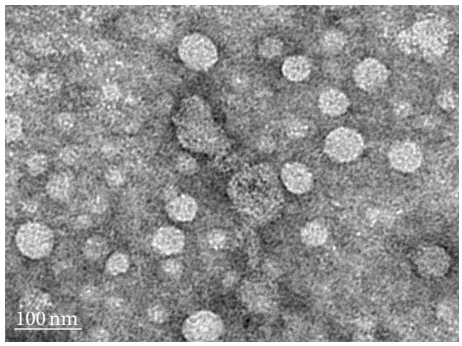


Figure 3: Transmission electron microscopic evaluation shows small vesicles within the expected range of exosomes (30–90 nm) in the sample isolated from the ADMSCs culture medium by ultracentrifugation.

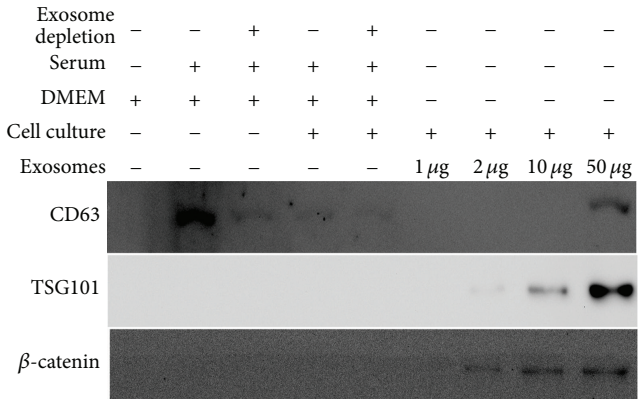


Figure 4: Western blot analysis of the culture medium, conditioned medium, and exosomes probed with antibodies against CD63, tumor susceptibility gene-101 (TSG-101), and β-catenin. Please note that CD63 is present in the culture medium, conditioned medium, and exosomes. TSG101 and β-catenin are absent in DMEM (Dulbecco's modified Eagle medium) without or with 10% serum but are present in the exosome fractions (1 μg, 2 μg, 10 μg, and 50 μg), particularly the 50 μg sample.

(8/11). Finally, eight rats in each group were included in the analysis.

3.2. Electron Microscopy and Exosome Protein Quantification and Characterization. Transmission electron microscopy revealed the presence of nanovesicles (30–90 nm) (Figure 3) in the sample isolated using ultracentrifugation. SDS-PAGE showed that the proteins in DMEM supplemented with 10% serum or 10% exosome-free serum before or after cell culture for 3 days were similar, including the presence of 70 kDa albumin and 34 kDa, 100 kDa, and 170 kDa proteins. The exosomal proteins were mainly in the 38 kDa, 60 kDa, 80 kDa, 100 kDa, and 180 kDa gel bands, confirming that the exosomal proteins were different from the serum proteins. Western blot analysis confirmed the expressions of CD63, TSG101, and β-catenin in the exosome fractions (1 μg, 2 μg, 10 μg, and 50 μg), particularly in the 50 μg sample (Figure 4).

3.3. Volume and ADC Measurements and Relationship. The tumor volume, volume ratios, tumor ADC and ASDC ratios at different time points, and comparisons are summarized in Table 2. For the control group, there was a rapid increase of tumor volume with significant differences in the values between D_{10} versus baseline, D_{20} versus baseline or D_{10} ($P < 0.05$ for all comparisons), and significantly higher D_{20}/baseline versus D_{10}/baseline volume ratios ($P = 0.012$). However, there were no significant differences in the absolute ADC values and D_{20}/baseline versus D_{10}/baseline ADC ratios at different time points (Figure 5). For the exosome-treated group, there was partial but nonsignificant decrease of tumor volume after the first exosome treatment; however, after the second treatment, there was a significant decrease in the tumor volume (D_{20} versus baseline or D_{10}, $P < 0.05$ for all comparisons) and significantly lower D_{20}/baseline versus D_{10}/baseline volume ratios ($P = 0.012$). However, there were

Table 2: Within-group and intergroup comparisons of tumor volumes, volume ratios, and ADC and ADC ratios of HCC between the control group and exosome-treated group.

	Control mean ± SD	Exosome-treated mean ± SD	*P*
Tumor volume (mm^3)			
Baseline	3816 ± 580	3905 ± 595	.798§
D_{10}	5320 ± 412*	3437 ± 632	.002§
D_2	6719 ± 625*†	1625 ± 587*†	<.001§
Tumor volume ratio			
D_{10}/baseline	1.38 ± 0.18	0.83 ± 0.08	<.001§
D_{20}/baseline	1.74 ± 0.21	0.42 ± 0.13	<.001§
P (D_{20}/baseline versus D_{10}/baseline)	.012‡	.012‡	
ADC ($\times 10^{-3}$ mm^2/sec)			
Baseline	0.71 ± 0.08	0.70 ± 0.07	.959§
D_{10}	0.72 ± 0.09	0.83 ± 0.11*	.028§
D_{20}	0.73 ± 0.08	1.01 ± 0.06*†	<.001§
ADC ratio			
D_{10}/baseline	1.01 ± 0.18	1.19 ± 0.12	.038§
D_{20}/baseline	1.04 ± 0.19	1.43 ± 0.17	<.001§
P (D_{20}/baseline versus D_{10}/baseline)	.674‡	.017‡	

D_B: baseline, D_{10}: posttreatment day 10, D_{20}: posttreatment day 20.
*$P < .05$ for comparison with baseline values (Tukey-Kramer multiple comparison test).
†$P < .05$ for comparison with day D_{10} values (Tukey-Kramer multiple comparison test).
‡Wilcoxon signed rank test, §Mann-Whitney *U* test.

significant increases in the absolute ADC values between D_{10} versus baseline, D_{20} versus baseline or D_{10} ($P < 0.05$ for all comparisons), and significantly higher D_{20}/baseline versus D_{10}/baseline ADC ratios ($P = 0.017$) of the tumors (Figure 6). Compared to the controls, the exosome-treated animals harbored significantly smaller tumors and volume ratios and significantly higher ADC and ADC ratios on D_{10} and D_{20} ($P < 0.05$ for all comparisons). Simple regression analysis revealed a significant correlation between the whole-tumor volume and ADC ratios ($P < 0.001$, $R^2 = 0.679$) (Figure 7).

3.4. NKT-Cell Changes in Circulating Blood. There were no significant differences in the percentages of NKT-cells for the circulating T-cells between the two groups prior to HCC induction and prior to treatment. However, the circulating NKT-cells in all rats increased (mean percentages from 0.5% to 1.3%) after N1S1-cell inoculation. Compared to the controls, the exosome-treated rats had significantly higher percentages of circulating NKT-cells on posttreatment day 5 and day 15. Notable, the controls showed decreased percentage of NKT-cells on posttreatment day 15 (Table 3).

3.5. Histopathological Analysis and Immunohistochemical Staining. Hematoxylin-eosin staining revealed that all tumors in the exosome-treated group were lower-grade HCC (two Edmondson-Steiner grade I and six grade II), whereas the majority of tumors in the control group were high grade (one grade II, four grade III, and three grade IV). The frequency of low-grade HCC in the exosome-treated group (8/8 rats) was significantly higher than the controls (1/8 rats) ($P < 0.001$) (Table 3). In addition, immunohistochemical examinations showed that the mean numbers of intratumoral CD8α+ NKT-cells were also significantly higher in the exosome-treated animals than the controls (Figure 8) (Table 3).

4. Discussion

In cell-based therapies, the use of embryonic stem cells is limited because of ethical issue, whereas bone marrow- (or hematopoietic-) derived stem cells (BMSCs) are commonly used. Although BMSCs do not play a role in hepatocarcinogenesis in rodent and hepatitis B virus transgenic mice models [22, 23], the involvement of BMSCs in many other malignancies, such as breast cancer, has been described [24]. In contrast to BMSCs with putative oncogenicity, ADMSCs would be advantageous because of anti-inflammatory and immunomodulating functions. In particular, the ethical and safety issues for ADMSCs are less concerning because they are somatic cells that do not undergo unwanted differentiation [20, 25].

Exosomes are nanovesicles secreted from intracellular multivesicular bodies with complex molecular compositions including common and cell type specific proteins and lipids, messenger RNA, and microRNA, acting as a vectorized multisignaling device [7, 8]. In the present study, electron microscopy, SDS-PAGE, and western blotting revealed the presence of protein-containing nanovesicles (30–90 nm) in

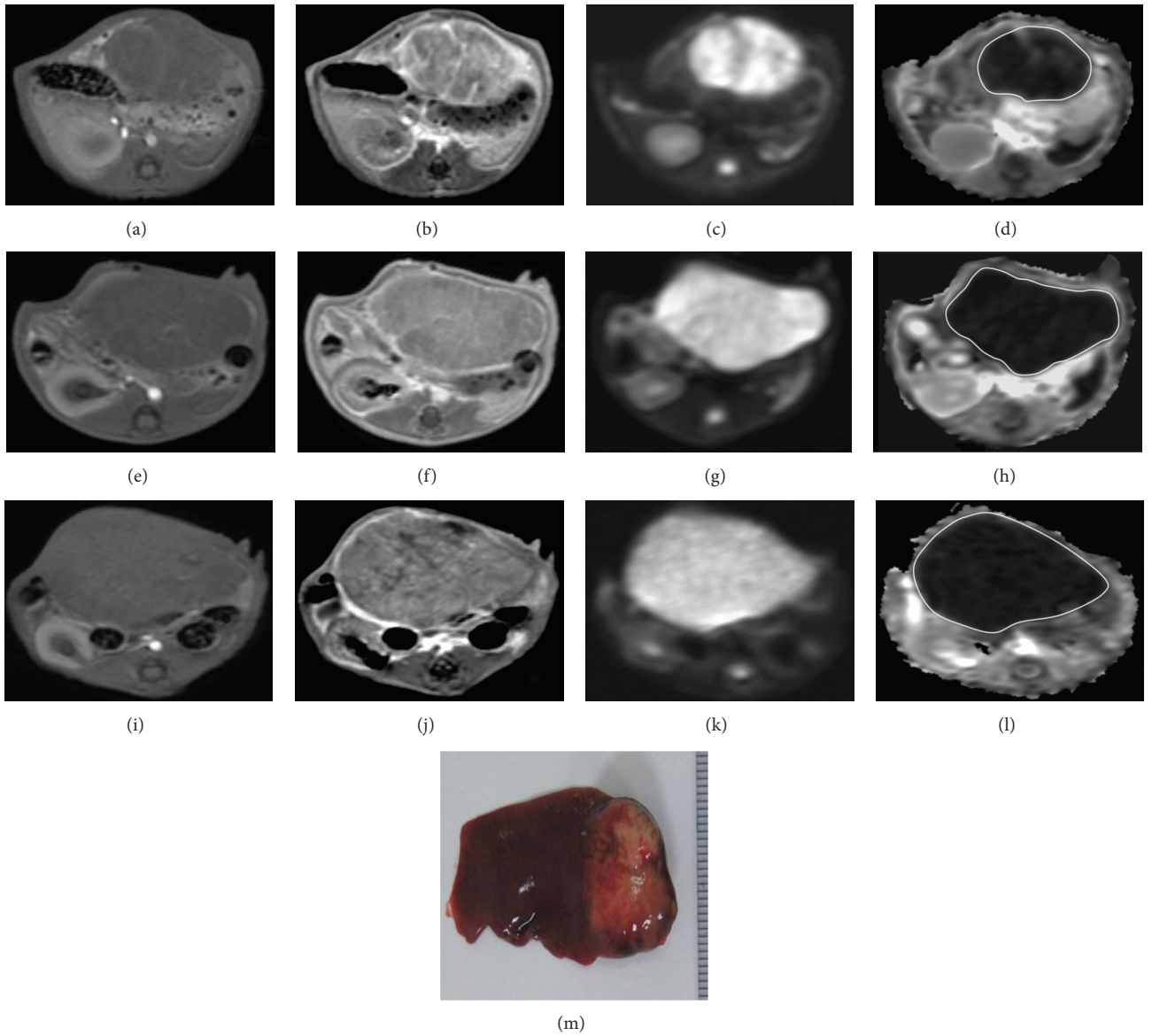


Figure 5: T1-weighted ((a), (e), and (i)), enhanced T1-weighted ((b), (f), and (j)), DW image (b value = 800 sec/mm^2) ((c), (g), and (k)), and ADC map ((d), (h), and (l)) of HCC at the level of greatest tumor diameter on baseline (a, b, c, d), posttreatment day 10 ((e), (f), (g), and (h)), and posttreatment day 20 ((i), (j), (k), and (l)). MR imaging of a control rat shows heterogeneously enhanced tumor with marked enlargement (whole-tumor volume ratios: D_{10}/baseline = 1.38, D_{20}/baseline = 1.85) whilst the ADC value (whole-tumor ADC ratios: D_{10}/baseline = 0.92, D_{20}/baseline = 1.04) remains low. Gross specimen (M) of the resected liver shows a large tumor in the left lobe with good correlation to MR imaging on posttreatment day 20.

samples with positive results for CD63, a specific marker of exosomes, and TSG-101, a cellular protein that functions in the secretion of multivesicular bodies, confirming that the nanovesicles are exosomes [8, 26]. Our results revealed that 8 of 11 exosome-treated rats (response rate 72.7%) had significant tumor reduction on day 20. To the best of our knowledge, this is the first animal study using ADMSC-derived exosomes for the treatment of HCC.

Rapid induction of orthotopic HCC in Sprague-Dawley rats via the ultrasound-guided implantation of N1S1-cells with 60% success rate has been described [27]. In the present study, a minilaparotomy approach was used to ensure successful N1S1-cells inoculation and rapid tumor induction in F344 rats. A high success rate of 90% was achieved. Histopathological confirmation of HCC after killing the rats further verified the feasibility of this model. In addition, the experimentally induced tumors had typical HCC features based on the MR imaging [28], suggesting that a 3.0 T imager can also be used in liver MR imaging studies of small animals.

Unlike the mRECIST and EASL criteria, which focus on enhanced components, whereas necrotic areas are not included [13, 14], the entire tumor was measured in our study

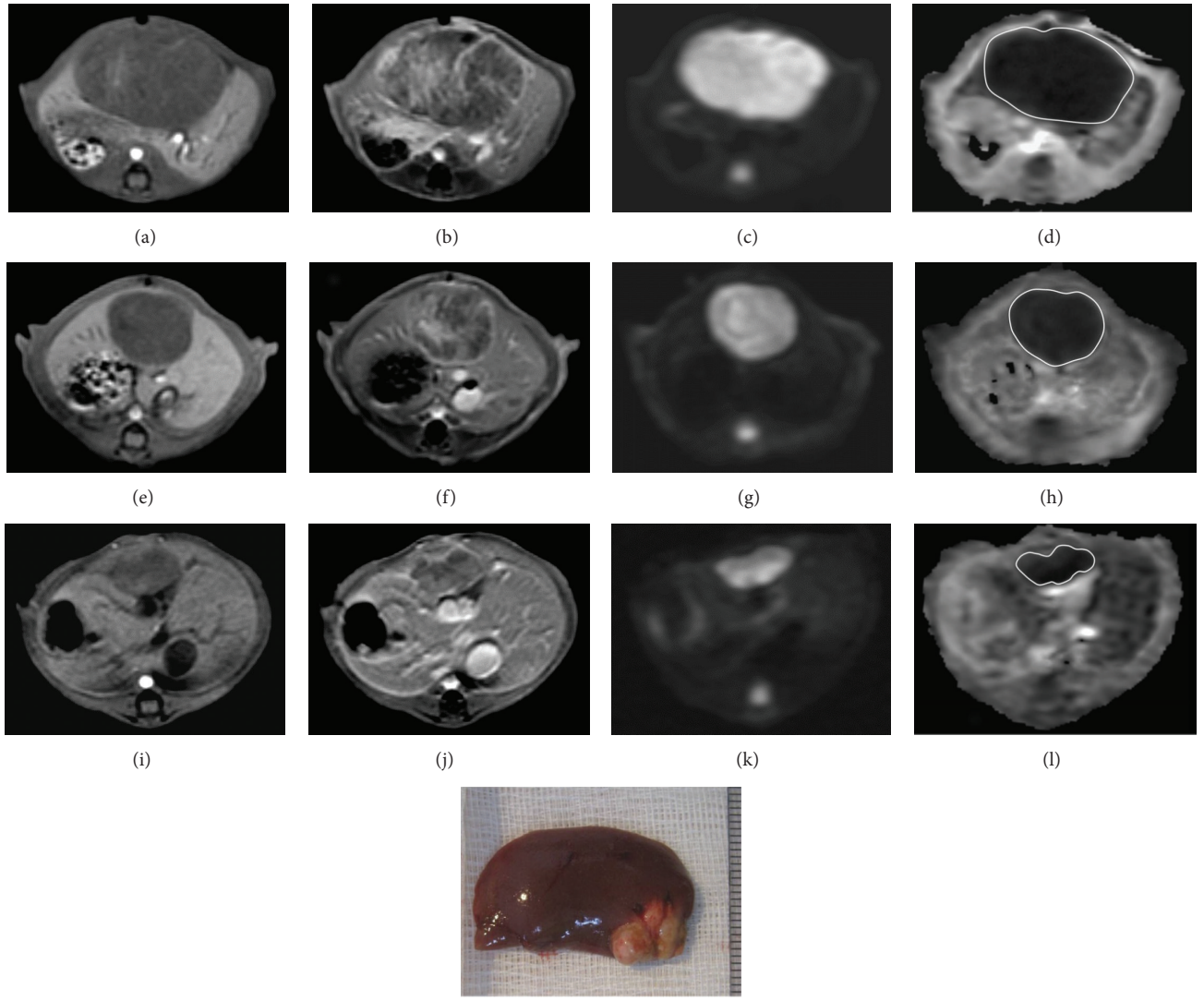


FIGURE 6: T1-weighted ((a), (e), and (i)), enhanced T1-weighted ((b), (f), and (j)), DW image (b value = 800 sec/mm^2) ((c), (g), and (k)), and ADC map ((d), (h), and (l)) of HCC at the level of greatest tumor diameter on baseline ((a), (b), (c), and (d)), posttreatment day 10 ((e), (f), (g), and (h)), and posttreatment day 20 ((i), (j), (k), and (l)). MR imaging of an exosome-treated rat shows partial but nonsignificant tumor reduction and significantly increased ADC ratio on posttreatment day 10 (D_{10}/baseline whole-tumor volume ratio = 0.72 and ADC ratio = 1.29). On posttreatment day 20, the exosome-treated rat harbored significantly smaller tumor and higher ADC ratio (D_{20}/baseline whole-tumor volume ratio = 0.29 and ADC ratio = 1.63). Gross specimen (M) of the resected liver shows a small lobulated tumor in the left lobe with good correlation to MR imaging on posttreatment day 20.

because we found that the tumors grew or shrank in an even manner without macroscopic necrotic changes. Rapid HCC growth (baseline, 3816 ± 580 mm^3; day 20, 6719 ± 625 mm^3) with persistent low ADC values (baseline, 0.71 × 10^{-3} mm^2/sec; day 20, 0.73 × 10^{-3} mm^2/sec) was observed in the controls, which indicated the persistent high cellularity of the tumors. By contrast, the exosome-treated animals rats showed significant tumor shrinkage (baseline, 3905 ± 595 mm^3; day 20, 1625 ± 587 mm^3) and ADC increment (baseline, 0.70 × 10^{-3} mm^2/sec; day 20, 1.01 × 10^{-3} mm^2/sec), indicating reduced tumor cellularity on posttreatment day 20. Notably, the exosome-treated rats showed partial but nonsignificant tumor reduction (D_{10}/baseline volume ratio = 0.83 ± 0.08), but significantly increased ADC and ADC ratios on posttreatment day 10, suggesting that a significant change of ADC precedes the change in tumor size and, therefore, ADC may be an early biomarker of treatment response.

Our results demonstrated that the ADC values of rat HCC (approximately 0.7–1.0 × 10^{-3} mm^2/sec) were lower than those reported for human HCC (approximately 0.9–1.3 × 10^{-3} mm^2/sec) [13–19, 28, 29]. Caution is required in the interpretation of absolute ADC values, which may be affected by MR instrument, choice of b-values, sequencing, location of lesion, and, as shown in the present study,

Table 3: Comparisons of percentages of circulating NKT-cells at different time points, HCC differentiation, and intratumoral CD8α+ NKT-cells between the control group and exosome-treated group.

	Control mean ± SD (n = 8)	Exosome-treated mean ± SD (n = 8)	P
Circulating NKT-cells (%)			
Before HCC induction	0.45 ± 0.35	0.55 ± 0.36	.574*
Pretreatment	1.28 ± 0.37	1.25 ± 0.33	.878*
Posttreatment day 5	1.43 ± 0.47	2.63 ± 0.59	.001*
Posttreatment day 10	0.69 ± 0.29	2.44 ± 0.57	<.001*
HCC tumor differentiation			
E-S grade (I-II : III-IV)	1 : 7	8 : 0	<.001†
Intratumoral NKT-cells			
Number of CD8α+ cells/HPF	5.1 ± 2.7	18.7 ± 3.5	<.001*

NKT-cells: natural killer T-cells, E-S: Edmondson-Steiner, HPF: high power field.
*Mann-Whitney U test.
†Fisher's exact test.

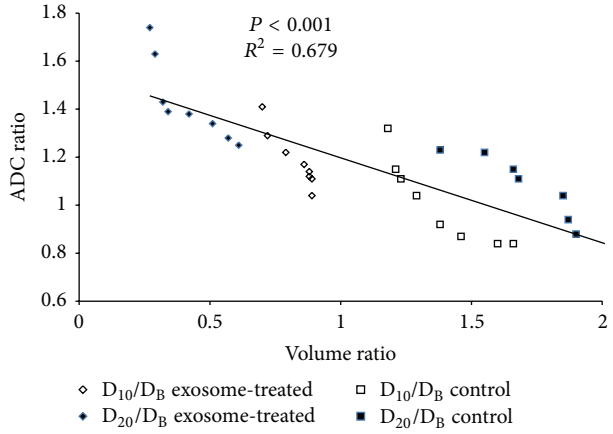


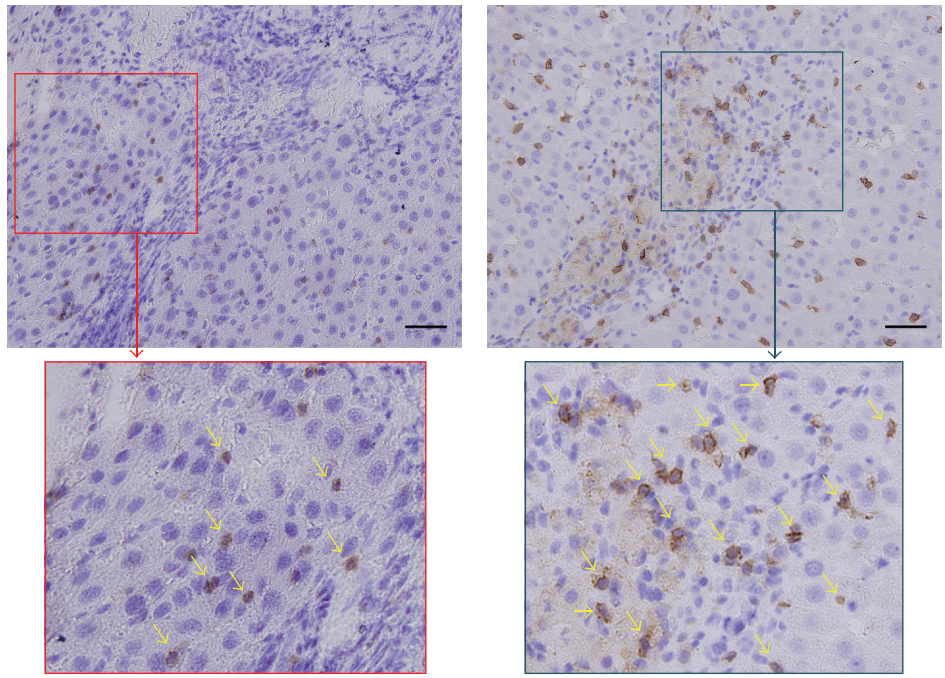
Figure 7: The graph shows the relationship between the whole-tumor volume ratio and ADC ratio, indicating a strong correlation ($P < 0.001$, $R^2 = 0.679$; simple linear regression analysis).

different species [15]. Conversely, the comparison between whole-tumor volume ratio and ADC ratio is based on individual changes relative to the baseline; therefore, the concern regarding the variations in absolute values is minimized. Our results showed that the exosome-treated rats have a significant tumor reduction with a lower mean volume ratio (0.42 ± 0.13 versus 1.74 ± 0.21) and higher mean ADC ratio (1.43 ± 0.17 versus 1.04 ± 0.19) compared to the controls. Furthermore, simple regression analysis revealed a significant inverse correlation between the ADC ratio and volume ratio ($P < 0.001$, $R^2 = 0.679$). Consistent with prior studies showing that the histopathological differentiation of HCC is inversely correlated with the ADC value [18, 19], our study showed that the ADC ratio of the controls, with more high-grade HCC, was significantly lower than the exosome-treated rats.

Western blotting confirmed the presence of β-catenin in ADMSC-derived exosomes in our study. β-catenin is a component of the Wnt/β-catenin signaling pathway, which plays an important role in T-cell immunity [30]. NKT-cells serve as a bridge between the innate and adaptive T-cell immune system by acting as first responders. Notably, type I (invariant) NKT-cells with an invariant T-cell receptor-α chain are protective, whereas type II NKT-cells with diverse T-cell receptors primarily inhibit antitumor responses [31]. In the present study, the initial increase of circulating NKT-cells in both groups may be an antitumor response provoked by N1S1 cell implantation. Further increases of circulating protective NKT-cells were observed in the exosome-treated rats with tumor reduction. However, NKT-cells antitumor immunity was overcome by on-going tumor progression in the controls. Consistent with prior studies demonstrating that increased intratumoral invariant NKT-cells are associated with HCC suppression, improved patient survival, and less tumor recurrence [32–34], the exosome-treated rats harbored significantly smaller tumors and more intratumoral invariant (CD8α+) NKT-cells and low-grade HCC than the controls.

This study has several limitations. First, the study sample size was small. Second, this animal study is only a short-term investigation that fails to show the long-term therapeutic impact of ADMSC-derived exosomes on HCC. Third, DW imaging was performed with two different b-values (0 and 800 sec/mm^2) as commonly used in clinical practice [15, 28], and the diffusion fraction of ADC would be more accurately estimated when the perfusion fraction is minimized. Additional studies with multiple b-values with less perfusion contamination and regional ADC variations should be performed. Fourth, the degree of HCC enhancement was not assessed because this study focused on the ADC changes. Finally, the reasons why several of the exosome-treated rats showed no treatment response have to be elucidated. Further studies are needed to investigate the complex mechanisms and cellular-molecular changes caused by ADMSC-derived exosomes.

In conclusion, ADMSC-derived exosomes promoted NKT-cell antitumor responses in rats, thereby facilitating



(a) (b)

Figure 8: Immunohistochemical staining (200x) with CD8α in the rat in the control group (a) and the rat in the exosome-treated group (b) with focal magnification view shows significantly higher number of intratumoral CD8α+ NKT-cells (arrows) in the exosome-treated rat than in the control. Scale bar = 50 μm.

HCC suppression, early ADC increase, and low-grade tumor differentiation. A significant change of ADC preceded the change in tumor size and, therefore, ADC may be an early biomarker of treatment response.

Conflict of Interests

All authors assert that there was no conflict of interests (both personal and institutional) regarding specific financial interests that were relevant to the study performed or reported in this paper.

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