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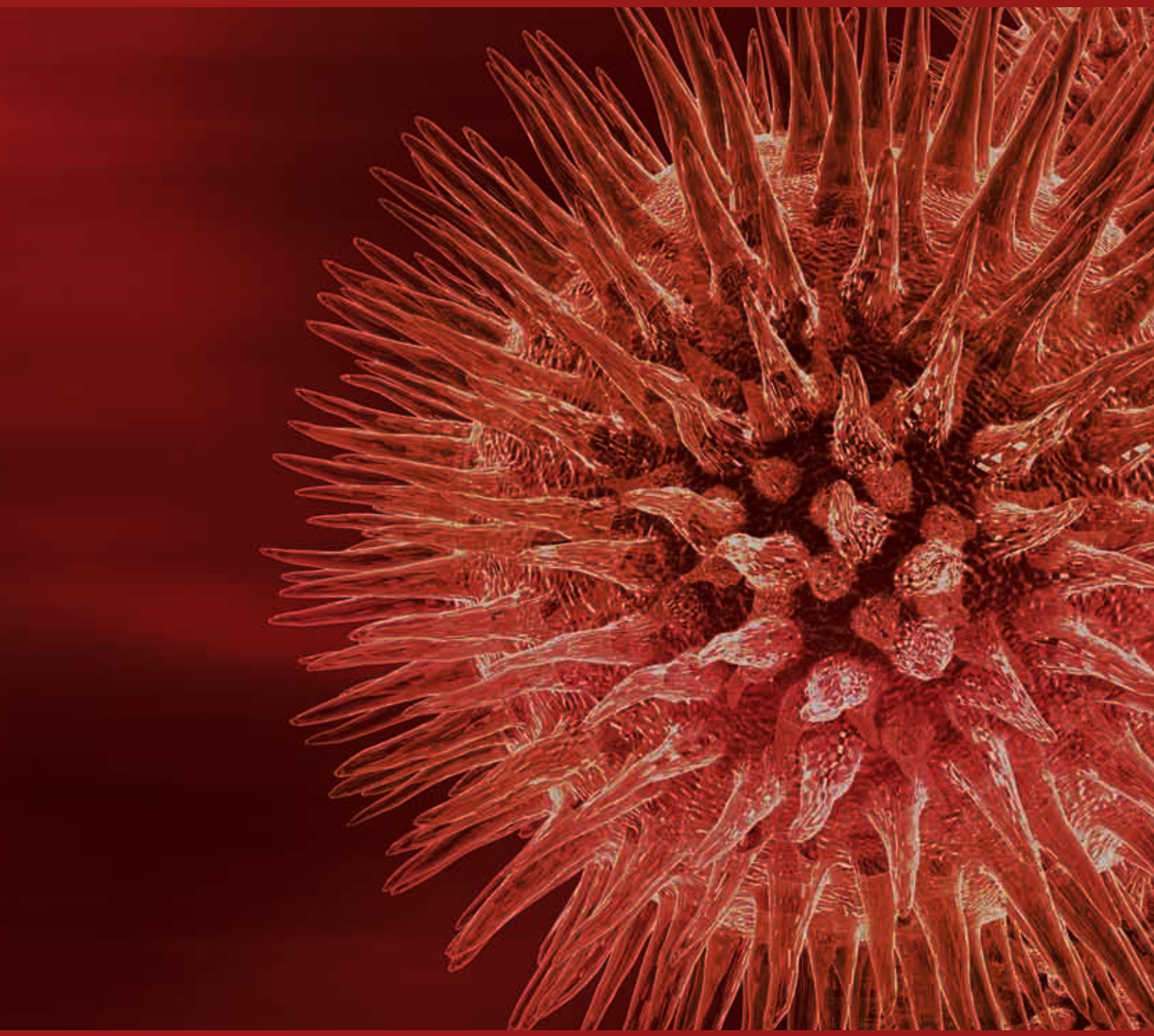
Toward Personalized Cell Therapies by Using Stem Cells 2013

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



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Editorial

Toward Personalized Cell Therapies by Using Stem Cells 2013

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The generation of induced pluripotent stem cells (iPSCs) from somatic cells by retrovirus transfection encoding Oct4, Sox2, cMyc, and Klf4 established a major landmark in the field of stem cell biology as it allows the establishment of patient-specific pluripotent cells. However, tumor formation and immunogenicity have been suggested if patients own iPSCs or differentiated cells are transplanted back to the patient. T. d. S. Fernandez et al. discussed why hiPSCs have the potential to induce tumors in host. Then they review the potential use of hiPSCs in clinical applications for cancer. In order to avoid oncogenic transformation, R. O'Doherty et al. reviewed the nonviral methods to induce pluripotency. S. Thanasegaran et al. clearly showed that iPSCs and differentiated tissue cells from iPSCs, which were established from retroviruses, had no immunogenicity by transplantation into syngeneic mice. Thus iPSCs have great possibility to treat many diseases such as neurological disorders summarized by N. Jongkamonwiwat and P. Noisa.

MSCs from bone marrow, skin, and adipose tissue have been used for the model experiments of stem cell therapy for many diseases. H. Kim et al., review the stem cell therapy in bladder dysfunction especially bladder outlet obstruction. Y. Gao et al. first isolated MSC from chicken fibroblasts. Y. Zhang et al. reported that MSC transplantation into diabetic rats reduced blood glucose level and prevented renal damages, which was enhanced by ultrasound-targeted microbubble destruction. MSCs have low proliferative potential, which may be a hurdle for their therapeutic use. Y. Hu et al. used nonviral rDNA vectors to transfer genes into hMSCs. They found that these vectors enhanced the proliferation of hMSCs and succeeded in gene transfer. H. He et al., succeeded in differentiating BM-MSCs to hepatocytes on decellularized ECM.

When we apply regenerative medicine, we have to think that transplanted tissue must function appropriately. X.-M. Fu et al. reported that sympathetic innervation could be effectively induced into engrafted engineered cardiomyocyte sheets using GDNF.

Acknowledgments

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Ken-ichi Isobe
Herman S. Cheung
Ji Wu

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Research Article

Intravenous Transplantation of Mesenchymal Stromal Cells to Enhance Peripheral Nerve Regeneration

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Peripheral nerve injury is a common and devastating complication after trauma and can cause irreversible impairment or even complete functional loss of the affected limb. While peripheral nerve repair results in some axonal regeneration and functional recovery, the clinical outcome is not optimal and research continues to optimize functional recovery after nerve repair. Cell transplantation approaches are being used experimentally to enhance regeneration. Intravenous infusion of mesenchymal stromal cells (MSCs) into spinal cord injury and stroke was shown to improve functional outcome. However, the repair potential of intravenously transplanted MSCs in peripheral nerve injury has not been addressed yet. Here we describe the impact of intravenously infused MSCs on functional outcome in a peripheral nerve injury model. Rat sciatic nerves were transected followed, by intravenous MSCs transplantation. Footprint analysis was carried out and 21 days after transplantation, the nerves were removed for histology. Labelled MSCs were found in the sciatic nerve lesion site after intravenous injection and regeneration was improved. Intravenously infused MSCs after acute peripheral nerve target the lesion site and survive within the nerve and the MSC treated group showed greater functional improvement. The results of study suggest that nerve repair with cell transplantation could lead to greater functional outcome.

1. Introduction

Common causes of disastrous nerve injuries include motor vehicle accidents, violence, sports-related injuries, and falls [1]. Traumatic nerve damages can lead to complete functional loss of the affected limb and are often combined with life threatening injuries which have to be treated first. During this time, the transected nerves undergo Wallerian degeneration [2] in parallel to irreversible muscle degeneration. After peripheral nerve injury, the duration of nerve transection before reinnervation of effected organ is critical; even after immediate nerve repair, clinical results are often disappointing. Therapeutic strategies to improve and especially accelerate axonal regeneration and remyelination are of great importance.

Cell-based therapies using mesenchymal stromal cells (MSCs) are being investigated in clinical trials for a number of neurological diseases including stroke [3] and peripheral nerve [4] and spinal cord [5] injuries. The rationale is that the transplanted MSCs provide neuroprotection, neovascularisation, and induction of axonal sprouting by their production of cytokines and neurotrophic factors [6]. Peripheral myelin-forming cells (Schwann cells and olfactory ensheathing cells) have been shown to improve survival when directly transplanted into peripheral nerve and lead to improvement in functional outcome [7–9]. However, harvesting of these cells requires nerve biopsy in the case of Schwann cells and biopsy from nasal mucosa both of which have some potential morbidity associated with them. A major issue preventing clinical use of OECs for intralesional cell transplantation

after nerve injury is the difficulty to harvest a sufficient amount of viable autologous cells in the injured individual. Resulting donor site morbidity such as impairment of smell or anosmia may limit clinical use. The harvesting of bone marrow derived MSCs in patients is a common procedure and has low morbidity, thus making these cells attractive as potential cell transplantation source.

While a relatively large number of experimental and clinical studies have been carried out with direct or intravenous infusion of MSCs (see [3, 6] for review) the repair potential of intravenously transplanted MSCs in peripheral nerve injury has not been addressed. The primary objective of this study was to determine if intravenously transplanted MSCs following peripheral nerve transection reach the lesion site and what impact the MSCs have on functional recovery.

2. Methods

2.1. Cell Preparation. MSCs were prepared as previously described with modifications [10, 11]. Cells were prepared from bone marrow aspirates (10 μL), which were isolated from femur and tibia of adult rats using a heparinized 24G needle. Cell material was diluted 1 : 1 with α-MEM (Invitrogen, Karlsruhe, Germany) and filtered through a 70 μm nylon mesh (Cell Strainer, BD Falcon; Becton Dickinson, Franklin Lakes, NJ, USA). The resulting cell suspension was layered on top of 15 mL Ficoll-Paque Plus (Amersham Pharmacia Biotech, Uppsala, Sweden) and centrifuged for 30 min at 800 ×g at room temperature. The supernatant and interface were combined, diluted to about 50 mL with PBS (0.1 M) and centrifuged for 10 minutes at 800 ×g. After discarding of the supernatant the pellet was resuspended in 1 mL medium. The nucleated cells were counted and suspended at a concentration of 1×10^7/mL in the growth medium (α-MEM) supplemented with 2 mg/mL L-glutamine, 50 μg/mL streptomycin, and 20% (v/v) of not heat-inactivated fetal calf serum) and plated at $3 \times 10^6/\text{cm}^2$ in 100 mm culture dishes (Falcon, Becton Dickinson). The cells were incubated for 3 days, and the nonadherent cells were removed by replacing the medium in three washing steps. After the cultures reached confluency, the cells were dislodged by incubation with Accutase (PAA, Cölbe, Germany) at 37°C for 3-4 min. They were diluted and replated at a density of 2000 cells/cm^2 in 100 mm culture dishes. Cells were used for transplantation after 7 days of cultivation. Immunostaining of the cells with an anti-CD-90 antibody (monoclonal mouse antibody, Abcam, Cambridge, UK, 1 : 800), Stro-1, and CD 44 demonstrated that the purity of the BM-MSC preparations was >90% (data not shown). The cells did not stain for CD34 and CD45.

2.2. Nerve Lesion and Cell Transplantation Procedure. All animal experiments were approved by the Lower Saxony district government and the Medical School of Hannover and conducted according to the German Law of Animal Protection and were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals, and the Veterans Affairs Connecticut Healthcare System Institutional Animal Care and Use Committee approved all animal protocols respectively.

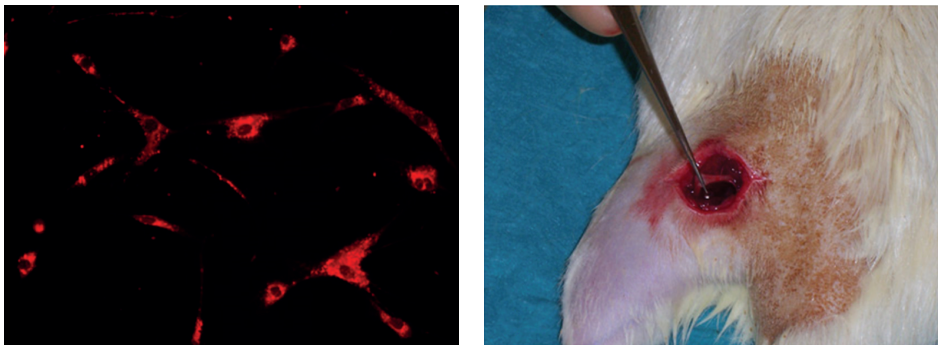
Adult wild Sprague Dawley rats (200–225 g) were used for this experiment ($n = 18$). The rats were anesthetized with ketamine (75 mg/kg i.p.) and xylazine (10 mg/kg i.p.). Preoperatively the rats underwent a splenectomy. The sciatic nerve was surgically exposed in anesthetized rats and completely sectioned by standardized nerve crushing the level of the piriformis tendon in the thigh. Cultured MSCs were detached from the culture flasks and resuspended in culture medium and prelabelled with PKH26. Using a syringe, 2 mL of the cell suspension, vehicle alone or negative control cells (fibroblasts), were injected via femoral vein directly after lesion induction. The animals survived for 21 days followed by scarification with removal of nerves for histological analysis.

2.3. Footprint Analysis. Determination of the walking track with analysis of the sciatic functional index (SFI) was performed according to the method described by De Medinaceli et al. [12]. To obtain footprints, hind paws were placed on an ink blotter and the animals were placed on a white piece of paper. Both feet produced five to six prints. Rats were tested weekly over the course of the experiment. Surgery was done on the left sciatic nerve of each animal and the right hind limb was used as internal control. Footprints were collected from the experimental (E, left) and normal (N, right) sides. Prints were measured for the following parameters: distance between foot prints (TOF), the entire plantar length (PL), the distance from the first to fifth toes, the toe spread (TS), the distance between the second and fourth toes, and the intermediary toe spread (IT). The SFIwas calculated according to the following formula:

$$\frac{(\text{ETOF} - \text{NTOF})}{\text{NTOF}} + \frac{(\text{NPL} - \text{EPL})}{\text{EPL}} + \frac{(\text{ETS} - \text{NTS})}{\text{NTS}} + \frac{(\text{EIT} - \text{NIT})}{\text{NIT}} \times \frac{220}{4}. \quad (1)$$

Calculated indices from this formula ranged between a score from zero to minus 100. Zero describes normal function and –100 complete transection of the sciatic nerve. Data are presented as means ± SE. Statistical evaluations were based on two-tailed t-test and χ^2 test (origin; criterion, $P < 0.05$).

2.4. Immunohistochemistry. Sciatic nerves from transplanted and control rats were processed for immunocytochemistry as described previously. Briefly, rats were deeply anesthetized with ketamine/xylazine and perfused transcardially, firstly with 0.9% saline and then with ice-cold 4% paraformaldehyde in 0.14 M Sorensen's phosphate buffer, pH 7.4. Sciatic nerves were removed and postfixed for 20 min in 4% paraformaldehyde. Tissue was then cryoprotected in 30% sucrose in 0.14 M Sorensen's phosphate buffer overnight at 4°C. Ten micrometer longitudinal cryosections of the sciatic nerves were cut and mounted on Silane Prep glass slides (Sigma, St. Louis, MO, USA). Sections were processed for immunostaining for monoclonal antibody neurofilament (NF, Sigma, St Louis, MO, USA; dilution 1 : 1000) followed



(a) (b)

Figure 1: Prelabelling of cells and of intravenous injection via femoral vein. Cultured MSCs were stained with the membrane dye PKH26 before cell transplantation (a). Sciatic nerve lesion was induced by nerve crush (b), followed by intravenous injection of MSCs via femoral vein.

by incubation with secondary antibody goat anti-mouse IgG-Alexa Fluor 594 (Invitrogen, Eugene, OR, USA; 1:1000) and coverslipped with DAPI-containing mounting media (VectaShield, Vector Laboratories, Burlingame, CA, USA). The sections were examined with a fluorescence microscope (Nikon Eclipse 800; Spot RT Color CCD camera; Diagnostic Instruments).

3. Results

3.1. PKH 26 Labelling and Crush Lesion. The sciatic nerve was surgically exposed in anesthetized rats and its axons were transected by nerve crush (Figure 1(b)). The transection site was standardized at the level of the piriformis tendon in the thigh. Isolated MSCs in culture showed characteristic flattened fibroblast-like morphology after cell attachment and removal of nonadherent cells. MSCs were immunopositive for the stem cell markers CD 90, CD 44, and Stro-1 and were immunonegative for the hematopoietic stem cell markers CD 34 and CD 45 (data not shown). Immediately before cell transplantation, cultured MSCs were detached from the culture flasks and resuspended in serum free culture medium and prelabelled with PKH26 (Figure 1(a)) for in vivo cell tracing after injection.

For intravenous systemic injection ($n = 18$), animals received MSCs (1.0×10^6) in 1 mL total fluid volume (DMEM) of the cell suspension or vehicle alone (sham control; $n = 6$) by using a syringe. The intravenous cell injection was performed via the femoral vein.

Three weeks after lesion induction and systemic cell delivery, the nerves were removed and prepared for histology. In both experimental groups there was evidence of increased axonal regeneration and improved functional outcome. However, the MSC transplantation group (Figures 2(c) and 2(d)) in comparison to the control group in low and high power images (Figures 2(a) and 2(b), resp.) had greater numbers and more axons proximal, within and distal to the repair site. In frozen sections of the nerve, PKH26-labelled MSCs could be found within the regenerated peripheral nerve after systemic delivery shortly after lesion induction indicating a homing effect of the MSCs to the peripheral nerve lesion site (Figures 2(c) and 2(d)). The MSCs (red) survived in the lesion site and distributed longitudinally across the lesion site in both proximal and distal directions with the regenerated axons. The regenerated axons are stained with neurofilament (green) in low and higher magnifications (Figures 2(a)–2(d)). The extended distribution of the transplanted PKH-labelled MSCs (red) demonstrates the homing effect into the lesioned peripheral nerve and subsequent regenerated nerve fibers in relation to the neurofilament stained axons (Figures 2(c) and 2(d)).

3.2. Functional Analysis of Stepping Behaviour. Footprint analysis using the sciatic nerve functional index (SFI) was carried out before lesion (day 0), beginning seven days after nerve crush and i.v. cell injection (MSCs or fibroblasts) and sham control (media infusion). The SFI score is zero for normal animals and a negative value for nerve impairment. At day seven the MSC group showed greater functional improvement (66.25 ± 3.75) than either the control cell injection (fibroblast) or the sham (vehicle alone) groups (98.8 ± 4.4 versus 106.0 ± 4.5). The locomotor improvement was observed in 14 days (MSCs 58.0 ± 3.0 versus fibroblast cell injection 86.2 ± 3.95 and vehicle alone 94.9 ± 3.85) and maintained in 21 days (MSCs 44.7 ± 2.5 versus fibroblast cell injection 77.5 ± 4.5 and vehicle alone 79.9 ± 4.0; see Figure 3). The functional improvement in the MSC group was significant in 7 days, but the rate of change in improvement from 14 to 21 days was comparable between the three experimental groups, suggesting that the effect of the MSCs was an early interventional event.

4. Discussion

The primary objective of this study was to investigate whether systematically administered MSCs target a site of a nerve crush lesion and lead to functional improvement. To this end MSCs were isolated from isogenetic rats and labelled with PKH26 which is known to have a long half-life in vivo and has been successfully used in experimental neurological settings

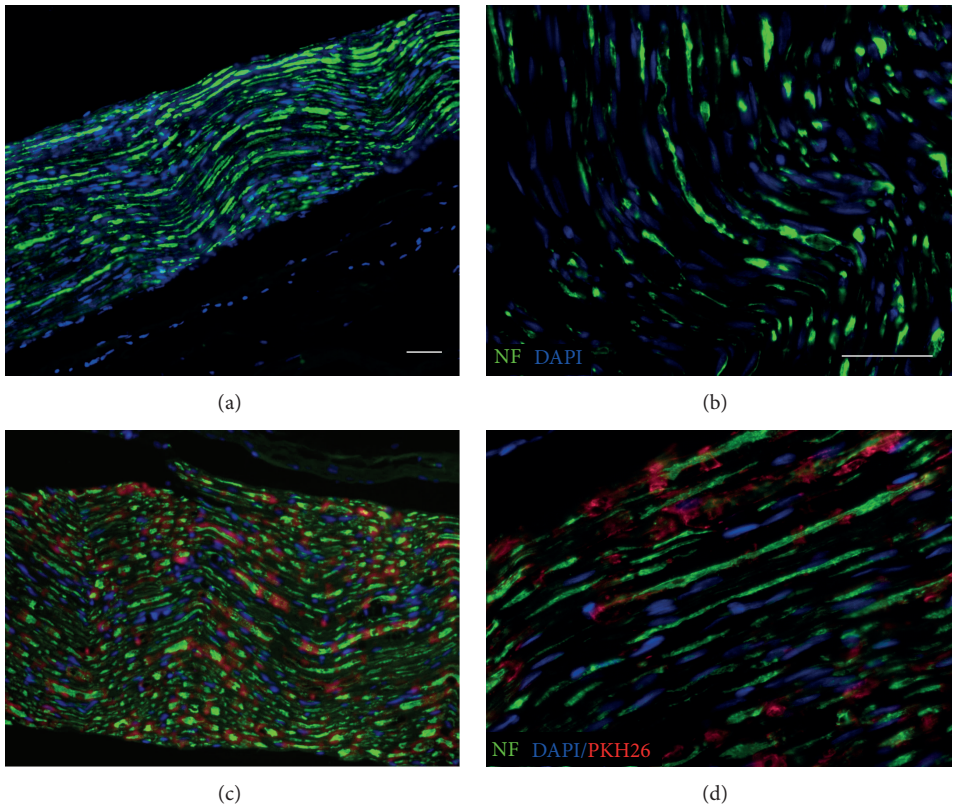


Figure 2: Longitudinal sections and immunohistology of sciatic nerve after peripheral nerve crush injury and intravenous transplantation of PKH-labelled MSCs. (a) and (b) Demonstration of axonal regeneration after peripheral nerve injury and cell transplantation: regenerated axons are stained with neurofilament (green) in low and higher magnifications. (c) and (d) Distribution of i.v. transplanted PKH-labelled MSCs (red) and homing effect into the lesioned and subconsequent regenerated nerve fibers in relation to the neurofilament stained axons and endogenous cells stained with DAPI present within the nerve (blue).

[13]. PKH26-labelled cells were found in the regenerated nerves three weeks after the transplantation demonstrating that at least part of the transplanted cells integrated into the lesion site where they associated with regenerating nerve fibers. Interestingly, labelled negative control cells were not found in the lesion site. Functional recovery in the MSCs transplanted animals as assessed using footprint analysis was significantly increased compared to untreated base levels and fibroblast transplanted negative cellular controls.

Using MSCs expressing firefly luciferase it has been demonstrated that MSCs selectively are incorporated into sites of inflammation such as cutaneous wounds and tumors while there was a bioluminescence clearance over 14 days in uninjured, nontumor-bearing animals [14]. It has been proposed that this tropism is due to inflammatory chemokines and hypoxic conditions in the respective microenvironment (reviewed in [15]). Intravenously injected MSCs have also been demonstrated to be incorporated into demyelinated and traumatic spinal cord injuries in rats where they induced a significant clinical improvement [11, 13]. Functional improvement after delivery of MSC has also been reported in several preclinical and clinical studies dealing with neurodegenerative diseases (e.g., reviewed in [16]). A recent study found structural changes and improved functional outcome following MSC infusions in a spinal cord injury model in the rat but did not detect MSCs in the lesion site [17]. Significant functional improvement was also observed following intravenous infusion of MSCs in a myocardial infarction model [18]. Interestingly, only a limited number of MSCs reached the lesion site and the vast majority of cells lodged in the lungs with a half-life of 24–48 hours. The MSCs were shown to produce the powerful multipotent anti-inflammatory compound and tumor necrosis factor-inducible gene 6 protein (TSG-6). Infusion of TSG-6 knockdown MSCs was much less efficacious. Thus, a potential mode of action of intravenous infusion of MSCs could be the "remote" production of anti-inflammatory or trophic factors in peripheral organs such as lung which then exert a systemic effect. These critical issues on the mechanism of action of MSCs require additional research.

Several biological characteristics of MSCs as applied to regenerative medicine [18] may contribute to enhanced neuronal regeneration. First, MSCs secrete a number of immunomodulatory factors [19, 20]. An appropriate inflammatory response is necessary to prevent infection and to start the repair process by removing myelin and cell debris, but extensive inflammation can also have negative influence [21]. As a second measure MSCs also provide angiogeneic factors stimulating an appropriate vascularization of the regenerating tissue [22]. Vascular damage and resulting undersupply

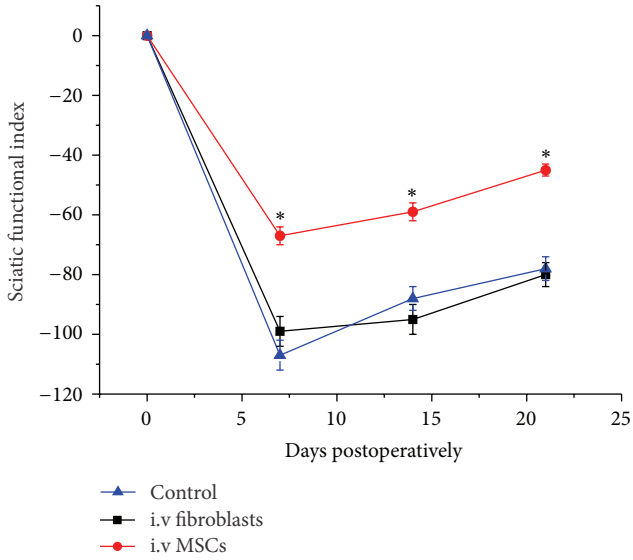


FIGURE 3: Foot print analysis after intravenous cell transplantation after sciatic crush lesion. The animals with MSC transplanted nerve showed greater functional recovery as scored using the Sciatic Functional Index (SFI) than in sham control or control cell transplantation indicating that the cells have an enhancing effect for axonal regeneration and remyelination which results in improved functional outcome. Data are presented as means ± SE. Statistical evaluations were based on two-tailed t-test, χ^2 test (Origin; criterion, *significantly different, $P < 0.05$).

of nutrients and oxygen often accompany peripheral nerve injuries. Administration of vascular endothelial growth factor in a nerve conduit used to bridge a 1 cm sciatic nerve defect led to enhanced axonal regeneration with 78% more myelinated axons after 180 days [23].

5. Conclusion

In summary our results indicate that intravenous MSCs can improve functional outcome in a peripheral nerve injury model and that some of the transplanted reach and survive in the lesion area for at least three weeks. The precise mechanism for this beneficial effect is not yet clear, but a number of mechanisms including immunomodulation, stimulating neovascularization, and neurotrophic influences may contribute. Given that intravenous infusion of MSCs have been used in a number of clinical studies with demonstrated safety (see [6]) the prospect of using MSCs as an adjunct therapy in nerve defect repairs should be considered.

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

Human Mesenchymal Stem Cells Provide Protection against Radiation-Induced Liver Injury by Antioxidative Process, Vasculature Protection, Hepatocyte Differentiation, and Trophic Effects

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[2] *UMRS-938, Equipe Prolifération et Différenciation des Cellules Souches du Pr Luc Douay: Application à la Thérapie Cellulaire, Faculté de Médecine Saint-Antoine, Université Paris VI Pierre et Marie Curie, 27 rue de Chaligny, 75012 Paris, France*
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To evaluate the potential therapeutic effect of the infusion of hMSCs for the correction of liver injuries, we performed total body radiation exposure of NOD/SCID mice. After irradiation, mir-27b level decreases in liver, increasing the directional migration of hMSCs by upregulating SDF1α. A significant increase in plasmatic transaminases levels, apoptosis process in the liver vascular system, and in oxidative stress were observed. hMSC injection induced a decrease in transaminases levels and oxidative stress, a disappearance of apoptotic cells, and an increase in *Nrf2*, *SOD* gene expression, which might reduce ROS production in the injured liver. Engrafted hMSCs expressed cytokeratin *CK18* and *CK19* and *AFP* genes indicating possible hepatocyte differentiation. The presence of hMSCs expressing *VEGF* and *Ang-1* in the perivascular region, associated with an increased expression of *VEGFr1, r2* in the liver, can confer a role of secreting cells to hMSCs in order to maintain the endothelial function. To explain the benefits to the liver of hMSC engraftment, we find that hMSCs secreted *NGF*, *HGF,* and anti-inflammatory molecules *IL-10*, *IL1-RA* contributing to prevention of apoptosis, increasing cell proliferation in the liver which might correct liver dysfunction. MSCs are potent candidates to repair and protect healthy tissues against radiation damages.

1. Introduction

Multipotent stromal cells, also named mesenchymal stromal cells or mesenchymal stem cells (MSCs), are capable of dividing and their progenies are further capable of differentiating into one of several mesenchymal phenotypes, such as osteoblasts, chondrocytes, myocytes, marrow stromal cells, tendon-ligament fibroblasts, and adipocytes [1]. Animal models have shown that MSCs can engraft and distribute to several tissues after systemic infusion [2–7] and engraft in several injured tissues [3, 4, 8–13], for example, the liver [14–16]. Previously, we showed that in a mice model the presence of intravenously injected MSCs increased in damaged tissues following radiation exposure [7, 8] and that in a nonhuman primate model MSCs could be detected in regenerating tissues [4]. Thanks to their relatively easy isolation from bone marrow (BM) and to their extensive capacity for in vitro expansion, MSCs have been considered for approaches in cell therapy and tissue engineering [17–19]. A number of clinical trials are ongoing to explore the effect of MSCs in vivo in several contexts, such as facilitation of hematopoietic recovery after hematopoietic stem cell transplantation (HSCT) [5, 20–22], prevention and treatment of graft-versus-host disease (GVHD) [23, 24], and treatment of osteogenesis imperfecta [25, 26] and metabolic disorders

[27]. It has been shown that MSCs infusion engraftment in the liver facilitates recovery from chemically induced acute liver damage as well as recovery by an indirect effect after radiation injury [28–32]. In addition, these MSCs differentiate in hepatocyte-like cells and secrete a variety of cytokines and growth factors that have both paracrine and autocrine activities. These secreted bioactive factors suppress the local immune system, inhibit fibrosis (scar formation) and apoptosis, enhance angiogenesis, and stimulate mitosis and differentiation of tissue-intrinsic reparative cells and stem cells [33]. MSCs are promising candidates for the repair of tissues altered by radiation exposure, as described for skin regeneration [8, 9]. Additionally, MSCs have antiproliferative, immune-modulatory, antioxidative, and anti-inflammatory effects [28–34]. MSCs have implications for treatment of allograft rejection, graft-versus-host disease, autoimmune inflammatory bowel disease, and other disorders in which immunomodulation and tissue repair are required.

Bone marrow transplantation (BMT) is a sophisticated therapeutic procedure consisting in high-dose chemoradiotherapy followed by intravenous infusion of hematopoietic stem cells to reestablish marrow function. BMT is largely used in treatments of hematologic malignancies, including leukemia and lymphomas [35]. This treatment requires conditioning consisting in a massive chemotherapy combined, or not, with total body irradiation (TBI). Before the BMT, the TBI is performed by ionizing radiation (IR) inducing the release of free radicals in tissues [36]. Thus, IR can damage both the healthy tissues and the tumoral cells which may induce secondary effects due to radiation exposure [37].

Liver disease is an important cause of morbidity among BMT recipients. A retrospective study realized on a group of 103 transplanted patients revealed that the incidence of liver failure attributed to hepatic GVHD was 22.3% and to venoocclusive disease (VOD) was 9.7% [38]. VOD in the liver is a major complication of BMT [39, 40]. GVHD of the liver after allogeneic hematopoietic stem cell transplantation classically presents with increased bilirubin and alkaline phosphatase (ALP) levels. A hepatitic variant presenting more than a 10-fold increase in aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels was recently recognized [41]. Finally, human mesenchymal stem cell transfusion has demonstrated to improve liver function in acute-on-chronic liver failure patients [42].

The purpose of this study was to reduce the liver toxicity associated with a traditional preparative regimen consisting in massive chemotherapy treatment combined with TBI before BMT. We used an immunotolerant mice model (NOD/SCID mice) receiving a sublethal dose (3.2 Gy) of TBI to observe the biological effect of hMSCs on the induced hepatitic dysfunction. We established a protective role of hMSCs on the liver by limiting the decrease in hepatic activity and the oxidative stress induced by TBI. HMSCs were preferentially localized around the blood vessels of the liver suggesting that hMSCs could ensure the protection of the vascular endothelium against toxic damage. The protection of organ vascular endothelium integrity against free radicals damage by means of MSC infusion could be a potential therapeutic treatment for preventing radiation-induced vascular complications. Therefore, MSC therapy should be considered early on to prevent the progression of liver disease induced by radiation exposure.

2. Material and Methods

2.1. Isolation, Purification, and Expansion of Human Bone Marrow-Derived MSC (hMSC). BM cells were obtained from iliac crest aspirates from ten healthy volunteers after informed consent was obtained and used in accordance with the procedures approved by the human experimentation and ethics committees of Saint Antoine Hospital. (The local ethics committee is named "Comité de Protection des Personnes- Ile-de-France V," Hôpital Saint-Antoine, Paris.) The ethics committee approved this study specifically. Oral patient agreements were obtained before study. This consent procedure was approved by the local committee. The ethics committee did not require the investigator to obtain signed informed consent from all participants because it considered there was no breach of confidentiality, minimal risk for participants, and no procedures involved for which written consent is normally required outside the research context.

As previously described [7], 50 mL of BM was taken from the donors over heparin (Choay by Sanofi-Synthélabo). Low-density mononuclear cells (MNCs) were separated on Ficoll Hypaque density gradient (d: 1.077). MNCs were plated at a concentration of 10^7 cells per milliliter in T-75 cm^2 tissue culture flasks in Dexter medium (McCoy 5A medium supplemented with 12.5% heat-inactivated fetal calf serum, 12.5% heat-inactivated horse serum, 1% sodium bicarbonate, 1% sodium pyruvate, 0.4% minimum essential medium (MEM) nonessential amino acids, 0.8% MEM essential amino acids, 1% MEM vitamin solution, 1% L-glutamine (200 mM), 1% penicillin-streptomycin solution (all from Invitrogen, Groningen, The Netherlands), 10^6 M hydrocortisone (Stem Cell Technologies, Vancouver, BC), and 2 ng/mL human basic recombinant fibroblast growth factor (FGFb; R & D Systems, Abington, United Kingdom) and incubated at 37°C and 5% CO_2 in a humidified atmosphere. After 1 week, nonadherent cells were removed with (the same) complete fresh medium (without hydrocortisone) and first passage hMSCs (P1 hMSC) were plated at a density of 4.10^5 per T-75 cm^2 flask. First pass hMSCs (P1 hMSC) were collected and counted when 80% of the cells were confluent. The viability was assessed by trypan blue assay.

After the second passage, hMSCs were plated at different densities using different culture conditions in order to induce osteogenic, chondrogenic, or adipogenic differentiation. Osteogenic differentiation of hMSCs was as follows: cells were plated at a density of 3.10^3 cells/cm^2 in Dulbecco's modification of Eagle's medium (DMEM) with L-glutamine supplemented with 10% of FBS (Life Technologies), containing 50 μg/mL of ascorbate-2-phosphate (Sigma) and 100 μM of β-glycerolphosphate (Sigma Chemicals Co., Saint Louis, MO, USA). Chondrogenic differentiation of hMSCs was as follows: cells were plated at 50.10^3 cell/cm^2 in DMEM with L-glutamine supplemented with 10% of FBS, containing 50 μg/mL of ascorbate-2-phosphate (Sigma) and 10 ng/mL

of transforming growth factor TGF-β1 (Sigma). Adipogenic differentiation of hMSCs was as follows: cells were plated at 20.10^3 cells/cm^2 in DMEM with L-glutamine supplemented with 10% of FBS until confluence. After confluence, the basic media were completed by 50 μg/mL of ascorbate-2-phosphate (Sigma). During culture differentiation, cells were incubated at 37°C with 5% of CO_2 for 21 days. The medium was renewed every 3 days.

Prior to transplant, a sample of the prepared hMSCs was analyzed by a fluorescence-activated cell sorter (FACS). Following trypsin treatment, human cells were washed and resuspended in aliquots of 2×10^5 cells in phosphate buffered saline (PBS) solution supplemented with 0.5% bovine serum albumin (BSA; Sigma). Staining was performed with phycoerythrin (PE)-conjugated monoclonal antibody against CD105 (SH2), CD73 (SH3), and CD45 (Becton-Dickinson) for 30 minutes at 4°C followed by 2 washes in PBS containing 0.5% BSA. Cells were resuspended in PBS 0.5% BSA and analyzed at 10,000 events/test by FACScalibur (BD Pharmingen). Mouse Immunoglobulin G1 (IgG1) was used as isotopic controls (IOTest). Before infusion when the hMSCs were collected at the second passage, the rates of viability to blue trypan were 98%.

2.2. BM-MSC Infusion into NOD/SCID Mouse Model. All experiments and procedures were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as published by the US National Institutes of Health (NIH Publications number 85-23, revised 1996) and with the European Directive number 86/609/CEE and were approved by the local ethics committee (P09-11). NOD-LtSz-*scid/scid* (NOD-SCID) mice, from breeding pairs originally purchased from Jackson Laboratory (Bar Harbor, ME, USA), were bred in our pathogen-free unit and maintained in sterile microisolator cages. TBI was carried out with a sublethal dose of 3.2 Gy (IBL 637, IRSN France) using a source of Cesium (^{137}Cs) with a dose rate of 1.85 Gy/minute. We initially investigated the deterioration of hepatic function induced by TBI to determine whether injection of hMSCs could decrease the damages. A total of 100 eight-week-old mice were used to study blood parameters, divided in 4 groups: 25 animals were used as controls (no irradiation, no injection—Group 1), 25 mice were injected with hMSCs but were not irradiated (Group 2), 25 mice received TBI of 3.2 Gy (Group 3), and 25 mice were irradiated (TBI at 3.2 Gy) and injected with hMSCs (Group 4). The mice were put down to collect the blood and the liver at different times (3, 7, 15, 30, and 60 days after TBI). For each of the five time points, 5 animals were used. The BM-MSCs were delivered to each mouse intravenously (IV) through the tail vein using a Myjector 1 mL syringe (TERUMO 29G X 1/2) 24 hours after TBI. The NOD/SCID mice were transplanted with a dose of 5.10^6 P2 hMSCs in 100 μL of PBS 1X. On average, the hMSCs derived from one bone marrow sample could be were used to inject 5 mice.

2.3. Blood Parameters. The plasmatic levels of urea, creatinine, and transaminase were measured to demonstrate the negative impact of TBI on the kidneys and/or the liver. In our first investigation, we observed that plasmatic urea levels decreased significantly between 7 and 15 days after TBI, suggesting a reduction in the hepatic activity (data not shown). We therefore examined the hepatic activity further. We measured the levels of transaminases, urea, and creatinine specifically at 7 days after TBI in the plasma of the 4 groups of mice. The quantitative determination of the plasmatic concentration of urea, creatinine, and transaminases was carried out by means of a Thermo Clinical Labsystems Analyzer (Konelab 20). The analyses were performed on plasma diluted to a final volume of 400 μL. Plasma was obtained from an intracardiac puncture carried out under anesthesia before the liver was carefully collected. The weight and water and food intake of the animals were monitored during the entire study.

2.4. Lipid Peroxidation Detection. Lipid peroxidation was assayed by the measurement of related substances that react with thiobarbituric acid (TBARS) [43]. The V79-4 cells were seeded in a culture dish at a concentration of 1×105 cells/mL and treated with esculetin at 10 μg/mL after 16 h plating and then hydrogen peroxide (H_2O_2) 1 mmol/L was added to the plate after 1 h, which was incubated for a further 24 h. The cells were then washed with cold PBS, scraped, and homogenized in ice-cold 1.15% potassium chloride (KCl). About 100 μL of cell lysates was combined with 0.2 mL of 8.1% SDS, 1.5 mL of 20% acetic acid (adjusted to pH 3.5), and 1.5 mL of 0.8% thiobarbituric acid (TBA). The mixture was adjusted to a final volume of 4 mL with distilled water and heated to 95°C for 2 h. After cooling to room temperature, 5 mL of a mixture of n-butanol and pyridine (15 : 1) was added to each sample, and the mixture was shaken vigorously. After centrifugation at 1,000 ×g for 10 min, the supernatant fraction was isolated, and the absorbance was measured at 532 nm.

2.5. Detection and Quantitative Analysis of Engrafted hMSCs: DNA Extraction and PCR Analysis. Biological samples (liver) were submitted for DNA extraction and PCR analysis to detect the presence of human cells in mice recipients. Genomic DNA for PCR analysis was prepared from tissues using the QIAamp DNA mini kit (Qiagen). Amplifications were performed following the standard recommended amplification conditions (Applied Biosystems, Foster City, CA, USA) as previously described by François and colleagues [7]. The amount of DNA contained in each somatic cell (diploid) was 6.16 pg as determined from a single-copy gene. This value was used to calculate the number of gene copies contained in a defined amount of human or mouse DNA (measured by PCR). Therefore, the DNA amount and gene copy number were proportional to the number of cells. The ratio of human DNA over mouse DNA represents the number of human cells in mouse tissues. Amplification of the human beta-globin gene was used to quantify the amount of human DNA in each mouse tissue sample. The endogenous mouse receptor-associated proteins of the synapse (RAPSYN) encoding gene were also amplified, as an internal control. Standard curves were generated for the human beta-globin and mouse RAPSYN genes and used to quantify the absolute amount of human DNA in each mouse tissue. The specificity of human beta-globin

amplification was evaluated using tenfold dilution from 100 ng to 0.05 ng of hMSCs DNA with mouse DNA and did not demonstrate cross-reactivity. One hundred nanograms of purified DNA from various tissues was amplified using TaqMan Universal PCR Master Mix (Applied Biosystems). The primer sequences for human beta-globin amplification were 5′GTGCACCTGACTCCTGAGGAGA3′ (forward) and 5′CCTTGATACCAACCTGCCCAGG3′ (reverse), with the fluorescently labeled probe: 5′FAM-AAGGTGAACGTGGATGAAGTTGGTGG-TAMRA-3′. The primer sequences for mouse RAPSYN amplification were 5′ACCCACCCATCCTGCAAAT3′ (forward) and 5′ACCTGTCCGTGCTGCAGAA3′ (reverse), with the fluorescently labeled probe: 5′VIC-CGGTGCCAGTGATGAGGTTGGTCA-TAMRA3′. In order to determine the efficiency of the amplification and the assay precision, calibration curves for human beta-globin and mouse RAPSYN genes were generated with a 0.99 correlation coefficient and efficiency greater than 98%. Mouse DNA was isolated from the identical tissues of nontransplanted NOD/SCID mice and used as a negative control. Likewise, human DNA was isolated from hMSC culture and used as a positive control. The results were expressed in number of human cells per 100 mouse cells in each tissue (directly related to the number of copies of human beta-globin and mouse RAPSYN genes).

2.6. Real-Time Quantitative RT-PCR and Detection of miRNA. After isolation of total RNA from cells with RNA mini kit (Qiagen, Courtaboeuf, France), mRNA integrity was checked by analysis with the Agilent 2100 Bioanalyzer (Agilent Technologies, Massy, France). Complementary DNA was constructed by reverse transcription (RT) with SuperScript (Invitrogen). Polymerase chain reaction (PCR) assays were performed using the SYBR PCR Master Mix or TaqMan PCR Master Mix (Life Technologies) and specific primers for selected genes (see Table 1) on an ABI Prism 7900 Sequence Detection System (Invitrogen). For each sample, the PCR fluorescent signal from each target gene was normalized to the fluorescent signal obtained from the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The miRNA expression was normalized with U6.

2.7. Immunohistochemistry. After paraformaldehyde fixation, organs were rinsed with distilled water and dehydrated. Blocks were cut at 5 μm on a Rotary Microtome (LEICA). For immunohistochemical staining of the paraffin embedded samples, microtomed sections were deparaffinized in xylene and rehydrated in ethanol baths and PBS. The sections were dipped into PBS-triton in order to increase the tissue permeability and were then rinsed for 5 minutes in a distilled water bath. Negative controls were incubated with rabbit IgG diluted to 1 : 100. Detection of bound primary antibody was performed by incubation with biotinylated secondary antibody for 8 minutes. The biotinylated antirabbit IgG secondary antibody composed was diluted to 1 : 200 in PBS1x. Immunohistochemistry (IHC) is a powerful tool allowing detection and visualization of human β2-microglobulin within the cell or on the cell surface by traditional light microscopy. The immunoreactivity was performed on a NEXES IHC automat (Ventana, Illkirch, France) using alkaline phosphatase reaction with a FARED substrate detection kit (Enhanced V-red Detection red, number 760031, Ventana) with a 2% trypsin digestion step for 30 minutes (exposing masked epitopes). Slides were incubated for 30 minutes. The polyclonal anti-β-2-microglobulin antibody (product NCL-B2Mp, Novocastra) was added at a dilution of 1 : 50. For antibody detection, the Ventana kit was used, followed by counterstaining with hemalyn for 4 minutes. This procedure was controlled by NEXES 8 software. On successive sections, we carried out a HES staining.

Table 1: Primer references of studied genes.

Gene name	Reference
GAPDH	Mm99999915_g1
CK18	Mm01601704_g1
CK19	Mm00802090_m1
Alb	Mm00802090_m1
AFP	Mm00431715_m1
Nrf2	Mm00477784_m1
PCNA	Mm00448100_g1
TGFb1	Mm01178820_m1
VEGFR1	Mm00438980_m1
VEGFR2	Mm01222421_m1
TNFa	Mm00443260_g1
miRNA U6	001973
mir-27b	000409
GAPDH	Hs00266705_g1
HGF	Hs01117422_g1
SOD	Hs00533490_m1
VEGF	Hs00900055_m1
NGF	Hs00171458_m1
Ang-1	Hs01042023_g1
IL10	Hs99999035_m1
IL1-ra	Hs00277299_m1

2.8. Sex Chromosomes FISH and Costaining. Cells were placed on slides and fixed with methanol and acetic acid (3 : 1). The slides were then denatured with 70% formamide in 2 × SSC buffer at 65°C, dehydrated, and air-dried. The probes for the mouse transcription centromere were denatured at 65°C, applied to the denatured slides, and allowed to hybridize in a humid chamber overnight at 37°C in a dark room. The hybridized slides were then washed, and the cells were permeabilized with 0.2% triton-X 100 and incubated with 10% normal serum. Detection of bound primary antibody (human β2-microglobulin) was performed by incubation with biotinylated secondary antibody for 8 minutes. The biotinylated antirabbit IgG secondary antibody composed was diluted to 1 : 200 in PBS1x. For controls, we omitted one or both primary antibodies.

2.9. Statistical Analysis. To determine the effect of infusion of hMSCs on hepatic activity after TBI, the rates of urea,

Table 2: FACS analysis of hMSCs at the second passage, before transplant from 10 bone marrows. Frequency of positive cells for specific markers of hMSCs: CD105 and CD73 and hematopoietic cell markers (CD45).

	BM mL	hMSCs (10^6 cells)	% CD73	% CD105	% CD45
Mean	15.4	25.9	84.2	61.3	0.18
SEM	0.8	1.6	1.3	1.2	0.02

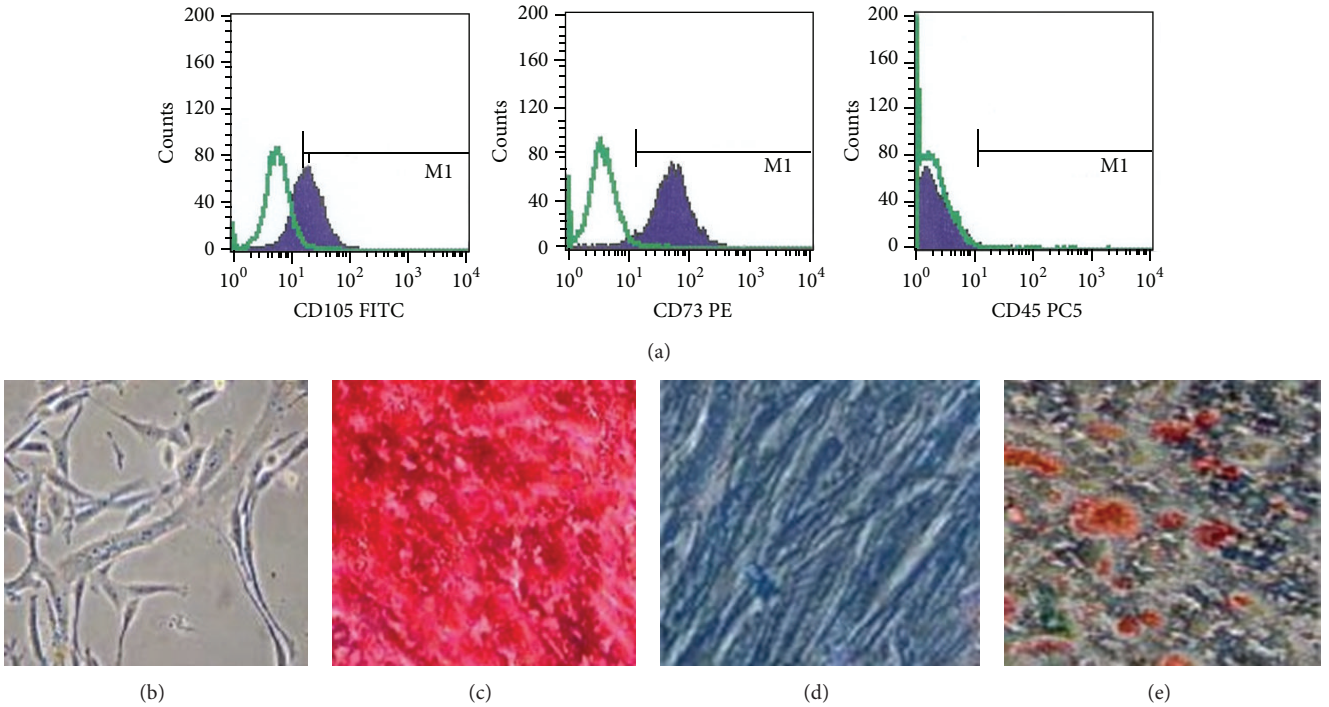

Figure 1: Phenotypic analysis at second passage and in vitro differentiation of hMSCs: FACS analysis of CD105, 73, 45 (a). The cultured hMSCs could enter different cell lineages such as osteogenic (c), chondrogenic (d), and adipogenic (e) lineages. Undifferentiated control (b).

creatinine, and transaminases were compared using Student's t-test in SigmaStat software. The significance for all analyses was set at $P < 0.05$. All values were expressed as the mean and standard error of the mean (SEM). Each group was composed of 5 to 10 mice according to the study.

3. Results

3.1. Mesenchymal Stem Cells Characterization. Phenotypic analysis showed that the hMSCs used in these experiments were strongly positive for the specific surface antigens CD73 and CD105, respectively, 84% ±1.3 and 61% ±1.2. Almost no contamination (0.2% ±0.02 CD 45+ cells) by hematopoietic cells was evidenced in the samples (Table 2).

MSCs were morphologically defined by a fibroblast-like appearance. Before use, each batch of MSCs was further characterized by confirming their specific ability to undergo osteogenic, chondrogenic, and adipogenic differentiations (Figure 1). Our results suggest that the hMSCs used for transplant have been expanded without significant loss in their differentiation capacities.

3.2. Blood Parameters after TBI. The weight and the water and food intake of the animals were monitored for 30 days after TBI. The animals lost weight significantly from 72 hours after radiation exposure. Their weight was normal again three weeks after TBI. No modification of water and food intake was observed for this period. The plasmatic urea level was measured every day following TBI over a period of 30 days. TBI induced a 1.3-fold decrease in blood urea at 7 and 15 days after TBI ($P < 0.001$) (Figure 1(a)). After 15 days after TBI, we noted that blood urea reached its preirradiation level. At 30 days after TBI, the urea level was comparable to that of the controls (7.26 ± 0.18 mmol/L). These observations suggest a decrease in hepatic activity induced by radiation exposure. Thus, we also measured the other blood biochemical parameters, that is, creatinine (Figure 2(b)) and transaminases at 7 days after TBI (Figures 2(c) and 2(d)). The biochemical analysis between irradiated animals and control animals 7 days after TBI showed an increase in blood AST and ALT, while blood creatinine did not vary (30.60 ± 0.87 μmol/L). The absence of an increase in blood creatinine concentration suggests that the TBI had not induced kidney damage 7 days after TBI. However, TBI induced a 2.7-fold increase in blood AST ($P < 0.001$) and a 2.5-fold increase in blood ALT ($P < 0.001$). These observations reveal a negative effect of radiation exposure on the metabolism of the animals. In nonirradiated NOD/SCID mice, the AST/ALT ratio was 3.0 ± 0.2. Seven days after TBI, the AST/ALT ratio was 3.3 ± 0.3. Both transaminases increased at the same time; no transaminase

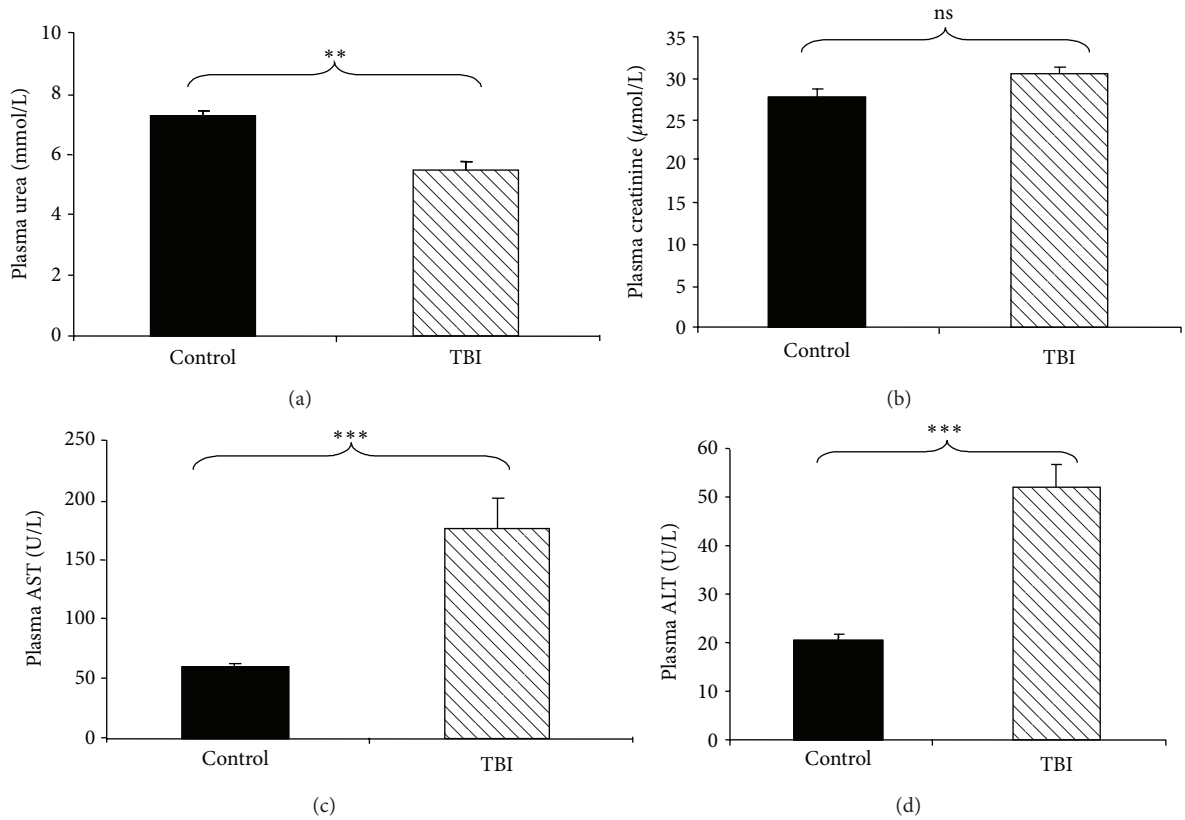

Figure 2: Biochemical analysis of plasma samples at 7 days after TBI. Biochemical analyses of plasmatic urea, creatinine, and transaminases were performed on 200 μL of plasma taken on NOD/SCID mice subjected to TBI at 3.2 Gy. Abbreviations: ALT: serum alanine aminotransferase; AST: serum aspartate aminotransferase. (a) Decrease of plasmatic urea levels at 7 days after TBI. (b) Plasmatic creatinine levels at 7 days after TBI. ((c) and (d)) Increase of plasmatic AST and ALT, respectively, at 7 days after TBI. All values are expressed as the mean and the standard error of the mean (SEM). The significance for all analyses was set at $P < 0.01$ (**) and $P < 0.001$ (***), and nonsignificance was noted as ns. Each group was composed of 5 animals ($n = 5$).

level increased alone. The comparison of the AST/ALT ratio between controls and irradiated animals showed that this ratio did not vary, suggesting that TBI involved hepatic and not muscular injuries. Previously, we reported that 15 days after TBI at 3.5 Gy TBI, cellular depletion of the spleen and hemorrhage in the bone marrow were observed, while no injury was observed in nonirradiated tissues [7]. Comparative histopathological analyses between nonirradiated and irradiated mouse livers at different times after TBI showed no morphological difference. Thus, no injury of hepatic tissue was observed from 3 to 30 days after TBI at 3.2 Gy (data not shown).

3.3. Blood Parameters after TBI and BM-MSCs Infusion. The BM-MSCs infusion in nonirradiated mice did not modify the blood urea and AST and ALT concentrations (7.84 ± 0.35 mmol/L; 72.67 ± 0.35 U/L and 17.20 ± 1.43 U/L, resp.) in comparison to the blood levels measured in nonirradiated and noninjected mice. No significant variation of these values was observed after hMSCs infusion in nonirradiated animals, suggesting the absence of toxicity of this graft. Biochemical analyses between irradiated noninjected animals and irradiated infused animals showed a decrease in blood AST and in ALT concentrations, 7 days after TBI (Figures 3(b) and 3(c)), while the blood urea concentration did not increase (Figure 3(a)). These observations suggested that systemic infusion of BM-MSCs restored preferentially the blood baseline levels of AST and ALT after TBI rather than the blood level of urea.

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3.4. hMSCs Decrease the Oxidative Stress Induced by Radiation. Oxidative stress in the cellular environment results in the formation of highly reactive and unstable lipid hydro-

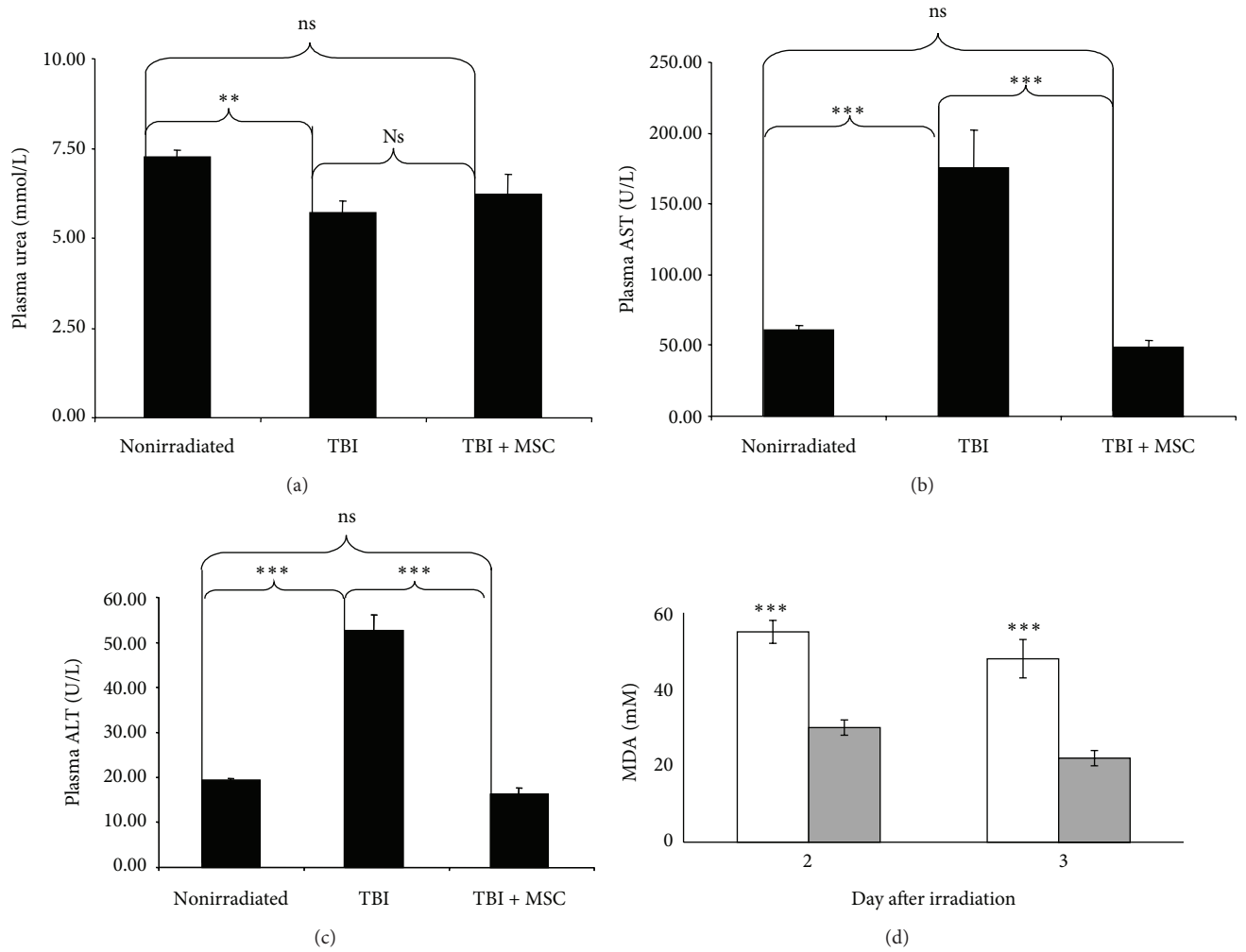

FIGURE 3: Biochemical analyses of plasmatic urea, creatinine, transaminases, and MDA. 200 μL of plasma was collected from NOD/SCID mice subjected to TBI at 3.2 Gy and injected with hMSCs intravenously. Abbreviations: ALT: serum alanine aminotransferase; AST: serum aspartate aminotransferase. (a) Blood urea concentrations. ((b) and (c)) Blood AST and ALT concentrations, respectively. (d) Lipid peroxidation was assayed by measuring the amount of thiobarbituric acid (TBARS) formation. Irradiation induced oxidative stress (white histogram) and hMSC injection (gray histogram) decreased oxidative stress. All values are expressed as the mean and the standard error of the mean (SEM). The significance for all analyses was set at $P < 0.01$ ($**$) and $P < 0.001$ ($***$) and nonsignificance was noted as ns. Each group consisted of 5 animals ($n = 5$).

peroxides. Decomposition of the unstable peroxides derived from polyunsaturated fatty acids results in the formation of malondialdehyde (MDA). Measuring MDA, we assayed lipid peroxidation in plasma in order to evaluate the modulation of oxidative stress after hMSC injection (Figure 3(d)). Irradiation-induced oxidative stress increased just after irradiation (days 2 and 3). hMSC injection decreased oxidative stress induced by irradiation significantly compared to irradiated mice noninjected mice ($P < 0.001$). These results imply that hMSCs have a protective effect against induced oxidative stress.

3.5. hMSC Engraftment in Liver. Stromal cell-derived factor 1 (SDF1), which is secreted by cells within injured tissues, and its receptor C-X-C chemokine receptor type 4 (CXCR4) are necessary for the migration of MSCs to damaged tissues. MSCs in the bone marrow express high levels of *CXCR4*. However, *CXCR4* expression is markedly reduced during the ex vivo expansion of MSCs. In our experiment, 3 days after irradiation only 20% of the mice had hMSCs engrafted in liver the frequency increased to 50% at 7 days and reached 100% at 15 days (Figure 5(f)). In order to determine whether engraftment is related to the quantity of SDF1 secreted by liver; we compared the level of SDF1 expression in liver containing hMSCs to liver without engraftment of hMSCs (Figure 4(a)). At 3 and 7 days after irradiation, the level of *SDF1* expression was significantly higher ($P < 0.05$) in liver engrafted with hMSCs compared to unimplanted liver. At 15 days, the comparison was not possible because all the livers tested contained hMSCs. Results are expressed as fold increase expression as compared to unirradiated liver not injected with hMSCs.

Since MSCs lost CXCR4 during culture, we tested whether MSCs may increase *CXCR4* expression in vivo, in

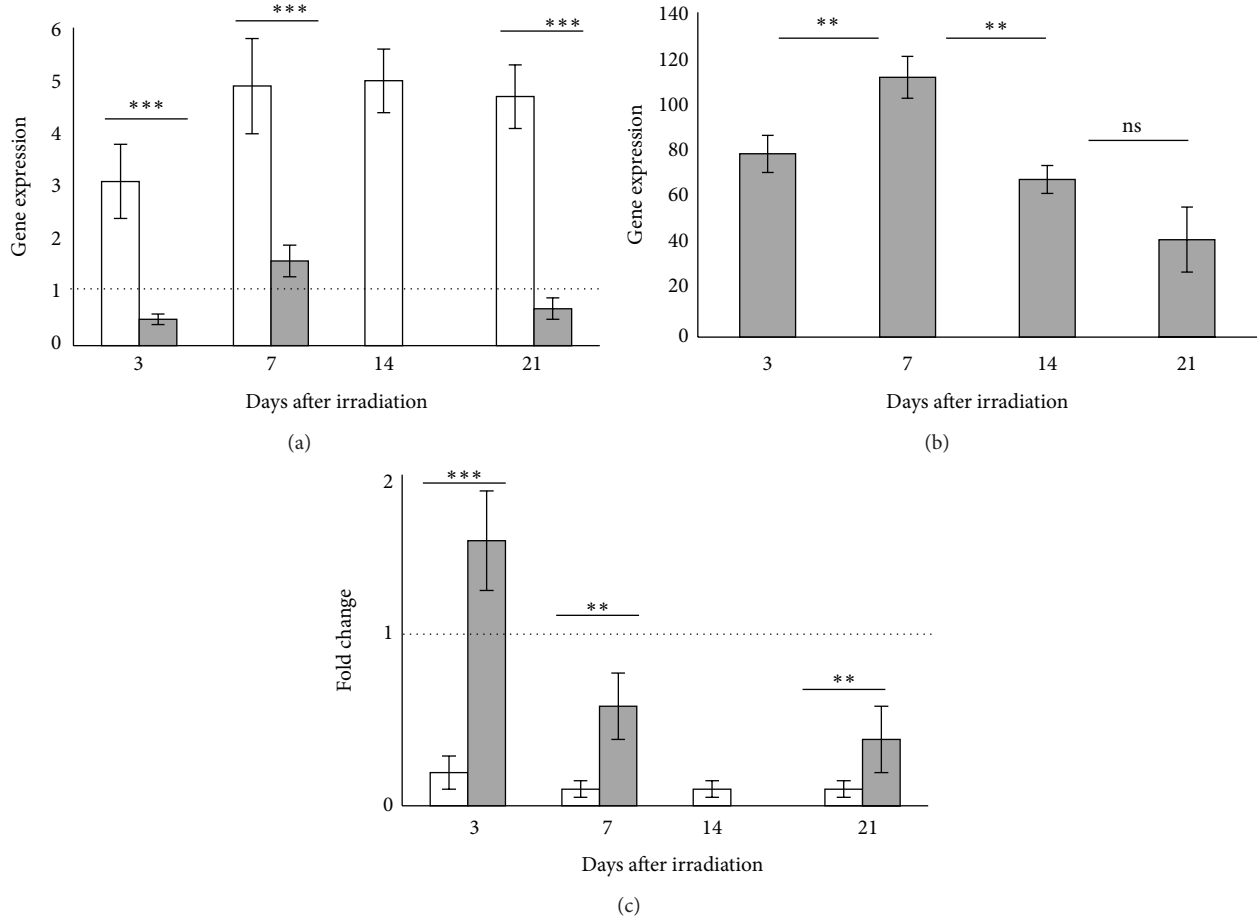

Figure 4: Expression of SDF1α and mir-27b in liver and CXCR4 in implanted hMSCs. (a) At 3 and 7 days after irradiation, the level of *SDF1* expression was significantly higher ($P < 0.05$) in liver engrafted with hMSCs (white histogram) compared to unimplanted liver (grey histogram). At 15 days, the comparison was not possible because all the livers tested contained hMSCs. (b) The level of *CXCR4* in hMSCs increased in vivo during the first days after injection and then decreased after 15 days. (c) The level of mir-27b in the liver of mice engrafted with hMSCs is less elevated than in liver ungrafted (Figure 4(c)). The level of mir-27b is more elevated in liver of mice ungrafted in comparison with liver containing hMSCs. The significance for all analyses was set at $P < 0.01$ ($**$) and $P < 0.001$ ($***$) and nonsignificance was noted as ns. Each group consisted of 5 animals ($n = 5$).

contact with organ secreting SDF1 such as irradiated liver in our experiment (Figure 4(b)). Results are expressed as fold increase expression of hMSCs engrafted in liver as compared to hMSCs before injection and normalized to *GAPDH*. The level of *CXCR4* increased rapidly and significantly in vivo during the first days after injection and then decreased after 15 days ($P < 0.05$).

A recent report describes that mir-27b can suppress the directional migration of MSCs by downregulating SDF1α expression by binding directly to the SDF1α 3′UTR [44]. We compared the level of mir-27b in liver of mice engrafted with hMSCs to ungrafted liver (Figure 4(c)). The level of mir-27b was more elevated in the liver of ungrafted mice in comparison to that of the liver containing hMSCs ($P < 0.05$).

In our conditions, hMSCs might have acquired in vivo a high level CXCR4 expression and migrated in irradiated liver expressing high levels of *SDF1α*. A high level of *SDF1α* might be related to a low level of mir-27b. We conclude that hMSCs engraftment is related to a low level of mir-27b.

Figure 5(f) displays the proportion of mouse livers where hMSCs were detected using quantitative PCR of the human β-globin gene. The human β-globin gene signal was detected at each time point studied from 3 to 60 days after TBI. Human DNA was detected in 20% of livers at 3 days after TBI. The highest number of livers in which human DNA was detected was observed at 15 days after TBI. Three weeks after TBI, the proportion of liver in which human DNA was detected decreased. The number of livers positive, in PCR, for human gene presence appeared to stabilize at approximately 50% and the second month after irradiation. The colonization of the liver by hMSCs appeared to be maximal 15 days after radiation exposure. As shown in Figure 5(f), an exponential increase in the number of livers containing human DNA was detected during the period of reduction in the hepatic activity, the critical phase. This correlation suggests that there is a larger mobilization of the BM-MSCs towards the liver of irradiated mice during the critical phase of this tissue. Thus, the hMSCs' engraftment kinetics in the liver could be correlated to the reduction in hepatic activity.

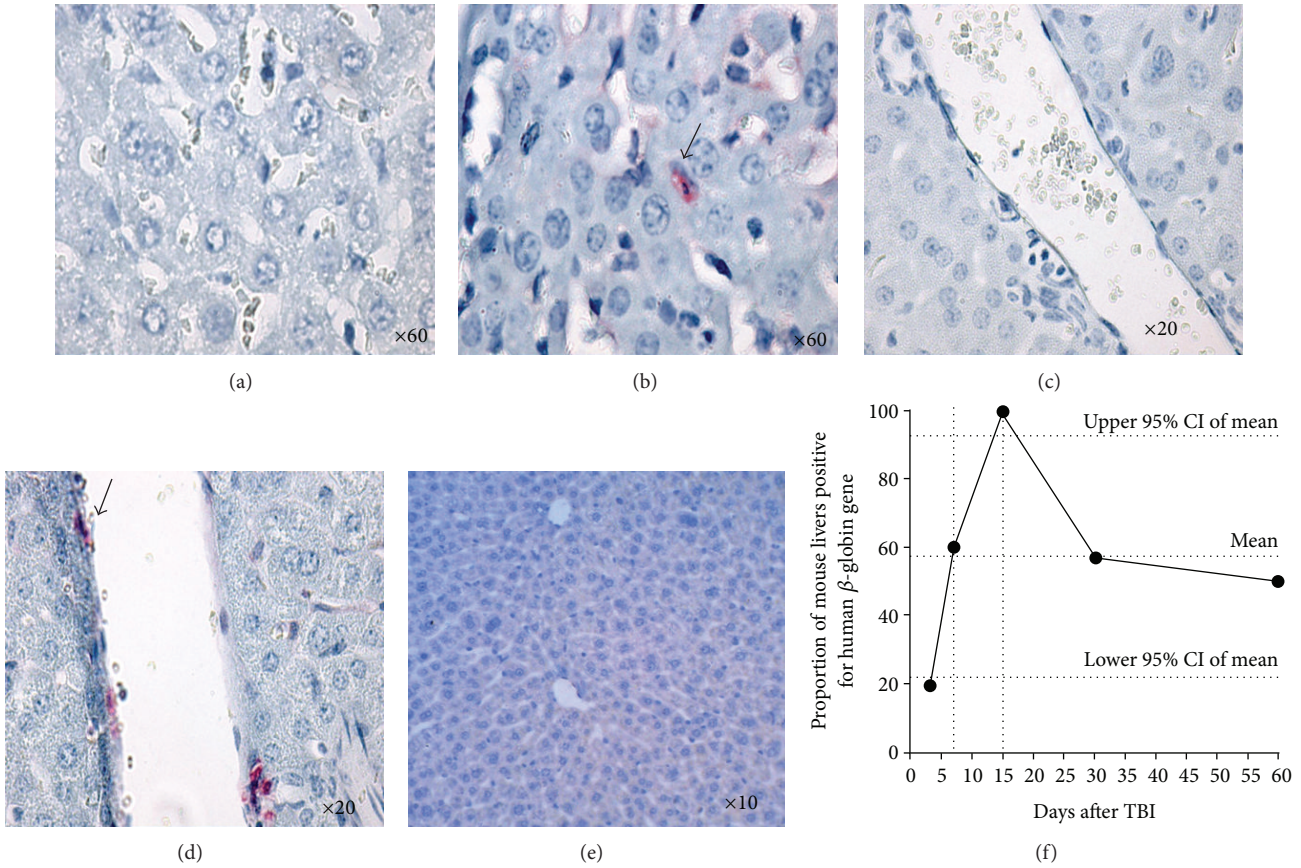

Figure 5: Human β-2-microglobulin immunostaining in mouse liver, 30 days after TBI. And percentage of mouse livers engrafted with hMSCs from 3 to 60 days after TBI. Immunostaining of hepatic cells (b), endothelium of portal vein (d) with human cells expressing beta-2-microglobulin stained in red, and respective negative controls ((a) and (c)). Isolated human cells can be seen between hepatic ((b) black arrow) and endothelial cells ((d) black arrow). (e) Negative control nonirradiated, noninjected with hMSCs (f).

Table 3: Frequency of human cells in livers of irradiated (TBI) NOD/SCID mice after hMSC infusion. The frequency of hMSCs engrafted in liver was quantified using a PCR detection of the human β-globin gene in mouse liver from 3 to 60 days after TBI. The hMSC engraftment rate was significantly increased at 30 days in the liver following TBI, when compared to the 15th day. All values are expressed as the mean and the standard error of the mean (SEM), $n = 10$.

Days after TBI	3	7	15	30	60
% human cells (mean, $n = 10$)	0.07%	0.09%	0.11%	3.08%	2.52%
SEM	0.01	0.04	0.12	1.12	0.91

To determine the quantity of human cells, quantitative PCR on the human β-globin gene was performed in livers from 3 to 60 days after TBI. The stippled lines represent the postirradiation period during which a reduction in the hepatic activity was observed (from 7 to 15 days after irradiation). From 20% (day 3) to 100% (day 15) of livers contain human DNA. A substantial engraftment of hMSCs in the mouse liver was measured during the period of reduction in the hepatic activity. hMSCs can have early beneficial effects on hepatic activity.

In a second step, we determined the frequency of hMSCs in the livers engrafted with hMSCs. Table 3 lists the frequency of hMSCs in irradiated NOD/SCID mouse livers. The quantitative detection of human β-globin gene in mouse liver from 3 to 60 days after TBI showed a significant increase of hMSC engraftment during this period (Table 3). Between 15 and 30 days after TBI, the quantity of human cells in liver increased significantly by 28-fold ($P = 0.001$). Thus, it appears that the percentage of livers positive for human bone marrow-derived stromal cells is not correlated linearly with the quantity of human DNA detected.

Moreover, the hMSC infusion involved a return to the basic levels of urea and transaminases 7 days after TBI (Figure 2). Altogether, these results suggest that the hMSCs can act very precociously and in very small quantity. If hMSCs have a beneficial effect on the liver before the 15th day after irradiation, this suggests that it is not the quantity of hMSC engrafted that is important. The important aspect would be that these adult cells are present in small quantity. At 30 days after irradiation, the hMSC quantity detected remains high, which suggests a strong presence of hMSCs and therefore a possible proliferation of them in this organ.

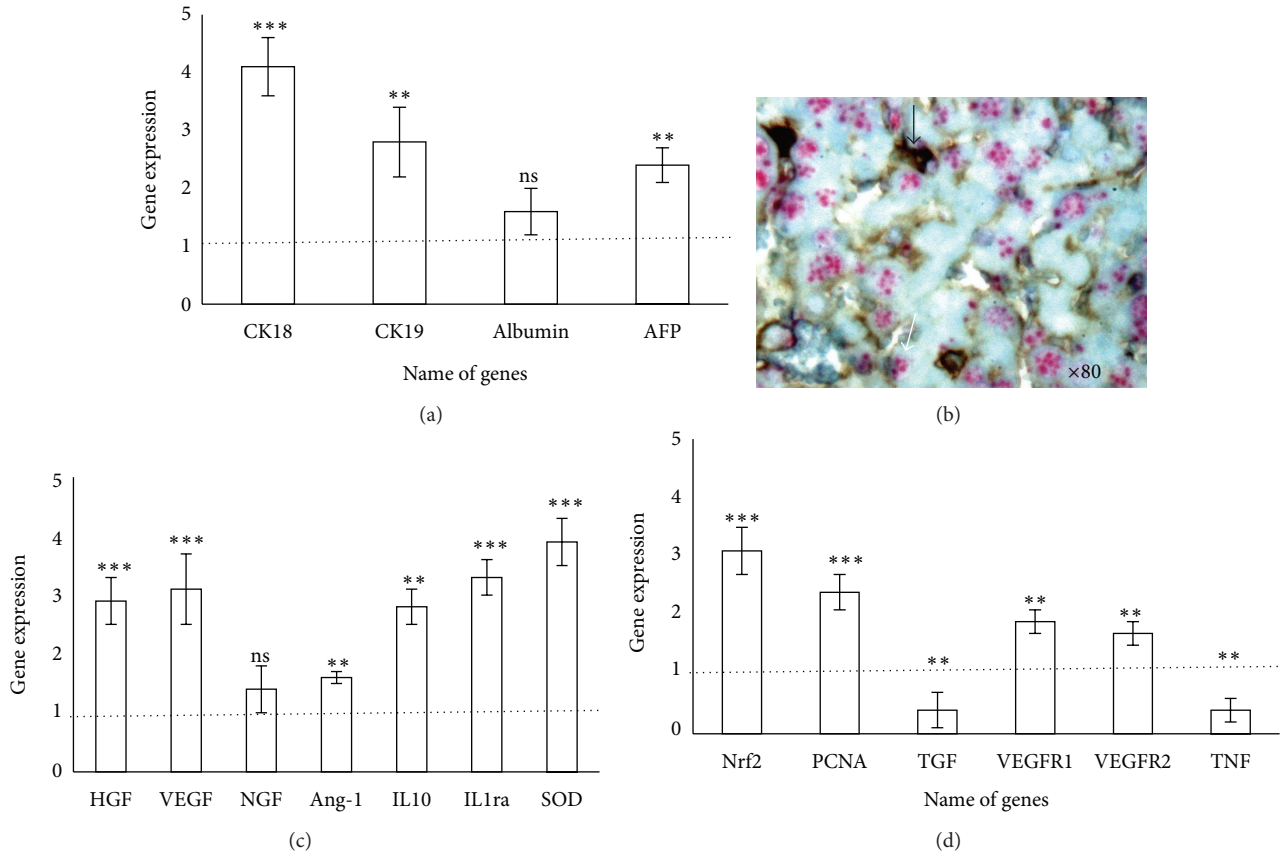

FIGURE 6: In situ hybridization analysis and gene expression of hMSCs implanted in liver. (a) To determine whether implanted hMSCs in vivo expressed liver specific genes hMSCs before injection was compared to hMSC gene expression 15 days after injection. RT-PCR values are representative of at least 5 replicates. Results are expressed as a fold increase in gene expression in hMSCs in liver compared to hMSCs before injection. Values greater than one indicate upregulation of the target genes and values less than one indicate their downregulation. (b) Determination of cell fusion. Black arrow indicates human cells beta-2-microglobulin stained. FISH analysis showing the mice transcription centers (white arrow). (Original magnification ×80.) (d) Determination of growth factors secreted by hMSCs implanted in mice liver. The significance for all analyses was set at $P < 0.01$ (**) and $P < 0.001$ (***) and nonsignificance was noted as ns. Each group consisted of 5 animals ($n = 5$).

3.6. Localization of hMSC Engrafted in the Liver by Immunohistology, 30 Days after TBI (Figures 5(a)–5(e)). To detect the hMSCs in situ, we used livers collected at 30 days after irradiation, when the highest quantity of human cells could be detected. To localize human cells in engrafted livers, we performed immunohistologic experiments using a human β-2-microglobulin specific antibody. Staining was carried out on livers collected in animals subjected to TBI, 30 days after exposure. Cells expressing the human β-2-microglobulin were observed either isolated between hepatic cells (Figure 5(b)) or in the endothelium of the portal vein (Figure 5(d)). Human cells were mainly localized in the perivascular region of the liver 30 days after TBI. In a previous study, we reported about the migration of hMSCs through the vascular wall and about an intravascular colonization under intima in the lungs [7]. These observations in the liver are in agreement with those published on the lungs and support the hMSCs' capacity to colonize the perivascular region of various tissues.

3.7. hMSC Differentiation in the Liver by RT-PCR. At 15 days after transplant (when 100% of liver possessed human cell implantation) to study the differentiation of human cells, we measured human mRNA of mice liver engrafted with human cells. To control that primers amplified specifically human mRNA, we examined liver total RNA from 5 control mice which had not received hMSCs. Values greater than one indicate upregulation of the target genes and values less than one indicate their downregulation. To determine whether implanted hMSCs in vivo expressed liver specific genes (Figure 6(a)), we measured human mRNA of *CK18, CK19,* albumin, and *AFP*. Results are expressed as fold increase expression as compared to hMSCs before injection and normalized to GAPDH. The hMSC engraftment significantly increased ($P < 0.05$) the expression of *CK18* (4.1-fold), *CK19* (2.8-fold), and *AFP* (2.4-fold). The hMSCs expressed a hepatocyte phenotype.

In a second step, we controlled whether a cell fusion occurred between hMSCs and hepatocytes. We determined cell fusion. Human cells were stained with beta-2-microglobulin (black arrow) and a fluorescence in situ hybridization (FISH) analysis showed the mice transcription centers (white arrow). No fusion of human cells with mice hepatocytes was observed.

In a third step, we investigated whether hMSCs induced liver regeneration by secretion of growth factors or limitation of inflammation (Figure 6(c)). The data was expressed as fold increase in the mRNA level of hMSCs before injection compared to human cells after implantation in liver. HGF is important in the proliferation phase of hepatocyte. An increased of 2.3 fold of HGF was observed. *VEGF* increased 3.1-fold, *NGF* 1.4-fold, and Ang-1 1.6-fold. Anti-inflammatory gene expression *IL10* and *IL1ra* increased 2.8- and 3.3-fold, respectively (significantly, $P < 0.05$). The secretion of growth factors and the modulation of inflammation promote liver regeneration. The hMSC engraftment exerts a paracrine proliferative effect on endogenous hepatocytes and endothelial cells.

In a fourth step, by measuring gene expression of mice liver gene (Figures 6(c) and 6(d)), we controlled whether hMSCs decrease oxidative stress (*SOD, Nrf2*) and promote hepatocytes (PCNA, TGF, TNF) and endothelial proliferation (VEGF-R1, VEGF-R2). The expression of *SOD* (3.9-fold), *Nrf2* (3.1), *PCNA* (2.4), and *VEGFR1* (1.9), *VEGFR2* (1.7) increased and *TGF* (0.4) and *TNF* (0.4) decreased significantly ($P < 0.05$).

In conclusion, we found no fusion of human cells with mice hepatocytes but an increase of gene expression specific of hepatocyte cells was observed. The hMSC liver regeneration process operated by different mechanisms: (1) tissue differentiation, (2) secretion of growth factors, (3) angiogenesis, (4) downregulation inflammation, and (5) limitation of oxidative stress.

4. Discussion

Irradiation induced an increase of plasmatic transaminases levels, an increase of the apoptosis process in the liver vascular system, and an increase of oxidative stress. hMSC injection decreased transaminases levels, oxidative stress, and apoptotic cells and may reduce ROS production in the injured liver. HMSCs corrected radio-induced liver dysfunction by a simultaneous effect on hepatocyte differentiation, protection of vascular functions, secretion of anti-inflammatory, and trophic factors (Figure 7).

Seven days after TBI, the urea level was significantly lower in irradiated mice than in non irradiated mice or in mice irradiated and injected with hMSCs. In addition, the absence of variation in water and food intake of the animals suggests that the reduction in the urea level is related to hepatic insufficiency. The modification of the plasmatic concentrations of urea and transaminases correlating with hepatic activity, without variation of the renal activity, shows that the liver is more radiosensitive than the kidneys. The systemic hMSC infusion 24 hours after TBI appears to be beneficial to the hepatic activity. In mice subjected to TBI, hMSC infusion allowed for the maintenance of the basic plasmatic levels of urea, AST, and ALT. The beneficial effects of an intravenous (IV) MSC injection have also been observed in other experimental models. IV infusion of MSCs soon after acute myocardial infarction (MI) in swine was shown to improve left ventricular ejection fraction and to limit wall thickening in the remote noninfarcted myocardium,

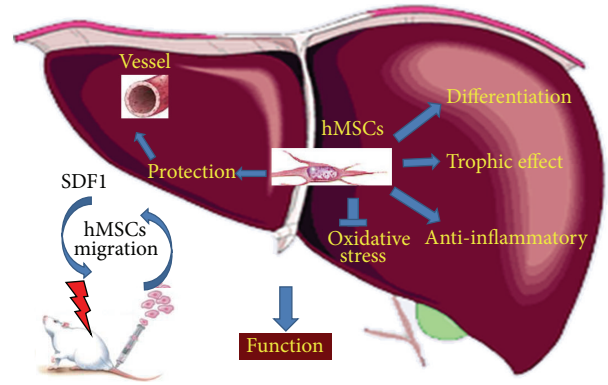

Figure 7: Effect of intravenous hMSCs injection on liver after total body irradiation (TBI). HMSCs migrate into liver, correcting radio-induced liver dysfunction by a simultaneous effect on hepatocyte, protection of vascular functions, differentiation, secretion of trophic, and anti-inflammatory factors.

consistent with a beneficial effect on post-MI ventricular remodeling [45]. IV delivery of MSC prepared from adult BM reduces infarction size and ameliorates functional deficits of cerebral ischemia and spinal cord injury in rat models [46]. In a nonhuman primate model, the route of MSC delivery (intratissue or IV) does not modify the capacity of MSC engraftment [47].

SDF1 and CXCR4 are necessary for migration of MSCs to damaged tissues. *CXCR4* expression is reduced during the ex vivo expansion of MSCs. We have shown that the level of SDF1 expression was higher in liver engrafted with hMSCs compared to unimplanted liver. We tested whether MSCs may increase CXCR4 in vivo in contact with organ secreting SDF1 such as irradiated liver. In hMSCs, the level of CXCR4 increased rapidly and substantially in vivo during the first days after injection. Furthermore, the level of mir-27b is more elevated in the liver of ungrafted mice in comparison with the liver containing hMSCs. These results are in accordance with a recent report that describes that mir-27b can suppress the directional migration of MSC by downregulating *SDF1α* expression by binding indirectly to the *SDF1α* 3′UTR [44]. We have shown that hMSCs acquired in vivo a high level *CXCR4* expression and migrated in irradiated liver expressing a high level of *SDF1α*. A high level of *SDF1α* might be related to a low level of mir-27b. We conclude that hMSC engraftment is related to a low level of mir-27b.

MSCs protect against injury by altering the oxidative microenvironment of the liver. *Nrf2* is a transcription factor that positively regulates the basal and inducible level of cytoprotective genes. *Nrf2* activation is protective against oxidative stress and induced SOD production which decreased ROS in liver [42]. We have shown that hMSC injection induced an increase in *Nrf2*, *SOD* gene expression which might reduce ROS production in the injured liver decreasing oxidative stress induced by irradiation. These results imply that hMSCs have a protective effect against induced oxidative stress.

To specifically identify human cells expressing β2-microglobulin, we determined that hMSCs preferentially localized

in the endothelium of the portal vein and between hepatic cells. In accordance with a previous report [48], no fusion of human cells with mice hepatocytes and an increase of gene expression specific of hepatocyte were observed. Engrafted hMSCs expressed *CK18*, *CK19,* and *AFP* genes indicating a possible hepatocyte differentiation. The transdifferentiation ability of hMSCs into hepatocytes may play a role in the repair of injured liver. Nevertheless, these cells were limited to a small portion of total liver mass and were not sufficient to reverse the injury (a percentage of 2.5 is necessary for this process). Differentiation of hMSCs in hepatocytes participates in liver regeneration as previously reported [28].

The human cells injected intravenously were mainly localized in the perivascular region of the liver 30 days after TBI, as already reported in the lung [7]. The presence of hMSCs expressing *VEGF* in the perivascular region can confer a role of secreting cells to hMSCs in order to maintain the endothelial vascular cell functions. Furthermore, hMSCs upregulated gene expression of *VEGF-R1, R2* in liver. hMSCs promote neoangiogenesis after liver irradiation as previously reported after liver resection [49].

Recent work suggests that vascular endothelial function is significantly impaired due to oxidative stress mediated by the generation of oxygen-derived free radicals in response to chronic or acute inflammation [50]. Moreover, the endothelium is described as a central regulator of vascular and body homeostasis. The vascular endothelium is versatile and multifunctional. In addition to its role as a selective permeability barrier, it is involved in many synthetic and metabolic properties including the modulation of vascular tone and blood flow, the regulation of immune and inflammatory responses, and the regulation of coagulation, fibrinolysis, and thrombosis. Gaugler and colleagues reported that exposure to radiation can alter endothelial cells functions [51]. These radiation-induced negative effects could be playing a critical role in mediating organ dysfunction. Thus, hMSC localization to the perivascular region could protect the vascular endothelium by the secretion of cytokines and/or chemokines, which in turn stimulate or maintain the integrity of this fundamental structure by preventing the appearance of secondary negative effects in the liver. This protective mechanism could also apply to other tissues, as hypoxia has been shown to influence the localization of hMSCs [52].

The expression of growth factors by hMSCs in liver including *NGF*, *HGF*, and *Ang-1* and anti-inflammatory molecules (*L-10, IL-1ra*) related to an increasing cell proliferation in liver (increase of *PCNA* gene expression) might correct liver dysfunction and promote liver regeneration as previously published [29, 48]. NGF, HGF, and VEGF are reported to increase the intrinsic ability of hepatocytes to proliferate or to facilitate the breakdown of scar tissue [48]. TBI induced negative effect on the liver. hMSC systemic infusion significantly prevented the secondary hepatic effect, while the hMSC infusion itself did not induce noticeable toxic effects. This correlates with the report that MSC infusion can increase the survival of patients developing GVHD [23, 53]. These findings and clinical observations reinforce the assumption that MSCs are potent candidates for protecting against and repairing various damages [42, 44].

Conflict of Interests

The authors have declared that no competing interests exist.

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Review Article

From Single Nucleotide Polymorphisms to Constant Immunosuppression: Mesenchymal Stem Cell Therapy for Autoimmune Diseases

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The regenerative abilities and the immunosuppressive properties of mesenchymal stromal cells (MSCs) make them potentially the ideal cellular product of choice for treatment of autoimmune and other immune mediated disorders. Although the usefulness of MSCs for therapeutic applications is in early phases, their potential clinical use remains of great interest. Current clinical evidence of use of MSCs from both autologous and allogeneic sources to treat autoimmune disorders confers conflicting clinical benefit outcomes. These varied results may possibly be due to MSC use across wide range of autoimmune disorders with clinical heterogeneity or due to variability of the cellular product. In the light of recent genome wide association studies (GWAS), linking predisposition of autoimmune diseases to single nucleotide polymorphisms (SNPs) in the susceptible genetic loci, the clinical relevance of MSCs possessing SNPs in the critical effector molecules of immunosuppression is largely undiscussed. It is of further interest in the allogeneic setting, where SNPs in the target pathway of MSC's intervention may also modulate clinical outcome. In the present review, we have discussed the known critical SNPs predisposing to disease susceptibility in various autoimmune diseases and their significance in the immunomodulatory properties of MSCs.

1. Introduction

Mesenchymal stromal cells (MSCs) are under investigation in clinical trials to treat autoimmune disorders and degenerative disorders due to their immunomodulatory and regenerative properties. Various sources of MSCs have been described in the literature, but the one widely studied source is the bone marrow derived MSCs. MSCs in bone marrow represent 1 in 100,000 nucleated cells, but they play a significant role in regulating the niche for hematopoietic stem cells and immune homeostasis and hypothetically can differentiate into cartilage, bone, and adipocytes [1]. Major limitation for the use of MSCs in clinical trials is their low frequency in the bone marrow aspirates. This specific challenge has been addressed by MSC expansion into large quantities by virtue of their *in vitro* mitogenic properties under standard cell culture conditions [2]. This rapid expansion favors robust translational inquiry to utilize these cells in cellular therapy. To minimize ambiguity, the International Society for Cellular Therapy (ISCT) proposed minimal criteria to define human MSCs as expressing CD105, CD73, CD90 and lack of CD45, CD34, CD14 or CD11b, CD79α or CD19, and HLA-DR surface molecules [3].

The therapeutic potential of transfused MSCs was well demonstrated in animal models of experimental autoimmune encephalitis, diabetes, rheumatoid arthritis, myocardial infarction, acute lung injury, retinal degeneration, acute renal failure, transplant rejection, liver fibrosis, inflammatory bowel diseases, and graft versus host diseases [4, 5]. Based on these preclinical observations, therapeutic applications of MSCs are currently being explored in more than 300 clinical trials (http://www.clinicaltrials.gov/). MSCs were

used for the first time to treat grafts versus host disease (GVHD) and later to treat autoimmune disorders such as Crohn's disease, multiple sclerosis, autoimmune rheumatic diseases, and autoimmune diabetes. The regenerative and anti-inflammatory properties eased their use to treat immune mediated disorders. MSCs suppress both innate and adaptive immune system as they inhibit the activation, proliferation, and also the function of lymphocytes, monocytes, dendritic cells, and natural killer cells. Previous reviews have already addressed the immunomodulatory properties of MSCs, and it is beyond the scope of current review [5–15].

The fundamental pathogenesis of autoimmune disorders lies on the loss of immune tolerance to self-antigens. In patients with autoimmune diseases, genetic changes in the genome of an individual affect the role of essential immunological pathways, leading to the breakdown of immune tolerance. The consequence of this effect is the inability of immune system to distinguish self- versus non-self-antigens. Genome wide association studies (GWAS) advanced the understanding of autoimmune diseases by identifying common single nucleotide polymorphisms (SNPs) and linking them to the cause of the diseases [16]. SNPs alter the phenotype and functionality of proteins in the immune system thereby affecting its function leading to disease. It is of essential interest to question the immunosuppressive properties of MSCs derived from the individuals bearing the disease causing SNPs.

Current clinical trials utilize MSCs obtained from autologous or allogeneic origin. In the autologous setting, MSCs acquired from the bone marrow of patients with autoimmune diseases are used in the suppressor therapy. While MSCs possess multiple immunoregulatory molecules to exert suppression, the question that remains unanswered is whether the SNPs in the immunomodulatory genes of MSCs affect the clinical outcome following MSC therapy. In the allogeneic setting, bone marrow-derived MSCs are expanded and banked from the universal healthy donor and subsequently administered to the patients. Since MSCs are derived from healthy donors, it is possible that these cellular products may not possess the genetic changes associated with the disease. However, MSCs specifically act on certain immune target pathways systemically or to the inflamed site and thereby execute immunosuppressive and regenerative functions. By considering the SNPs in these targets of MSC intervention, it raises the question if SNPs in the recipient's immune pathway affect the clinical outcome. In the present review, we analyzed the common SNPs identified in the autoimmune diseases that are under investigation for MSC therapy and their significance in the mechanism of MSCs immunosuppressive effect and clinical outcome.

2. Multiple Sclerosis

Multiple sclerosis (MS) is an autoimmune disorder of the central nervous system where myelin and oligodendrocytes are targeted by cell mediated and humoral immunity [17]. The beneficial effects of MSC therapy have been well described in autoimmune encephalomyelitis (EAE) mouse model that provided a basis for further exploration in clinical trials [18–20]. Currently, close to 15 clinical trials are registered to use MSC therapy for the treatment of multiple sclerosis (http://www.clinicaltrials.gov/). MSCs have been well tolerated and were deemed safe in patients with MS in the early phase clinical trials. Mallam et al. described that MSCs derived from MS patients show expansion, differentiation, and surface marker expression similar to the MSCs from healthy individuals [21]. In contrast another study reported, although MSCs from MS patients exhibit normal growth, phenotype and immunomodulatory properties, they secrete higher levels of lipopolysaccharide-stimulated IP10 compared to MSC from healthy controls [22]. These contradictory results suggest the functional differences in the MSC populations, rooted from the changes in their genetic profile, isolated from MS patients. However, the efficacy results demonstrated the evidence of structural, functional, and physiological improvement and are suggestive of neuroprotection [23, 24]. The most relevant SNPs linked with the pathogenesis of MS are harbored in the genes *HLADRB1, IL2RA, IL7R, CLEC16A, CD226, CYP27B1, MMEL1, SH2B3, CD40, CD80, CD86, and CD58* [25]. The relevance of *IL2RA, IL7A, CYP27B1, SH2B3, and MMEL1* for MSC therapeutic activity is subtle. MSCs do not express CD40 [26]. CD80 and CD86 do not present on MSCs, and addition of IFNg does not upregulate these costimulatory molecules. Although HLADR is absent on the MSCs, IFNg upregulates its expression [27]. Considering the IFNg dependency of MSC's suppressive activity, the current unknown factor is the differences in HLADR alleles on the immunosuppressive activity of MSCs. MSCs upregulate the adhesion molecule CD58 (lymphocyte function-associated antigen) after coculture with the T cells [28]. MSCs ability to bind to the inflamed tissues is important to execute their immunosuppressive effect [29]. The significance of SNPs on adhesion molecules on MSC's engraftment potential requires further investigation.

3. Autoimmune Rheumatic Diseases

MSCs are under clinical investigation for the treatment of autoimmune rheumatic diseases such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE), Sjögren's syndrome, and systemic sclerosis. The animal models of collagen-induced arthritis reported varied results. Although an earlier study demonstrated that MSCs do not have any beneficial effect on mice with collagen-induced arthritis (CIA), subsequent studies demonstrated therapeutical effect [30–32]. Few other combination approaches were suggested such as conditioning of MSCs with the drugs such as bortezomib by modulating the microenvironment and thereby enhancing the therapeutic efficiency of MSCs [33]. Results of early phase ongoing clinical trials are not available to evaluate the clinical impact of MSCs in RA. Although a study suggested that stromal cell function is defective in patients with RA, another study demonstrated the immunosuppressive functions of MSCs derived from three RA patients [34, 35]. The common SNPs identified in RA and relevant to MSC biology and immunomodulation are in the genes of CD58 (adhesion molecule), IL6ST (CD130), and chemokine

(C-C motif) receptor 6 [36]. IL6ST is the signal transducer in the IL-6 receptor complex to initiate the downstream signals. MSCs secrete high levels of IL-6, and it has been demonstrated that IL-6-dependent secretion of PGE2 by MSCs inhibits local inflammation in the mouse model of arthritis [31]. In addition, autocrine effect of IL-6 has been demonstrated as this cytokine enhances the survival of MSCs after serum starvation-induced apoptosis [37]. IL-6 secreted from MSCs accelerates intestinal epithelium recovery in the animal model of total body irradiation [38]. These studies highlight the significance of IL-6 in MSC immunobiology and support the need for further studies to evaluate the breadth of the signal induction with the CD130 receptor complex. MSCs have been shown to bind to Th17 cells via CCR6 and thereby induce regulatory T cell phenotype in these cells [39]. It is necessary to further investigate the SNPs in CCR6 on T cells and the immunomodulatory properties of MSCs.

Although MSCs from the SLE patients show immunosuppressive activity, they undergo senescence relatively faster than MSC from age matched healthy controls [40, 41]. Autologous MSC therapy in two SLE patients was safe but did not reduce the disease activity [42]. A direct link between the SLE and SNPs in genes of the inflammasomes has been demonstrated [43]. However, its relevance to MSC's immunomodulatory functions has not been studied so far to rationalize and improve future clinical trials for SLE.

Few trials evaluating the safety and efficacy of MSCs in Sjögren's syndrome and systemic sclerosis/scleroderma are ongoing. MSCs from the patients with Sjögren's syndrome show impaired immunosuppressive activity, and allogeneic MSC treatment improves disease outcome [44]. Ice et al. reviewed several SNPs linked to Sjögren's syndrome, but the relevance of these genes to MSCs therapeutic properties may be subtle [45]. Unlike Sjögren's syndrome, MSCs from scleroderma patients preserve their immunosuppressive functions [46]. SNPs associated with scleroderma and specifically SNPs in *FAS* gene is of further interest in MSC biology [47]. FAS/FASL interaction has been described in MSC's immunomodulatory properties [48]. Altogether MSC biology from autoimmune disease patients requires further investigations with the linkage to the genetic changes in the key immunomodulatory molecules.

4. Crohn's Disease

The etiology of Crohn's disease (CD), an inflammatory bowel disease, is presumed alteration of genetic factors or gut microbiota or the host immune system [49]. Despite the etiology, all these factors share the common clinical manifestation of excessive intestinal inflammation. Clinical trials using MSCs as cell based inflammatory bowel suppressive therapy for CD are promising [50–53]. GWAS scan of nonsynonymous SNPs in CD has identified a mutation in the genes of autophagy [54, 55]. Autophagy is a cellular homeostatic process in which the cell compartments are recycled under stressful conditions. Recent developments have highlighted a balancing role of autophagy in the immunity and inflammation [56]. Defects in this homeostatic autophagy process may cause the basic pathogenesis of many infectious and inflammatory diseases [56]. A majority of studies indicate that autophagy plays a major role in the CD pathogenesis [57–60]. ATG16L1 protein is an important player in the autophagy process by forming the autophagosomes. In the colitis mouse model, autophagy knockout mice (ATG16L1 deficient mouse) die after Dextran Sodium Sulfate (DSS) treatment due to the excessive production of proinflammatory cytokines IL-1 beta and IL-18 [61]. In addition, CD patients with T300A SNP in ATG16L1 gene show many abnormalities in the intestinal paneth cells, which are the producers of alpha-defensins in the intestine [62]. Peripheral blood mononuclear cells from the CD patients with T300A SNP in ATGL161 gene secrete high levels of proinflammatory IL-1 beta and IL-6 upon *in vitro* stimulation [63]. Altogether these studies demonstrate the potential linkage between phenotype and SNPs in autophagy genes in CD patients. Two important studies describe the relevance of autophagy in MSC's biological properties. The first study suggested that MSCs utilize autophagic mechanism to provide tumor stromal support [64]. The second study demonstrated the role of autophagy in MSC mediated hepatic regeneration in the animal model of liver diseases [65]. The role of autophagy in the MSC's immunomodulatory properties and the significance of these SNPs in MSC's biology is currently unknown. Utilization of autologous MSC treatment and the influence of SNPs in the genes of autophagy on the clinical outcome of crohn's diseases require further investigation.

5. Autoimmune Diabetes

Type I diabetes results from the immune destruction of insulin producing beta cells in the pancreatic islets of Langerhans. Although transplantation of islets of Langerhans helps to maintain the insulin levels, immunosuppressive therapy is a requirement. MSCs are under clinical investigation to treat autoimmune diabetes due their immunosuppressive and angiogenic properties and the ability to regenerate beta cells [66]. Reversal of hyperglycemia with MSC therapy has been demonstrated in a number of diabetic animal studies [67–70]. Type I diabetes associated SNPs were reported in the genes such as *IFIH1 (*interferon-induced helicase), *CTLA4, IL2RA, CLEC16A (C type lectin), and PTPN2* [71]. Of these genes, *PTPN2 is* of significant interest to the immunomodulatory properties of MSCs. *PTPN2* regulate signaling events by dephosphorylating multiple JAK and STAT molecules, and MSCs immunosuppressive properties are highly depend on the signal induction through IFNg [72]. Thus, the role of SNPs in *PTPN2* gene on the suppressive properties of MSCs and IFNg signaling events require further investigation.

6. Common SNPs in the Immunoregulatory Pathways of MSC's Intervention

The unique feature of MSCs is their array of immunoregulators, which collectively mediate the immunosuppressive and regenerative functions that impact the clinical outcome. The

most important T cell regulators defined by MSCs are IDO and PDL1/PDL2-PD-1 pathways.

6.1. Indoleamine 2,3 Dioxygenase. Indoleamine 2,3 dioxygenase (IDO) is an enzyme of tryptophan degradation pathway which converts tryptophan to kynurenine and suppresses the T cell responses [73]. IFNg upregulates IDO in MSCs thereby not only suppresses T cell proliferation but also induces the differentiation of monocytes into suppressor phenotype [74]. IDO expression by MSCs is considered as a standard readout for the functionality of the cellular product [75]. Impairment of IDO activity in the patients with autoimmune primary biliary cirrhosis has been reported, suggesting the possible role of IDO to maintain immune tolerance [76]. Arefayene et al. reported the genetic variants of IDO-1 gene with SNPs and associated altered enzyme activity, but this study does not include any disease specific SNPs in IDO [77]. A subsequent study demonstrated that SNP rs7820268 (C6202T) in the IDO gene is statistically more frequent in systemic sclerosis patients than in controls. In addition, patients bearing this SNP in IDO show impaired CD8+ T reg function [78]. This is an important functional study, which establishes a relationship of IDO SNPs with T cell responses. Future investigations are required to study the influence of SNPs in IDO on the immunomodulatory properties of MSCs.

6.2. PDL1/PDL2-PD-1 Pathway

6.2.1. T Cell Mediated Immune Responses. T cell activation is not only controlled by major histocompatibility complex (MHC) and T cell receptor (TCR) engagement but also by the interaction with other costimulatory molecules. PDL1/PDL2-PD-1 pathway is one such pathway which regulates the T cell tolerance in various conditions [79]. This pathway is implicated in negatively regulating T cell immunity in tumor microenvironment and chronic viral infections [80]. PD-1 is the receptor on the T cells with immunoreceptor tyrosine-based inhibiting motif (ITIM). Upon its engagement with the ligands PDL1 (B7H1) or PDL2 (B7DC), it provides negative signal to the T cells [81]. PDL1-PD1 pathway is implicated in MSC's suppression of T cell proliferation upon licensing with proinflammatory cytokine IFNg [20, 82–84]. Some studies reported that there is no correlation with SNPs in PDL1 gene and autoimmune diseases in Japanese patients. However, one study suggested that A/C polymorphism at position 8923 in PDL1 gene is associated with Graves diseases [85–87]. Similarly, another study demonstrated that SNPs in the gene for PDL2 is associated with SLE in Taiwan [88]. Since SNPs in the genes of PDL1 and PDL2 are not explicitly reported, it is possible to conclude that these ligands are intact on the surface of MSCs to execute the suppressive functions in the autologous therapy. However, the SNPs in the gene of PD-1 (*PDCD1*) are widely reported and associated with the autoimmune diseases [87]. Association between SNPs in *PDCD1* and disease susceptibility to autoimmune diseases were demonstrated in SLE [89, 90], Type I diabetes [91], RA [92, 93], MS [94], and Graves disease [95]. Kroner et al. specifically showed the functional relevance of SNPs in PDCD1 polymorphism by demonstrating the deficit in PD-1 mediated inhibition of cytokine secretion in T cells from the multiple sclerosis patients [94]. These studies clearly suggest the role of dysfunctional PDL1/PDL2-PD-1 pathway in the autoimmune patients. In the allogeneic cellular therapeutic situation, although the ligands PDL1 and PDL2 on MSCs are intact, it is possible that the SNPs in PD-1 may compromise the delivery of negative signals to T cells. Hence, PDL1/PDL2 mediated therapeutic effect by allogenic or autologous MSCs may depend on the PD1 polymorphism of the recipient which could predict the treatment responsiveness.

6.2.2. Humoral Immune Responses. T helper cells and B cell interaction plays an important role in the breakdown of peripheral tolerance in the autoimmune disorders. The helper T cells that are not sensitive to self-tolerance mechanisms secrete proinflammatory cytokines, resulting in expansion of the autoreactive B cells which produce autoantibodies to cause the self damage [96]. MSCs also affect B cell differentiation into plasma cells and subsequent immunoglobulin production [97–100]. MSCs affect the plasma cell differentiation through contact independent pathway by cleaving CCL2 in a unique mechanism [101]. Additionally, there is data suggesting that MSCs suppress B cells through PDL1/PD1 pathway [102]. Another study by Liu et al. demonstrates that periodontal ligament stem cells inhibit B cell activation through PDL1/PD1 [103]. These results suggest that stem cells act on the humoral immune responses through the PDL1/PD1 pathway. PD-1 is upregulated on the B cells after stimulation with ani-IgM and PMA/ionomycin [104]. Bertsias et al. reported that homozygous PD-1.3 SNP on the SLE patients causes lower expression of PD-1 on CD19+ B cells [105]. It is possible to speculate that lower expression of PD-1 on B cells due to PD-1.3 SNP may compromise MSC's inhibitory effect in B cells in SLE patients. Further studies are required to study the role of PD-1.3 SNP on the B cell interaction with MSCs.

7. Conclusion

MSCs are attractive to researchers due to their wide spectrum of immunomodulatory and regenerative properties, which collectively constitute their therapeutic activities. It is arguable that even if genetic changes such as SNPs affect one pathway, compensatory pathways may balance the functional machinery of MSCs. However, in certain situations, target pathways are crucial for the maintenance of immune tolerance, and in those conditions, MSCs could be considered as supplemental therapy along with the other immune suppressive molecules. SNPs are suggested as biomarkers for disease susceptibility in certain autoimmune disorders. Further studies are warranted in the direction of utilizing these SNPs as biomarkers for prediction of treatment responsiveness to MSC therapy.

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Research Article

Mesenchymal Stem Cell-Like Cells Derived from Mouse Induced Pluripotent Stem Cells Ameliorate Diabetic Polyneuropathy in Mice

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Background. Although pathological involvements of diabetic polyneuropathy (DPN) have been reported, no dependable treatment of DPN has been achieved. Recent studies have shown that mesenchymal stem cells (MSCs) ameliorate DPN. Here we demonstrate a differentiation of induced pluripotent stem cells (iPSCs) into MSC-like cells and investigate the therapeutic potential of the MSC-like cell transplantation on DPN. *Research Design and Methods*. For induction into MSC-like cells, GFP-expressing iPSCs were cultured with retinoic acid, followed by adherent culture for 4 months. The MSC-like cells, characterized with flow cytometry and RT-PCR analyses, were transplanted into muscles of streptozotocin-diabetic mice. Three weeks after the transplantation, neurophysiological functions were evaluated. *Results*. The MSC-like cells expressed MSC markers and angiogenic/neurotrophic factors. The transplanted cells resided in hindlimb muscles and peripheral nerves, and some transplanted cells expressed S100β in the nerves. Impairments of current perception thresholds, nerve conduction velocities, and plantar skin blood flow in the diabetic mice were ameliorated in limbs with the transplanted cells. The capillary number-to-muscle fiber ratios were increased in transplanted hindlimbs of diabetic mice. *Conclusions*. These results suggest that MSC-like cell transplantation might have therapeutic effects on DPN through secreting angiogenic/neurotrophic factors and differentiation to Schwann cell-like cells.

1. Introduction

The majority of research on mesenchymal stem cells (MSCs) has focused on their unique ability of their likelihood of differentiating to mesenchymal cells and an immunomodulation [1]. MSCs are obtained from various kinds of tissues: bone marrow, adipose tissue, and umbilical cord blood. Because of this accessibility, MSCs are clinically used for cell-based transplantation therapy for various diseases: stroke, coronary artery disease, graft-versus-host disease, and arteriosclerosis obliterans. The achievements of these cell-based therapies precipitate extended applications in further human diseases.

In the current study, we deal with diabetic polyneuropathy (DPN). DPN, which is one of the most common peripheral neuropathies as well as one of the most frequent diabetic complications, has not yet been conquered. There is no established therapy for DPN, although many researchers have proven that multiple factors are involved in the pathogenesis: polyol pathway [2], PKC activation [3–6], endoneurial nutritive blood flow [7, 8], advanced glycation end-product

Table 1: Primer sequences.

Accession number	Gene	Forward primer ($5' \rightarrow 3'$)	Reverse primer ($3' \rightarrow 5'$)
NM_001025250.3	Vegfa	CAGGCTGCTGTAACGATGAA	TTTCTTGCGCTTTCGTTTTT
NM_008006.2	Fgf2	GTGGATGGCGTCCGCGAGAA	ACCGGTTGGCACACACTCCC
NM_008808.3	Pdgfa	GAGATACCCCGGGAGTTGAT	TCTTGCAAACTGCAGGAATG
NM_001048139.1	Bdnf	GCCACCGGGGTGGTGTAAGC	CATGGGTCCGCACACCTGGG
NM_001112698.1	Ngf	GTGAAGATGCTGTGCCTCAA	GCGGCCAGTATAGAAAGCTG
NM_010275.2	Gdnf	CGGACGGGACTCTAAGATGA	CGTCATCAAACTGGTCAGGA
NM_001164034.1	Ntf3	CGAACTCGAGTCCACCTTTC	AGTCTTCCGGCAAACTCCTT

formation [9–14], and mitochondrial oxidative stress [15–17]. In the past decade, an increasing number of regenerative therapies, especially cell-based transplantation therapies, in rodent models of DPN have been reported [18–22].

Additionally, regenerative research has been accelerated owing to the discovery of induced pluripotent stem cells (iPSC) [23]. iPSCs possess a pluripotency of differentiation into not only terminally differentiated cells but also multipotent somatic progenitor/stem cells [24–26]. Due to the expectancy for clinical applications, MSC-like cells have also been derived from iPSCs [26].

Having already indicated the beneficial effects of transplantation of bone-marrow-derived MSCs in diabetic polyneuropathy of type 1 diabetic mice, in this paper, we investigate the effects of cell-based therapy utilizing MSC-like cells derived from mouse iPSCs [18]. Needless to say, this investigation would be the first report of the novel cell therapy, and additionally, this contains two original approaches: one is that we employed iPSC derived from aged mouse with consideration for the feasibility of clinical applications, and another is that endocrine abilities of the obtained MSC-like cells were compared with those of not only mouse MSCs but also mouse Schwann cells.

2. Research Design and Methods

2.1. Cell Culture. Mouse iPSCs were derived from bone marrow myeloid cells of GFP expressing 21-month-old EGFP-C57BL/6 mice (C14-Y01-FM131Osb), as previously reported [27]. The iPSCs were maintained at 37°C with 5% CO_2 in DMEM (Life Technologies, Carlsbad, CA) containing 10% Knockout Serum Replacement (Life Technologies), 1% fetal bovine serum (FBS), 1000 U/mL leukemia inhibitory factor (LIF) (EMD Millipore, Billerica, MA), nonessential amino acids, 5.5 mmol/L 2-mercaptoethanol (2ME), 50 U/mL penicillin, and 50 mg/mL streptomycin on feeder layers of mytomycin C-inactivated SNL76/7 cells (the European Collection of Cell Cultures, Salisbury, UK), which were clonally derived from an STO cell line transfected with a G418 resistance cassette and a leukemia inhibitory factor expression construct [28].

2.2. Induction of Differentiation into Mesenchymal Stem Cell (MSC)-Like Cells. For the induction into MSC-like cells, iPSCs were maintained in differentiation medium (DM), which is an α-minimum essential medium (Life Technologies) supplemented with 10% FBS and 5×10^{-5} mol/L 2ME. At day 0 of induction of differentiation, iPSCs were dissociated with the use of trypsin. To promote embryonic body (EB) formation, the cells were cultured at a concentration of 10^3 cells per 20 μL of DM, utilizing the hanging drop method in petri dishes. After 2 days, the EBs were cultured in DM supplemented with 40 ng/mL of all trans retinoic acid (Sigma-Aldrich Co., St. Louis, MO) for 3 days. The colonies were enzymatically passaged into tissue culture dishes containing the DM and were continuously passaged every 3 or 4 days for 4 months.

2.3. Senescence-Associated Beta-Galactosidase (SA β-Gal) Staining. SA β-Gal staining was utilized to evaluate cell senescence. MSC-like cells were fixed with 4% PFA and incubated with the staining solution from a Senescence Detection kit (Bio Vision, Mountain View, CA, USA) at 37°C for 24 hours. Stained cells were stored in 70% glycerol and the cells stained light blue were counted under a microscope.

2.4. FACS Analysis. The iPSC-derived EBs differentiated by retinoic acid were harvested and incubated in a buffer containing antibodies to selected putative MSC surface antigens. The antibodies used for FACS analysis were phycoerythrin (PE) conjugated to anti-CD11b, anti-CD31, anti-CD34, anti-CD44, anti-CD45, anti-TER119, anti-PDGFRα, anti-Sca-1, and unconjugated antibodies against CD105 (BD Biosciences, San Diego, CA). A total of 2×10^5 cells from different treatments were resuspended in 200 μL of Dulbecco's PBS containing 2% FBS and incubated for 30 minutes at 4°C with the antibodies. The cells incubated with the anti-CD105 antibody were followed by PE-conjugated secondary antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). The proper isotype-identical immunoglobulins served as controls. After staining, the cells were fixed in 2% paraformaldehyde, and quantitative FACS analysis was performed on a FACSCalibur flow cytometer (BD Biosciences, San Diego, CA).

2.5. Real-Time Reverse Transcription PCR (RT-PCR) Analysis. Total RNA was isolated from cultured MSC-like cells. RNA was reverse transcribed into cDNA by ReverTraAceqPCR RT kit (Toyobo, Osaka, Japan) according to the manufacturer's instructions. Primers were designed by Primer3 software (http://frodo.wi.mit.edu/) and tested for specificity with NCBI-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). Primer sequences are shown in Table 1. Real-time quantitative PCR was performed and monitored using

the Mx3000P QPCR System (Stratagene Agilent Technologies, Santa Clara, CA) with SYBR Green I as a double-stranded DNA-specific dye according to the manufacturer's instructions (Applied Biosystem, Foster City, CA). The PCR products were analyzed with agarose gel containing ethidium bromide to confirm these predicted lengths. Relative quantity was calculated using the ΔΔCt method.

2.6. Cell Differentiation into Osteocyte, Chondrocyte, and Adipocyte. The MSC-like cells were differentiated as previously reported [29]. For the induction to osteoblast, MSC-like cells were seeded at 1×10^5 cells/cm^2 in DMEM supplemented with 10% fetal calf serum (FCS), 100 nM dexamethasone (Sigma-Aldrich), 2 mM β-glycerophosphate (Sigma-Aldrich), and 150 μM ascorbate-2-phosphate (Sigma-Aldrich). Cells were incubated until the 21st day, with a medium change every 4th day. After the induction, alkaline phosphatase staining was performed on the cells utilizing a Leukocyte Alkaline Phosphatase Kit (Sigma-Aldrich).

For the induction into chondrocyte, 3×10^5 MSC-like cells were centrifuged in a 48 well plate to form a pellet and incubated for 21 days in 500 μL of DMEM with 170 μM ascorbic acid-2-phosphate, 100 nM dexamethasone, 350 μM proline, 1 × insulin-transferrin-selenium (Gibco), 1 mM sodium pyruvate (Sigma-Aldrich), and 10 ng/mL transforming growth factor-β1 (Sigma-Aldrich). The medium was changed every 4 days. For Alcian blue staining, the pellets were fixed with 4% PFA for 8 hours and then embedded in O.C.T. compound (Sakura Finetechnical, Tokyo, Japan). The embedded blocks were sectioned and incubated with 10% Alcian blue (Sigma-Aldrich) in 0.1 N HCl for 30 minutes.

Adipogenic differentiation was induced by culturing in DMEM supplemented with 20% FCS, 1 μM dexamethasone, 350 nM hydrocortisone, 500 μM isobutyl-methylxanthine (Sigma-Aldrich), 100 ng/mL insulin (Sigma Aldrich), and 60 μM indomethacin (Sigma-Aldrich). Cells were seeded at 2×10^4 cells/cm^2 and cultured for 12 days at 37°C. The medium was changed every 4 days. For Oil Red-O staining, the cells were fixed with 4% PFA and stained with Oil Red-O (Sigma-Aldrich) dissolved in 60% isopropyl alcohol for 15 minutes.

2.7. Western Blotting. The MSC-like cells were lysed in detergent lysis buffer (Cell Lysis Buffer, Cell Signaling Technology, Boston, MA) supplemented with 1 mM phenylmethanesulfonylfluoride (Sigma-Aldrich), followed by centrifugation. After the concentration of proteins was measured with a BCA assay (Sigma Chemical), the proteins were transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA) after SDS-PAGE. The membranes were blocked and incubated with rabbit anti-p19 and anti-p16 antibodies (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Antigen detection was performed using ECL Plus Western Blotting Detection Reagents (Amersham Pharmacia Biotech, Piscataway, NJ) with horseradish peroxidase-conjugated anti-rabbit IgG antibody (Cell Signaling Technology). Images were scanned and their densities were determined by ImageJ (National Institutes of Health, Bethesda, MD). The expressions of the proteins were corrected by beta-actin density, and the expression in tissues of normal mice was arbitrarily set at 1.0.

2.8. Animals and Induction of Diabetes. Five-week-old male C57BL/6 mice (Chubu Kagaku Shizai, Nagoya, Japan) were allowed to adapt to the experimental animal facility for 7 days. The animals were housed in an aseptic animal room under controlled light/dark and temperature conditions with food and water available ad libitum. Diabetes was induced by intraperitoneal injection (i.p.) of streptozotocin (STZ) (150 mg/kg; Sigma-Aldrich). Control mice received an equal volume of citric acid buffer. One week after STZ administration, the mice with plasma glucose concentrations of >16 mmol/L were classified as diabetic group. Twelve weeks after the induction of diabetes, 1×10^5 cells/limb of the MSC-like cells in 0.2 mL saline were injected into the right thigh and soleus muscles of normal and diabetic mice. The corresponding left hindlimb muscles were treated with saline alone. Three weeks later, the mice were harvested after evaluation of hemodynamic and neurophysiological parameters: blood flow of plantar skin, current perception threshold (CPT), nerve conduction velocity (NCV), and the thermal plantar test (TPT). Before the transplantation of the MSC-like cells, fasting blood glucose levels and hemoglobin A1c were examined by a FreeStyle Freedom Glucose Meter (Nipro, Osaka, Japan) and a RAPIDIA Auto HbA1c-L assay kit, using latex agglutination (Fujirebio Inc., Tokyo, Japan), respectively. The Nagoya University Institutional Animal Care and Use Committee approved the protocols of this experiment.

2.9. Measurement of CPTs Using a Neurometer. To determine a nociceptive threshold, CPTs were measured in 12- and 15-week diabetic and age-matched nondiabetic mice using a CPT/LAB neurometer (Neurotron, Denver, CO) according to the method by Shibata et al. [18] with minor modifications. The electrodes (SRE-0405-8; Neurotron) for stimulation were attached to plantar surfaces of the mice. Each mouse was kept in a Ballman cage (Natsume Seisakusho, Tokyo, Japan) suitable for light restraint to keep the mice awake. Three transcutaneous-sine-wave stimuli with different frequencies (2000, 250, and 5 Hz) were applied to the plantar surfaces of the mice to determine the CPT of sensory perceptions (pressure, pain, and pain and temperature, respectively). The intensity of each stimulation was gradually and automatically increased (increments of 0.01 mA for 5 and 250 Hz, increments of 0.02 mA for 2000 Hz). The minimum intensity at which each mouse withdrew its paw was defined as the CPT. Six consecutive measurements were conducted at each frequency.

2.10. NCV. After intraperitoneal injection of sodium pentobarbital (5 mg/100 g), mice were placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature of 37°C. Motor NCV (MNCV) was calculated between the sciatic notch and the ankle using a Neuropak NEM-3102 instrument (Nihon-Koden, Osaka, Japan), as previously described [18, 30, 31]. The sensory NCV (SNCV)

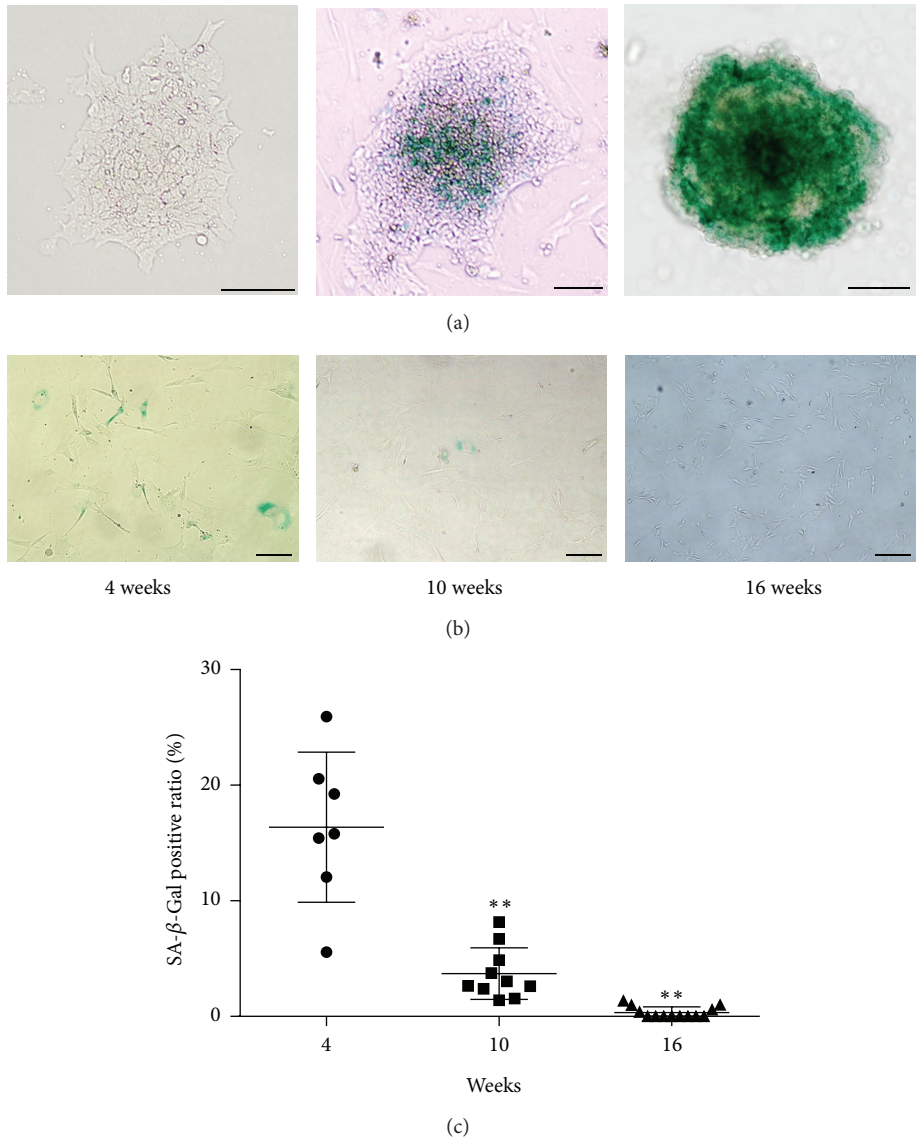

Figure 1: Senescence-associated beta-galactosidase (SA β-Gal) staining. (a) The induced pluripotent stem cell colonies obtained from aged mice colonies were heterogeneously expressed with SA β-Gal (green). Scale bar = 50 μm. (b) After long term culture, differentiated MSC-like cells expressing SA β-Gal (green) decreased. Scale bar = 100 μm. (c) The ratio of SA β-Gal positive mesenchymal stem cell (MSC)-like cells decreased significantly in 10 weeks or 16 weeks cultured MSC-like cells compared with 4 weeks cultured cells. 4 wks: the MSC-like cells cultured for 4 weeks, 10 wks: the MSC-like cells cultured for 10 weeks, and 16 wks: the MSC-like cells cultured for 16 weeks. $^{**}P < 0.005$ compared with 4 wks.

was measured between the hindlimb knee and ankle using retrograde stimulation.

2.11. TPT. Three weeks after the transplantations, hind paw withdrawal response against thermal stimuli of radiant heat was measured using a plantar test 7370 device (Ugo Basile, Comerio, Italy). Radiant heat was beamed onto the plantar surface of the hind paw. The paw withdrawal latencies were measured six times per session, separated by a minimum interval of 5 minutes. Paw withdrawals due to locomotion or weight shifting were not counted. Data are expressed as paw withdrawal latency in seconds.

2.12. Blood Flow of Plantar Skin. Before the evaluation of the MNCV and SNCV, blood flow of the plantar skin was measured by a laser Doppler probe (laser-Doppler flowmetry FLO-N1; Omegawave Inc., Tokyo, Japan) placed 1 mm above the skin. During this measurement, the mouse was placed on a heated pad in a room maintained at 25°C to ensure a constant rectal temperature of 37°C.

2.13. Histochemistry. Three weeks after the transplantation, the mice were anesthetized with sodium pentobarbital (5 mg/100 g) and perfused with 20 ml of 4% PFA fixative. After perfusion, soleus muscles with a sural nerve were

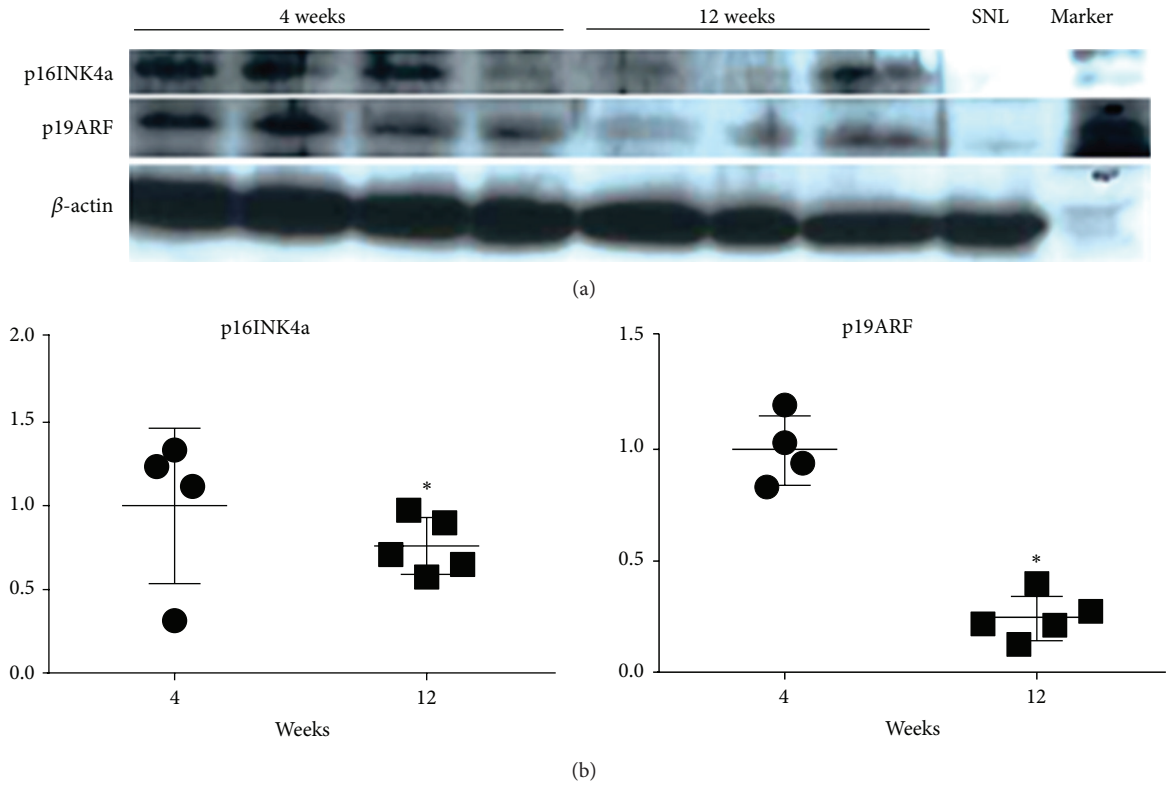

Figure 2: The protein expression levels of p16INK4a and p19ARF. (a) Upon Western blotting, the senescence marker proteins, p16INK4a and p19ARF, were expressed in the mesenchymal stem cell (MSC)-like cells after 4 weeks culture. Both levels decreased in the cells after 12 weeks culture. (b) In a densitometric analysis with correction of β-actin intensity, both protein levels in the cells cultured for 12 weeks decreased significantly compared with the levels in the 4-week cultured cells. 4 wks: the MSC-like cells cultured for 4 weeks, 10 wks: the MSC-like cells cultured for 10 weeks, $^{*}P < 0.05$ compared with 4 wks.

excised and fixed in 4% PFA at 4°C overnight. Specimens were immersed in PBS containing 20% sucrose, embedded within an O.C.T. compound (Sakura Finetechnical) and cut into 6 μm sections, which were used for staining as previously reported [18, 19] with minor modifications. The vascular capillaries were stained by Alexa Fluor 594 conjugate isolectin GS-IB4 (Life Technologies) and were counted under a fluorescence microscope (BX51, Olympus Optical) and images were obtained by a CCD camera (DP70, Olympus Optical). Five fields from each section were randomly selected for the capillary counts. GFP-expressing cells representing the transplanted MSC-like cells were observed under a microscope. Nuclei were stained with DAPI (Merck).

2.14. Statistical Analysis. All of the group values were expressed as means ± SD. Statistical analyses were made by one-way ANOVA, with the Bonferroni correction for multiple comparisons. All analyses were performed by personnel unaware of the animal identities.

3. Results

3.1. The Aging Markers in MSC-Like Cells Obtained from Aged-Mouse iPSC Decreased with Time. The iPSCs derived from a 21-month-old mouse contained the aging marker SA β-Gal. However, the levels of SA β-Gal varied among iPS cell colonies (Figure 1(a)). After a 16-week culture, MSC-like cells kept their fibroblast-like morphology, adherent to a dish bottom and flatly spread, and the ratio of SA β-Gal positive cells decreased over time (Figures 1(b) and 1(c)).

Although p16INK4a and p19ARF, other markers of cell aging, were highly expressed in the early stages of the culture compared with immortalized SNL feeder cells (Figure 2(a)), the expression levels of p19ARF significantly decreased and those of p16INK4a presented a reduction tendency after 3 months (Figures 2(a) and 2(b)).

3.2. The MSC-Like Cells Expressed MSC Markers and Differentiated into Mesenchymal Derived Cells. The MSC-like cells expressed cell surface markers of MSC, that is, CD105, CD140a, Sca-1, and CD44, and expressed no haematopoietic lineage markers, that is, CD34, TER119, CD31, CD45, and CD11b (Figure 3(a)). After their induction into three mesenchymal derived cells, that is, chondrocyte, osteoblast, and adipocyte, each of the differentiated cells was stained with specific dyes. The cells induced into chondrocytes exhibited stainability with Alcian blue, the cells into osteoblast were confirmed their alkaline phosphatase activity, and the cells into adipocytes were proven that they contained lipid

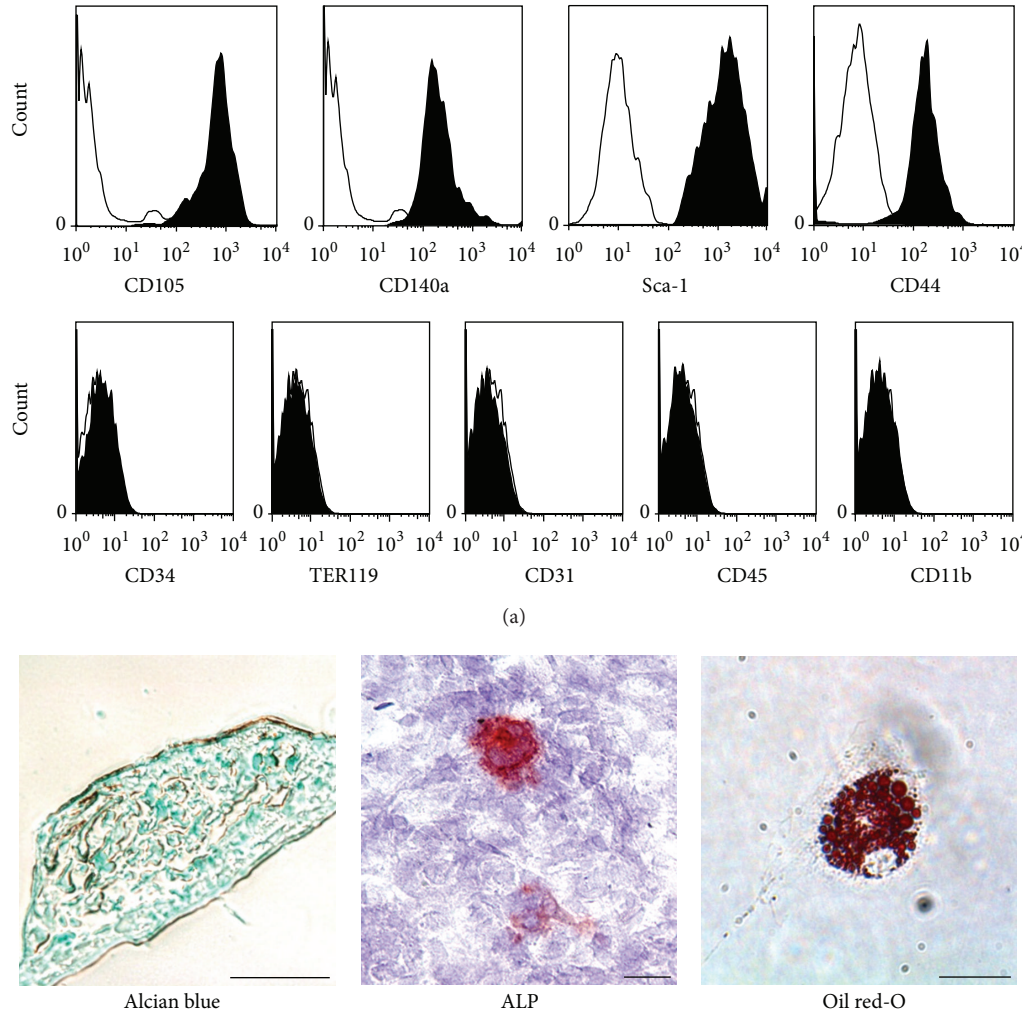

Figure 3: The characterization of the mesenchymal stem cell (MSC)-like cells. (a) Flow cytometry analyses of mesenchymal stem cell (MSC) and hematopoietic cell lineage markers in the MSC-like cells. The MSC-like cells expressed MSC markers, CD105, CD140a, Sca-1, and CD44, in spite of no expression of hematopoietic markers, CD34, TER119, CD31, CD45, and CD11b. Open curves: control, filled curves: each of target antibodies. (b) After differentiation of the MSC-like cells *in vitro*, each differentiated cells into chondrocyte, osteoblast, and adipocyte exhibited staining abilities with Alcian blue, alkaline phosphatase, and Oil Red-O, respectively. ALP: alkaline phosphatase staining, scale bar = 50 μm.

droplets within their cytoplasm utilizing Oil Red O staining (Figure 3(b)).

3.3. MSC-Like Cells Expressed Angiogenic and Neurotrophic Factors. To verify the endocrine effect of the MSC-like cells, the following mRNA expression levels of angiogenic and neurotrophic factors were evaluated in the cells: VEGF-A, PDGF-A, FGF2, NGF, brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), Neurotrophin-3 (NT-3), and ciliary neurotrophic factor (CNTF) (Figure 4). For comparison, immportalized mouse MSC cell-line PA6 cells were evaluated. Furthermore, because Schwann cells have been established as an intrinsic neuroprotective cell, the endocrine ability of IMS cells, immortalized mouse Schwann cells, was also compared to that of MSC-like cells. The MSC-like cells indicated a similar pattern of endocrine ability as PA6 MSC except for PDGF-A, in which the MSC-like cells expressed significantly high transcript levels compared to PA6. Compared to the IMS cell, the MSC-like cells expressed a comparable level of FGF2, higher levels of VEGF-A and BDNF, and lower levels of GDNF, NT3, NGF, and PDGF-A (Figure 4).

3.4. Body Weight and Blood Glucose Levels. Twelve weeks after the STZ injection, the diabetic mice showed severe hyperglycemia and significantly reduced body weight gain, and after the transplantation, there was no significant change in either group (Table 2).

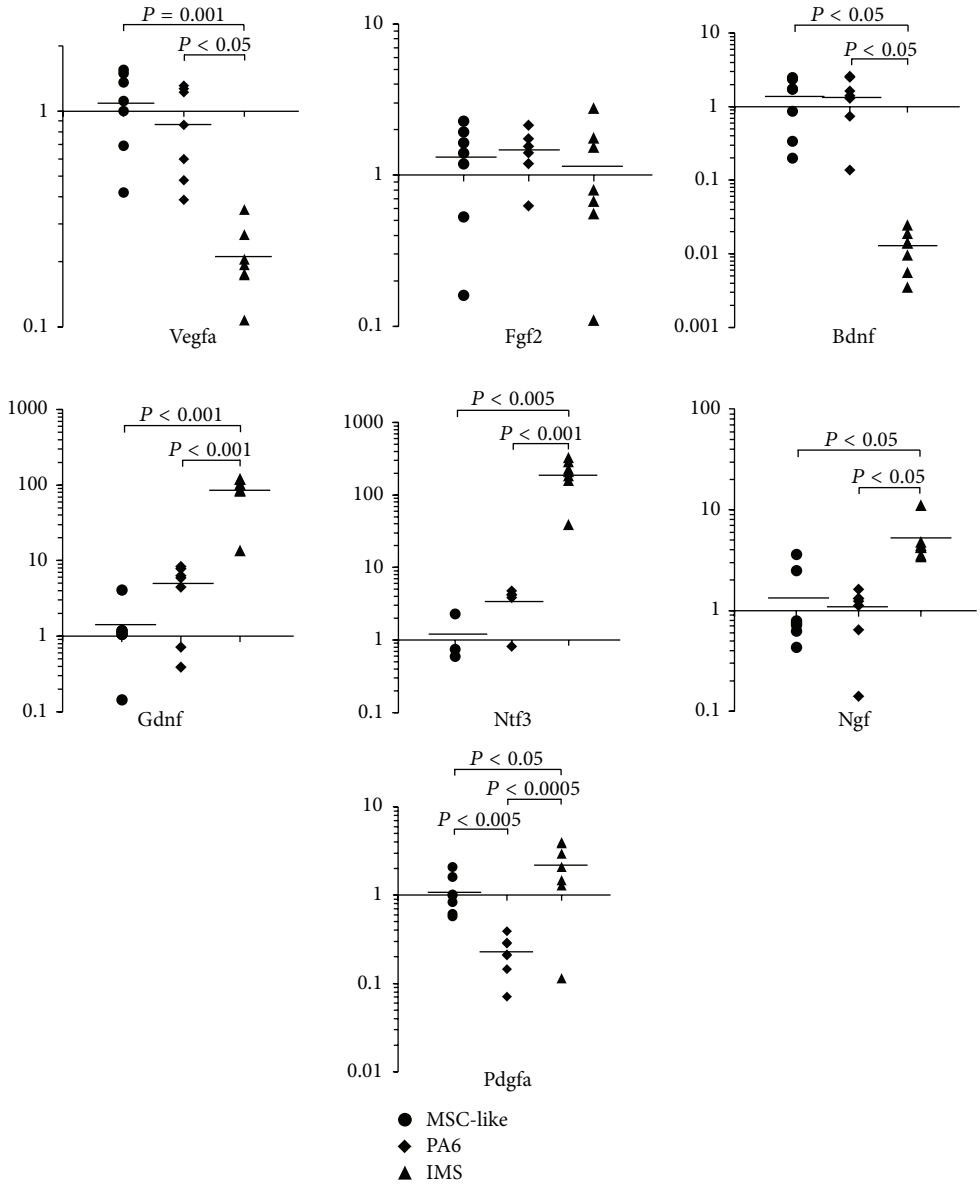

Figure 4: The expression ability of growth factors in the mesenchymal stem cell (MSC)-like cells. Transcript levels of growth factors were examined in the MSC-like cells, mouse MSC cell-line PA6, and mouse immortalized Schwann cell-line IMS. Each data was presented as a fold change of each expression level in the MSC-like cells. Filled circle: the MSC-like cells, filled diamond shape: PA6, filled triangle: IMS. $n = 5$ in each group.

Table 2: Body weights and blood glucose levels.

	Non-diabetic mice		Diabetic mice	
	Pretransplantation	Posttransplantation	Pretransplantation	Posttransplantation
Number	10	10	8	8
Blood glucose (mmol/L)	9.1 ± 1.5	8.2 ± 1.6	23.1 ± 2.8*	22.3 ± 2.3#
Body weight (g)	30.6 ± 2.7	31.3 ± 3.0	26.1 ± 1.0*	28.3 ± 0.7#

Results are means ± SD. *$P < 0.05$ versus pretreatment non-diabetic mice. #$P < 0.05$ versus posttreatment non-diabetic mice.

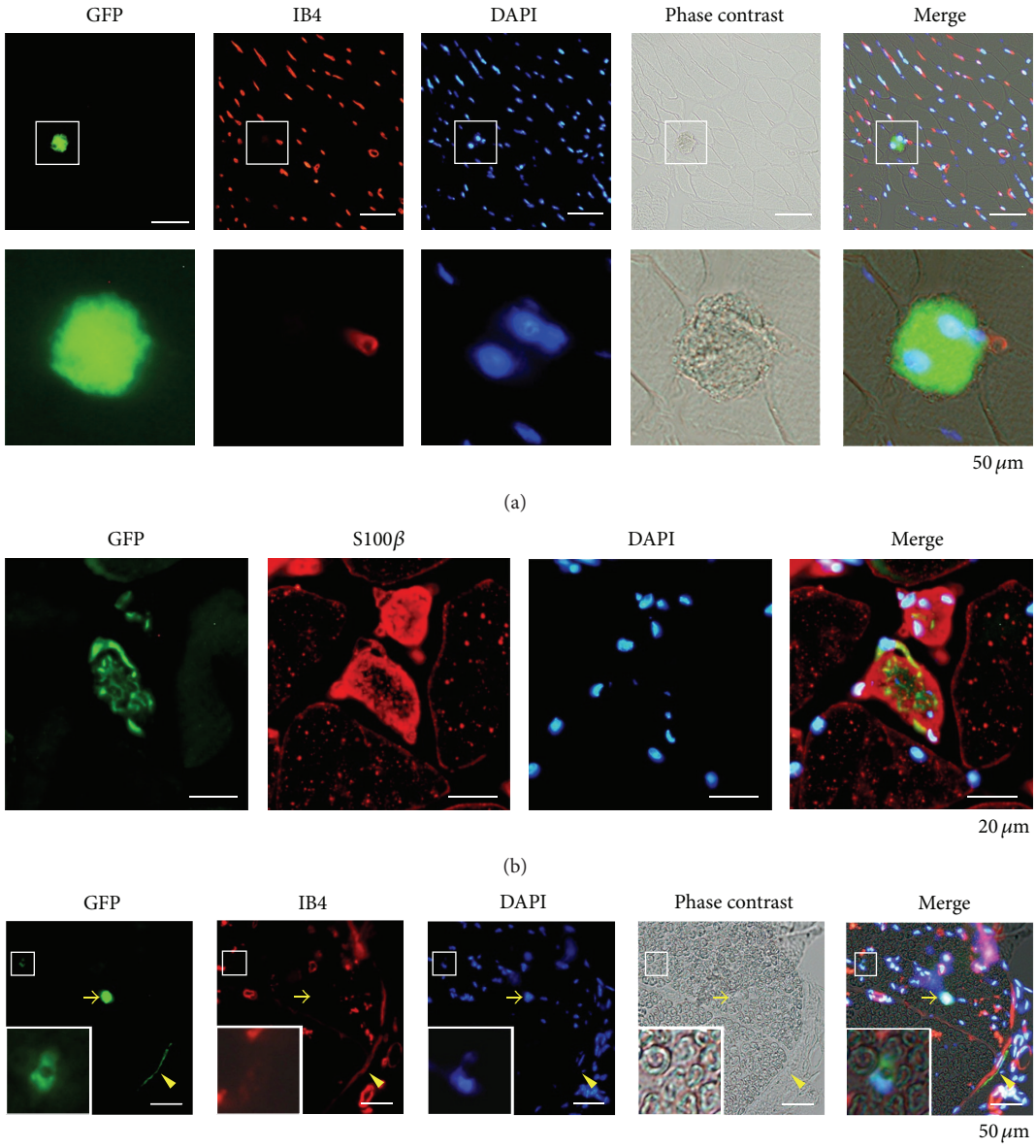

Figure 5: Distribution of the transplanted mesenchymal stem cell (MSC)-like cells. (a) The transplanted MSC-like cells expressing GFP (inset) engrafted in a soleus muscle (upper panels). Lower panels are magnified images of insets. Scale bar = 50 μm. IB4: isolectin GS-IB4. (b) In a soleus muscle, the MSC-like cells engrafted in one of the peripheral nerve branch and expressed S100β, a Schwann cell marker. Scale bar = 20 μm. (c) The GFP expressing MSC-like cells (yellow arrow and inset) engrafted in a sural nerve. Some engrafted cells surrounded a nerve fiber (inset). Some engrafted cells resided close to a perineurium (yellow arrow head). Scale bar = 50 μm. IB4: isolectin GS-IB4.

3.5. The Transplanted Cells Were Found within Skeletal Muscles and Peripheral Nerves. Two months after the transplantation, some treated mice were harvested to verify the engraftment of the GFP-expressing (GFP$^+$) transplanted cells within the tissues of the recipients. No teratoma was detected in the rough sectioned tissue slices of the soleus muscles, brains, hearts, lungs, or livers, and GFP$^+$ cells were nonexistent except in the muscles and nerves of the transplanted hindlimbs (data not shown). Some GFP$^+$ cells, which resided among muscle fibers, appeared not to form any functional tissue structure (Figure 5(a)), and the other GFP$^+$ cells, residing within or around peripheral nerves, expressed S100β, one of the Schwann cell markers (Figures 5(b) and 5(c)). A relationship between vessels and GFP$^+$ engrafted cells was not obviously observed in sural nerves (Figure 5(c)).

3.6. The Blood Flow of Plantar Skin and the Capillary Number to Muscle Fiber Ratios Increased in Treated Limbs. By 12 weeks after the onset of diabetes, the blood flow of plantar skin in diabetic mice decreased significantly compared with that in nondiabetic mice, but the decrease was ameliorated in the limbs treated with MSC-like cells (Figure 6(a)).

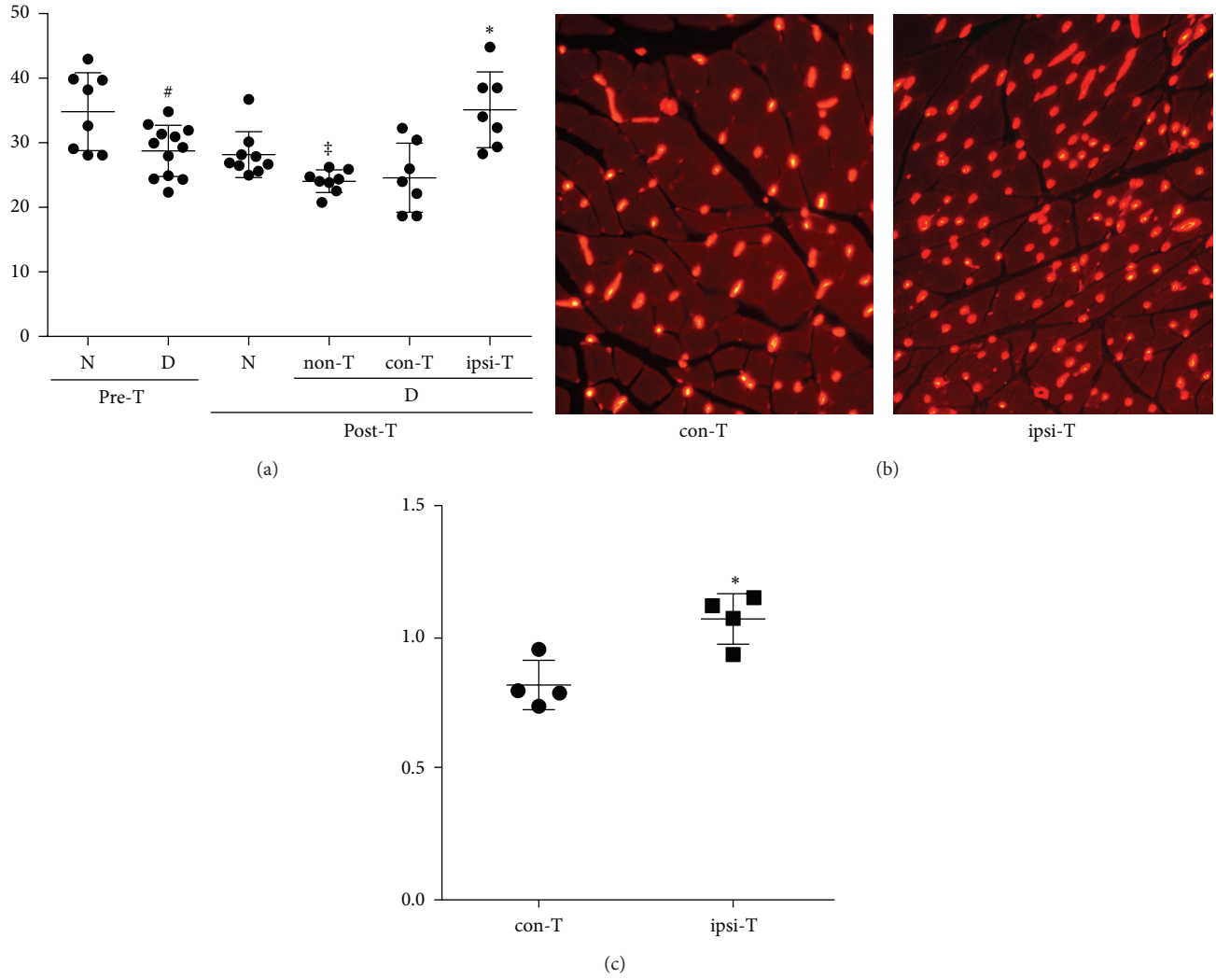

FIGURE 6: Plantar skin blood flow and capillary number in a soleus muscle. (a) Plantar skin blood flow in diabetic mice significantly decreased compared with that in nondiabetic mice. The flow was ameliorated in transplanted mice. $^{\#}P < 0.05$ compared with pretransplanted nondiabetic mice, $^{\ddagger}P < 0.05$ compared with posttransplanted mice, $^{*}P < 0.05$ compared with non-T. D: diabetic mice, N: nondiabetic mice, con-T: contralateral limbs of transplanted mice, ipsi-T: ipsilateral limbs of transplanted mice. $n = 7$–12 in each group. (b, c) In a soleus muscle, capillaries were visualized with isolectin GS-IB4 (red). Quantification of the capillary-to-muscle number ratio revealed the increase of the ratio in transplanted limbs. $^{*}P < 0.05$ compared with con-T. con-T: contralateral limbs of transplanted mice, ipsi-T: ipsilateral limbs of transplanted mice. $n = 4$ in each group.

The vasculatures were visualized by Alexa594-conjugated isolectin IB4, a marker for endothelial cells (Figure 6(b)). Transplantation of MSC-like cells significantly augmented the capillary number to muscle fiber ratios in the transplanted limbs (ipsi-T) compared with the ratio in the saline-injected side limbs (con-T) in diabetic mice (Figures 6(b) and 6(c)).

3.7. Reduced Sensory Perception in Diabetic Mice Was Ameliorated by the MSC-Like Cell Transplantation. After 12 weeks of diabetes, current perception thresholds (CPTs) at 5, 250, and 2000 Hz had significantly increased compared with those in normal mice, representing hypoalgesia in diabetic mice. Three weeks after the transplantation, these deficits in sensation had significantly improved in diabetic mice compared with saline-treated diabetic controls (Figure 7(a)). To strengthen the existence of the perception dysfunction, TPT was performed. The actual perception of thermal stimuli was also impaired in diabetic mice after the 12-week diabetic duration, and, consistent with the result of CPT, the impairment was also ameliorated in the transplanted limbs (Figure 7(b)).

3.8. MSC-Like Cells Improved Delayed SNCV in Diabetic Mice. Motor nerve conduction velocity and sensory nerve conduction velocity of diabetic mice were significantly delayed compared with those of normal mice (Figure 7(c)). The delay of SNCV was significantly restored in the transplanted limbs three weeks after the treatment.

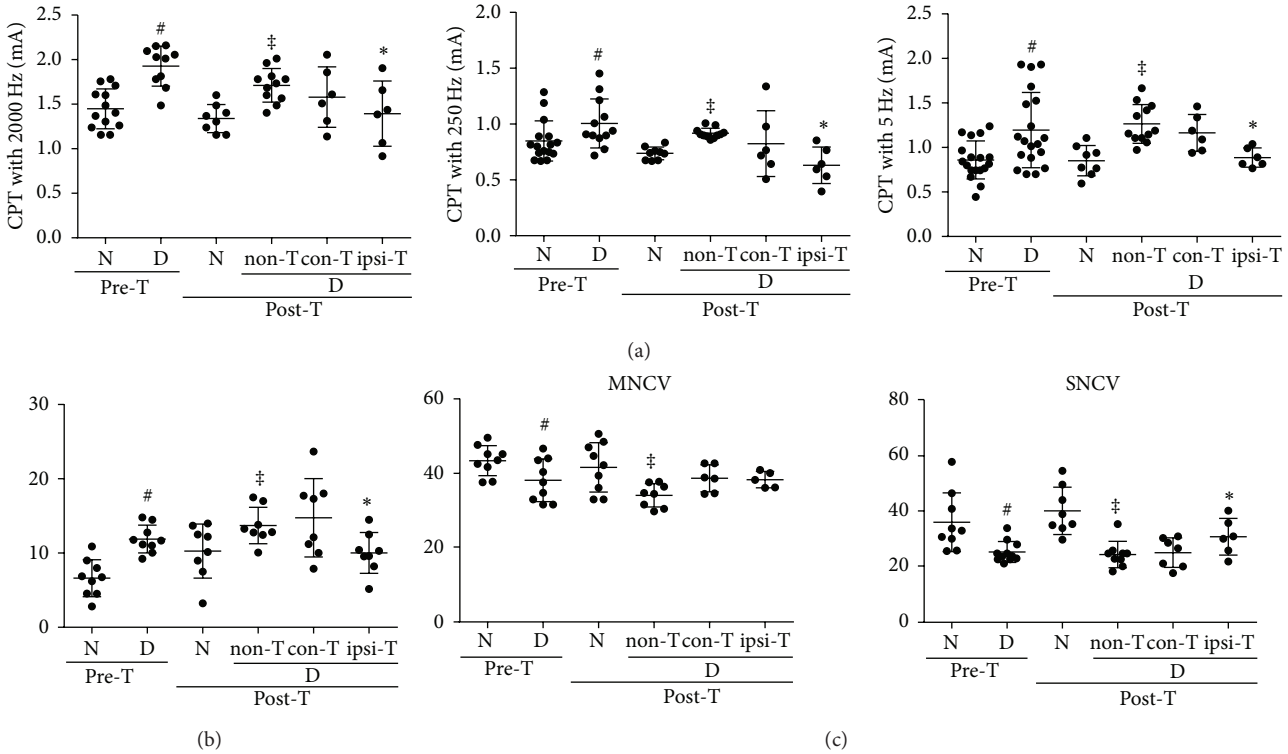

Figure 7: Neurophysiological evaluations. (a) All of current perception thresholds (CPTs) were impaired in diabetic mice and the impairments were ameliorated in the transplanted limbs. (b) The thermal plantar test clarified the impairments of thermal perception in diabetic mice and the impairments were ameliorated in the transplanted limbs. (c) Motor and sensory nerve conduction velocity (MNCV and SNCV, respectively) decreased in diabetic mice compared with those in nondiabetic mice. The SNCV increased in the transplanted limbs of diabetic mice. $^{\#}P < 0.05$ compared with pretransplanted nondiabetic mice, $^{\ddagger}P < 0.05$ compared with posttransplanted mice, $^{*}P < 0.05$ compared with non-T. D: diabetic mice, N: nondiabetic mice, con-T: contralateral limbs of transplanted mice, and ipsi-T: ipsilateral limbs of transplanted mice. $n = 6$–19 in each group.

4. Discussion

The present study demonstrated that MSC-like cells could be obtained from aged mouse iPSCs and the transplantation of the MSC-like cells ameliorated physiological impairments and reduced blood flow of plantar skin in DPN. The histological appearance revealed that the capillary number to muscle fiber ratios increased in the skeletal muscles of the transplanted lower limbs. Furthermore, the transplanted cells were grafted around the injected sites, and some of them differentiated to S100β expressing cells in peripheral nerves.

MSCs, which might contain heterogeneous subpopulations of cells, are generally characterized as having the ability of differentiation into chondrocytes, osteoblasts, and adipocytes, and have no cell surface markers of hematopoietic cell lineages [32]. In addition, they adhere to the bottom of tissue culture dishes and expand into the shape of fibroblast-like spindle cells. In this study, we acquired adherent spindle cells and demonstrated their *in vitro* multilineage differentiation abilities and their lack of hematopoietic lineage markers. As a cell surface marker specific to mouse MSCs remains undefined, we examined a combination of markers, that is, CD105, CD140a, Sca-1, and CD44, according to a previous paper [33], and confirmed the existence of these markers on the MSC-like cells.

In spite of widespread research on MSCs, the physiological roles of MSCs in adult animals and humans have not been elucidated sufficiently. Therefore, most transplantation therapies of MSCs are expected to exert their immunosuppressive properties and cytoprotection or tissue regeneration through their paracrine effects [34–36]. As we employed the MSC-like cells in anticipation of their paracrine effect, the transcript levels of trophic growth factors in these cells were assayed and compared with those in murine cell-lines of a MSC and a Schwann cell, which supports peripheral neurons physically and chemically. The transcript levels of MSC-like cells were comparable to those of the MSC cell-line, while the expression pattern in the MSC-like cells was distinguished from the pattern in the Schwann cell-line. Experiments comparing cell therapy using Schwann cell precursors with therapy using MSC-like cells could be inspected in the future.

In general, stem cells acquired from aged animals also display phenomena of cell senescence, and many researchers have tried to achieve cell rejuvenation [37, 38]. Recent papers have indicated that reprograming somatic cells into pluripotent cells might facilitate the rejuvenation [39, 40]. Consistent with these previous reports, we presented the reduction of cell senescence related proteins, that is, SA-β-Gal, p16INK4a, and p19ARF, in the long-cultured reprogrammed

cells. However, these proteins were not suppressed immediately after the reprogramming. We speculate from the heterogeneous staining properties of the iPS cell colonies with SA-β-Gal that incompletely reprogrammed iPSCs would not proliferate and be negatively selected through the lengthy culturing period, and only completely reprogrammed iPSCs would expand. Further experiments including telomerase assay and DNA methylation analysis to confirm the accuracy of cell senescence should be developed in the future.

Although our original purpose was to investigate the paracrine/endocrine effect of MSC-like cells in DPN, the MSC-like cells engrafted in peripheral nerves, surpassed our expectation, by expressing S100β, a Schwann cell marker. This fact could indicate that the grafted cells might directly construct peripheral nervous tissues. Although it has been reported that MSCs differentiated to Schwann cell-like cells *in vitro* [41], *in vivo* differentiation of MSCs into Schwann cells has not yet been documented. On the other hand, cell fusion between proinsulin-producing bone marrow-derived cells and dorsal root ganglion neurons in DPN has been described [42]. Therefore, further experiments to exclude the possibility of cell fusion and to elucidate the Schwann cell function of the grafted cells should be considered.

In conclusion, we have demonstrated the beneficial effects of transplantation of MSC-like cells derived from iPSCs on DPN. Although additional studies to reveal the safety of the transplantation on DPN would be required, this cell transplantation appears to be a novel therapeutic strategy for DPN.

Conflict of Interests

The authors declare that they have no conflict of interest.

Acknowledgments

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Review Article

Adipose-Derived Mesenchymal Cells for Bone Regereneration: State of the Art

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Adipose tissue represents a hot topic in regenerative medicine because of the tissue source abundance, the relatively easy retrieval, and the inherent biological properties of mesenchymal stem cells residing in its stroma. Adipose-derived mesenchymal stem cells (ASCs) are indeed multipotent somatic stem cells exhibiting growth kinetics and plasticity, proved to induce efficient tissue regeneration in several biomedical applications. A defined consensus for their isolation, classification, and characterization has been very recently achieved. In particular, bone tissue reconstruction and regeneration based on ASCs has emerged as a promising approach to restore structure and function of bone compromised by injury or disease. ASCs have been used in combination with osteoinductive biomaterial and/or osteogenic molecules, in either static or dynamic culture systems, to improve bone regeneration in several animal models. To date, few clinical trials on ASC-based bone reconstruction have been concluded and proved effective. The aim of this review is to dissect the state of the art on ASC use in bone regenerative applications in the attempt to provide a comprehensive coverage of the topics, from the basic laboratory to recent clinical applications.

1. Introduction

Multipotent mesenchymal stem cells (MSCs) are non-hematopoietic cells of mesodermal derivation residing in several postnatal organs and connective tissues. They were first described in the early 1960s, as an adherent, fibroblastoid cell population with inherent osteogenic properties [1]. Since then, an overwhelming number of studies have demonstrated that MSCs are endowed with a higher plasticity, being able to differentiate into cells of mesenchymal lineages, such as adipogenic, osteogenic, and chondrogenic [2]. MSCs are also capable of transdifferentiation towards epithelial cells, such as alveolar epithelial cells [3], hepatocytes [4–7], epithelial cells from the gastrointestinal tract [8, 9], and kidney cells [10]. The question of possible neural transdifferentiation of MSCs is still debated and controversial [11–13]. Nonetheless, converging evidence has indicated the capability of MSCs to pursue a functionally and morphologically actual glial fate [14–17]. The common origin of both mesenchymal cells and neural cells from the neural crest, in the vertebrate embryo, may in part explain the high degree of plasticity of MSCs [18].

Bone Marrow (BM) was originally considered the reference source for MSCs isolation; to date they have been isolated from a multitude of adult tissues, including muscle, adipose tissue, connective tissue, trabecular bone, synovial fluid [19], and perinatal tissues, such as umbilical cord, amniotic fluid, and placenta [20–24]. In particular, the ubiquity, the ease of retrieval and the minimally invasive procedure required for harvesting the adipose tissue (AT), make it an ideal source for high yield MSCs isolation. Moreover, adipose tissue-derived MSCs (ASCs) can be maintained longer in culture and possess a higher proliferation capacity compared to BM-derived MSCs. Indeed ASCs and BM-MSCs exhibit virtually identical transcription profiles for genes related to the stem cell phenotype, supporting the concept of a common origin of the mesenchymal lineage from a wide variety of tissues [2, 25].

2. Fat as a Source of ASCs

Adipose tissue is a highly complex tissue comprising mature adipocytes (>90%) and a stromal vascular fraction (SVF), which includes preadipocytes, fibroblasts, vascular smooth muscle cells, endothelial cells, resident monocytes/macrophages, lymphocytes, and ASCs [26–28]. The density of the AT stem cell reservoir varies as a function of location, type, and species. Within the white fat, a highest number of ASCs reside in subcutaneous depots compared to visceral fat, with the highest concentrations occurring in the arm region and the greatest plasticity described in cells isolated from inguinal AT [29]. Studies in the canine model showed that the proliferative capacity of ASCs appears to inversely correlate with donor age, while stemness, self-renewal, and multipotency are progressively lost with culture passages [30, 31]. Moreover, significant differences in molecular profiles and immunophenotype have been described in subcutaneous and visceral fat-derived ASCs [31, 32]. The significant sexual dimorphism of adipose tissue distribution and function reflect gender- and hormone-related differences in cellular composition and molecular profiles, which should be taken in due account [33, 34]. Finally, ASCs have been described also in brown fat depots and are able to easily undergo skeletal myogenic differentiation [35, 36].

3. Isolation and *Ex Vivo* Expansion of ASCs

Human ASCs can be isolated from adipose tissue collected tissue through liposuction or during reconstructive surgery through resection of tissue fragments. Current methods used for isolating ASCs rely on collagenase digestion followed by centrifuge separation of the SVFs from primary adipocytes. ASCs are selected *in vitro* based on their plastic adherence properties and display typical spindle-shaped fibroblastoid morphology. They can be extensively subcultivated in monolayer culture on standard tissue culture plastics with a basal medium containing 10% of fetal bovine serum [2, 4, 37].

Once a primary culture is established, ASCs are easily and rapidly expanded *ex vivo* [2, 38]. The average frequency of ASCs in processed lipoaspirate is 2% of nucleated cells and the yield of ASCs is approximately 5,000 fibroblast colony-forming units (CFU-F) per gram of adipose tissue, compared with estimates of approximately 100–1,000 CFU-F per milliliter of bone marrow [39], making AT an excellent candidate source for regenerative therapy.

4. Characterization of ASCs

Although a minimal set of cell surface markers to be analyzed for MSCs identification has been defined in 2006 [40], the correct immunophenotype characterization of ASCs has been debated for a long time. Due to the inherent SVF heterogeneity, a multiparameter flow cytometric analytic and sorting strategy have been developed. Based on the hematopoietic marker CD45, the endothelial marker CD31, the perivascular marker CD146, and the stem-stromal markers CD34, CD90, CD105, and CD117 (c-kit), four distinct populations have been defined in the SVF fraction in uncultured conditions: putative ASCs (CD31−, CD34+/−, CD45−, CD90+, CD105−, CD117− and CD146−), endothelial-progenitor cells (CD31+, CD34+, CD45−, CD90+, CD105−, CD117+ and CD146+), vascular smooth muscle cells or pericytes (CD31−, CD34+/−, CD45−, CD90+, CD105−, CD117+ and CD146+), and hematopoietic cells (CD45+) [41, 42]. Studies on whole AT have revealed that the stem/progenitor components, organized around small vessels in an annular fashion, are dominated by a prevalent supra-adventitial layer of CD34+ cells displaying MSCs-like multipotentiality [41–43]. These supra-adventitial adipose stromal cells (SA-ASC) surround arterioles and venules, which are colonized on their surfaces by CD146+ perivascular cells or pericytes [42, 44]. A component of proliferative CD34+ and CD31+ endothelial progenitor cells is associated with the luminal layer [45].

Compared to extensively cultivated ASCs, freshly isolated SVF cells and early passage ASCs express higher levels of CD117 (c-kit), human leukocyte antigen-DR, and stem cell-associated markers such as CD34, along with lower levels of stromal cell markers such as CD13, CD29, CD34, CD54, CD73, CD90, CD105, and MHC I [46, 47]. It seems that CD34+ ASCs have a greater proliferative capacity, while CD34− ASCs exert higher plasticity [48, 49].

Recently, the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT) have provided initial guidance for the scientific community working with adipose-derived cells defining the minimal criteria for the identification of ASCs [50]. In the SVF, cells are identified by the combination of the following markers: CD45−, CD31−, and CD34+. Added information should be given with the analysis of stromal/stem cell markers: CD13, CD73, CD90, and CD105. In culture, like BM-MSCs, ASCs are positive for CD90, CD73, CD105, and CD44, while negative for CD45 and CD31. Unlike BM-MSCs, ASCs are positive for CD36 and negative for CD106. Finally, to allow the identification of ASCs a multilineage differentiation assay should be performed.

5. Osteogenic Potential of ASCs and Their Role in Bone Regeneration

Cell-based approaches for bone formation and regeneration are widely considered the most effective, as they are able to efficiently sustain the physiologic osteogenic process *in vivo*. Indeed, the most promising field for ASCs application is represented by bone reconstruction/regeneration [38, 51]. Bones are dynamic organs, undergoing continuous remodeling to maintain tissue homeostasis, modify shape and morphology, and repair fractures [52]. The therapeutic options clinically available are currently restricted to allografts, microvascular bone, and osteomyocutaneous flaps taken from an autologous donor site, and bone distraction for reconstructive purposes [53–55]. In particular, bone "free flaps" harvested from fibula, scapula, iliac crest, or rib represent the therapeutic gold standard because they contain all the components needed for regeneration, including differentiated bone cells, their cellular precursors, and appropriate growth/differentiation factors. The main disadvantage of this technique relates to

the morbidity of the donor site, where a skeletal defect is created. Furthermore, the complexity of autograft procedures raises other technical issues: the maintenance of the arterial and venous flow of the flap in the case of inadequacy of the receiving site (e.g., previous radiation therapy); excessive extension of the bone defect in need of repair; peripheral vasculopathies; and poor general clinical condition [56]. Therefore, scientific research aims to bypass the need for allografts or autologous tissue grafts in repairing large bone defects (either posttraumatic or as a consequence of surgical resection), for which a spontaneous recovery cannot be expected. *In vitro* and *in vivo* models suggest that the use of expanded ASCs improve bone healing through direct differentiation into mature osteoblasts and paracrine effects that facilitate migration and differentiation of resident precursors. The secretome of the SVF [57, 58] and of the ASCs [59, 60] contains different endocrine factors (adipokines) with bone remodeling activity [61–63]. Specifically, the vascular endothelial growth factor (VEGF), present in the secretome of both SVF and ASCs, plays a major role in the repair of fractures or bone defects. The VEGF is able to activate the formation of a new network of blood capillaries, which is required during the physiological process of bone regeneration [64]. In addition, VEGF plays a direct role in the recruitment of hematopoietic stem cells leading to the formation of new bone [65, 66].

The cell osteogenic potential can be assessed *in vitro*, through an induction assay based on a widely standardized protocol, employing a culture medium supplemented with ascorbic acid, dexamethasone, and beta-glycerol phosphate [4]. Thereafter, to verify the acquisition of an osteogenic phenotype, staining protocols are used to detect calcium deposits and matrix mineralization (namely, Von Kossa and alizarin red methods) [67].

6. ASCs-Based Gene Therapy Osteoinductive Approaches

In recent years, cell-based osteoinductive gene-delivery techniques have produced the most convincing results both *in vitro* and *in vivo* models. Such methods use cells genetically-engineered to express selected osteogenic factors to be implanted into the anatomical site where bone regeneration is required. To date, recombinant bone morphogenetic proteins (BMPs) have been the most frequently studied and used osteoinducing agents [51, 68–74]. Lately, several new transcription factors involved in the osteogenic process have been reported, including Runx2, vascular endothelial growth factor (VEGF), the LIM mineralization protein (LMP), Sonic Hedgehog (SHH), and Nell-1 [56, 75–81]. In a study performed by Lee and colleagues [75], BMP-2 and RunX2 were coexpressed in ASCs, demonstrating that BMP2/Runx2-ASCs show a significant increase in bone formation compared to ASCs and BMP2-ASCs. Recently, Zhang et al. [77] studied the osteogenic differentiation of ASCs in presence of VEGF, BMP-6, or VEGF plus BMP-6, showing that the combination of VEGF and BMP-6 significantly enhance the expression of osteospecific genes like Dlx5 and osterix and suggesting a cross-talk between VEGF and BMP-6 signaling pathways during the osteogenic differentiation of ASCs. Also, two pro-osteogenic cytokine, Sonic Hedgehog (SHH) and Nell-1, have been studied by James et al. [76], revealing the additive effects of SHH and NELL-1 on the osteogenic differentiation of ASCs.

7. Scaffolds for ASCs in Bone Repair

Scaffolds for osteogenesis should mimic bone morphology and structure in order to optimize integration into the surrounding tissue and to provide a suitable microenvironment for MSCs adhesion, proliferation, and differentiation. The micro- and macroarchitecture of the scaffold is known to be highly dependent on the production process [82, 83]. A well-characterized biomaterial is hydroxyapatite (HA), $Ca_{10}(PO_4)_6(OH)_2$, which is currently used in clinical applications in different forms. HA is suitable for substituting or integrating diseased or damaged bone tissues since it resembles the mineralized bone phase and supplies fundamental ions for the newly forming bone during resorption [84, 85]. Also, beta-tricalcium phosphate (β-TCP), $Ca_3(PO_4)_2$, was thought suitable for clinical use as a carrier for MSCs because of its chemical and crystallographic similarities to the inorganic phase of native bone [86, 87]. Biphasic calcium phosphate (BCP) refers to homogenous composites of HA and β-TCP [88]. Properties like solubility and resorption capacity of BCP formulations vary widely among different ratios of HA and β-TCP. Unfortunately, calcium phosphate ceramics tend to have poor mechanical properties, predisposing them to brittleness and fractures [89, 90]. In the last years, several *in vitro* and *in vivo* studies highlight the osteoinductive role of biomimetic scaffold on ASCs [91, 92]. In particular, a study performed by Marino and collaborators [92] revealed that β-TCP matrix alone is sufficient to trigger the differentiation of ASCs toward an osteoblastic phenotype, regardless of whether the cells are grown in a proliferative or a differentiative medium. Also, Liao et al. [91] compared the osteogenic potential of porcine ASCs (P-ASCs) among three scaffold (polycaprolactone, PCL; polycaprolactone and β-tricalcium phosphate, PCL-TCP; collagen I coated-PCL-TCP, PCL-TCP-COL), in order to find an optimal scaffold for bone tissue engineering. The *in vitro* study demonstrated that pASCs display the best osteogenic differentiation rate on PCL-TCP-COL group scaffolds, as demonstrated by the highest ALP activity, osteocalcin expression and mineralization [91]. Also, the experiment in nude mice showed better woven bone and vascular tissue formation in the PCL-TCP-COL group than in the PCL group. In addition, the osteogenic ability of pASCs was found to be enhanced by coating COL onto the PCL-TCP scaffolds, both *in vitro* and *in vivo*. Moreover, Arrigoni et al. [93] compared the neoformed bone tissues achieved by treating critical tibial defects with either hydroxyapatite alone (HA, group I) or hydroxyapatite–autologous ASC constructs (ASCs-HA, group II), investigating their histomorphometric, immunohistochemical, and biomechanical properties. The study displayed that tibial defects treated with rabbit ASCs-HA showed an improved healing process when compared to naked scaffold-treated ones [93].

Calcium-, magnesium-, and silicon-containing ceramics, such as akermanite ($Ca_2MgSi_2O_7$), show better mechanical properties and degradation rates than other bioceramics and are reported to enhance osteogenic commitment of MSCs [86, 87, 94–96]. As shown by Liu and colleagues, human ASCs attachment and proliferation were similar on akermanite and β-TCP *in vitro*, and osteogenic ASCs differentiation was enhanced on the akermanite over the β-TCP after 10 days of culture [86]. Recently, Zanetti and collegaues observed that ASCs cultured for 21 days in osteogenic medium prior to be seeded onto akermanite-based scaffolds produce greater calcium deposition and osteocalcin expression, compared to cells seeded on β-TCP and PCL [94].

Taken together, these data highlight the advantage of using ASCs in combination with biomimetic scaffold providing a most effective strategy for treating bone defects.

8. Dynamic Culture Systems for Cell-Scaffold Constructs

Tissue formation in three-dimensional scaffolds is significantly affected by nutrient transport, physical stress, cell density, and gas exchange [97, 98]. For the best possible tissue regeneration, postimplantation cell viability and homogenous cell distribution throughout the scaffold are crucial [99]. Dynamic systems like perfusion bioreactors facilitate optimal seeding under controlled conditions [99]. The term "bioreactor" refers to a wide variety of culture systems that provide a mechanism to maintain cell-scaffold constructs in a biocompatible environment during application of defined chemical and physical stimuli. Perfusion bioreactors are culture systems in which nutrient medium is repeatedly forced or "perfused" through cell-scaffold constructs. Therefore, these are referred to as "dynamic" culture systems in order to distinguish them from "static" cultures in which there is no fluid motion (i.e., standard culture flask or plate). Such culturing systems are aimed at allowing tridimensional cell adhesion on the scaffold and inducing specific cell behavior under controlled and repeatable conditions. This situation mimics a complex natural environment, as the cell-scaffold compound is exposed to common mechanical stimuli, deriving from the shear forces from nutrient medium motion and enables generating constructs with increased functionality and engraftment capacity [99, 100].

So far, few studies have described the possibility to establish a 3D culture model for bone cells using mineralized porous scaffolds as templates, which relies on the use of a perfusion-based bioreactor device, highlighting the synergism between a bioactive scaffold and the effect of perfusion on cells and indicating the differentiation into an osteogenic phenotype [100, 101]. In particular, in the study by Fröhlich and collaborators [102] ASCs were seeded on decellularized native bone scaffolds, providing the necessary structural and mechanical environment for osteogenic differentiation, and cultured in a perfusion bioreactor. After 5 weeks of culture, the addition of osteogenic supplements (dexamethasone, sodium-beta-glycerophosphate, and ascorbic acid-2-phosphate) to culture medium significantly increased the construct cellularity and the amounts of bone matrix components (collagen, bone sialoprotein, and bone osteopontin), indicating that medium perfusion markedly improved the distribution of cells and bone matrix in engineered constructs [102]. Also, in the study performed by Declercq and colleagues [103], After 6 weeks of dynamic culture, scaffolds were highly colonized and the osteogenic gene expression was higher compared to static cultures. Recently, Silva and colleagues [104] demonstrated that ASCs differentiate towards the osteogenic phenotype when cultured in a bioactive glass scaffold, with the osteogenic Leibovitz L-15 medium and a perfusion bioreactor, as indicated both the significant increase in cell proliferation and viability, the increased ALP activity, and the expression of osteospecific protein (i.e., osteocalcin and osteopontin) 2-to-3 weeks after culture. Furthermore, a coculture model of human osteoblast and endothelial lineage cells has been established by seeding and culturing cells freshly isolated from the SVF of AT within porous 3D ceramic scaffolds [105]. This system was reported to generate 3D constructs that, upon implantation into nude mice, were able to generate bone tissue and fully functional blood vessels [105, 106]. Also, a study performed by Güven and colleagues [107] remarks the efficiency of SVF cells to generate 3D-osteogenic constructs, compared to ASCs, supporting the concept that vascular progenitors derived from human SVF cells accelerate the engraftment of critically sized osteogenic constructs, ultimately improving the efficiency and uniformity of bone tissue formation.

9. Preclinical Evaluation of ASC Osteoregenerative Potential

A huge amount of data in the literature demonstrates the efficacy of ASC-based approaches for inducing bone regeneration/healing *in vivo*. Critical size-calvarial defects are widely employed to study bone healing in animal models, mostly rodents, allowing an easy quantification of the amount of newly formed bone within a bidimensional defect [74, 108–127]. An initial proof of principle of the *in vivo* osteogenic potential of experimental constructs may be achieved using local intramuscular injection to induce ectopic bone formation [75, 121, 128–133]. Also, segmental defects in long bones of large animals are widely used as clinically relevant models, as resembling the fracture healing process [93, 112, 134–147].

A number of published report indicates that the combination of recombinant human BMP2 (rhBMP2) with ASC may increase the osteogenic potential *in vivo* (see Table 1), although recent evidences are retracting this consolidated dogma, suggesting that combining rhBMP2 with ASCs, should not be considered the best viable strategy for inducing bone healing.

Overall, the number of published data obtained from animal models employed to study the bone healing properties of ASCs is constantly growing. Although a comprehensive and systematic categorization of all publications on this topic may be quite impossible, Table 1 attempts to summarize the study design of relevant preclinical studies. It is noteworthy that successful results, in terms of bone healing, have been achieved in different animal models, using either undifferentiated ASC (i.e., in the absence of any prior *ex vivo* osteogenic

Table 1: Preclinical studies on ASC osteoregenerative potential.

Experimental model	Species	Scaffold/administration	Additional *ex vivo*/*in vivo* treatment	Graft type	Reference
Calvarial defect	Rat	PLGA	Alendronate	Xenogeneic	[74]
	Rabbit	HA-PLGA, collagen sponge	BV-BMP2/TGF β3	Allogeneic	[120]
	Mouse	PLGA	Dura mater	Xenogeneic	[117]
	Rat	β-TCP	Lenti-miR-31	Allogeneic	[110]
	Mouse	Custom scaffold	NOGGIN shRNA-Knockout	Xenogeneic	[119]
	Dog	HA-PLGA	None	Xenogeneic	[122]
	Mouse	Systemic injection	None	Allo/xenogeneic	[115]
	Mouse	Local injection	None	Xenogeneic	[116]
	Rat	DBM, PLA	None	Xenogeneic	[123]
	Rat	MAP-coated PCL/PLGA	None	Xenogeneic	[111]
	Rat	HA-β-TCP	None	Xenogeneic	[126]
	Rat	PLGA	None/osteogenic medium	Xenogeneic	[125]
	Dog	Coral	Osteogenic induction	Autologous	[109]
	Dog	Coral	Osteogenic induction	Allogeneic	[121]
	Pig	Collagen sponge	Osteogenic induction	Autologous	[127]
	Rat	DBX	Osteogenic induction	Allogeneic	[112]
	Rat	PCL-PLGA-β-TCP	Osteogenic induction + HUVEC	Xenogeneic	[113]
	Mouse	pDA-PLGA	rhBMP-2	Xenogeneic	[114]
	Rabbit	Collagen sponge	rhBMP-2	Allogeneic	[124]
	Mouse	HA-PLGA	Sonic hedgehog signaling Induction	Xenogeneic	[118]
	Rat	Local injection	VEGFa	Xenogeneic	[108]
Ectopic bone formation	Mouse	PLGA	BMP2/RUNX2 bicistronic vector	Xenogeneic	[75]
	Mouse	PRP + alginate microsphere	None	Allogeneic	[131]
	Mouse	β-TCP	None	Xenogeneic	[121]
	Rat	HA	None	Xenogeneic	[128]
	Rat	Matrigel	Osteogenic induction	Xenogeneic	[133]
	Rat	DBM	Osteogenic induction	Xenogeneic	[132]
	Mouse	Carbon nanotubes	rhBMP2	Xenogeneic	[130]
	Rat	PLDA	rhBMP2	Xenogeneic	[129]
Segmental defect	Rabbit	Local injection	Bovine BMP	Allogeneic	[135]
	Rat	Fibrin matrix	rhBMP2	Allogeneic	[139]
	Rat	β-TCP	Lenti-BMP2/7	Allogeneic	[134]
	Rabbit	PLA/PCL + vascularized periosteum	Ad-Cbfa1	Allogeneic	[140]
	Rabbits	HA-PLA-COL	Ad-hBMP2	Allogeneic	[137]
Segmental defect	Mouse	Systemic injection	None	Allogeneic	[140]
	Rat	Collagen gel	None	Xenogeneic	[145]
	Rabbit	PLGA	None/osteogenic medium	Xenogeneic	[112]
	Dog	β-TCP	None	Allogeneic	[138]
	Rabbit	HA	None	Autologous	[93]
	Rabbit	Ceramics, biphasic materials	None	Allogeneic	[136]
Vertebral defect/fusion	Mouse	Local injection	rhBMP6 nucleofection	Xenogeneic	[143]
	Rat	Lyophilized human cancellous bone	Gal-KO + osteogenic induction	Xenogeneic	[142]
	Rat	Fibrin gel	rhBMP6 nucleofection	Xenogeneic	[144]
Mandible defect	Pig	Local-systemic injection	None	Allogeneic	[147]
	Rat	HA/COL	None	Xenogeneic	[146]

HA: hydroxyapatite; PLGA: poly(lactic-co-glycolic acid); PLA/PCL: polylactic acid/polycaprolacton; Ad-Cbfa1: adenoviral expression vector carrying the Cbfa1 gene; DBM: demineralized bone matrix; β-TCP: beta-tricalcium phosphate; Lenti-miR-31: lentivirus expression vector carrying the microRNA-31; p-DA: polydopamine; PRP: platelet-rich plasma; Lenti-BMP2/7: lentivirus expression vector carrying either the BMP2 or the BMP7 gene, MAP: mussel adhesive proteins, NOGGIN shRNA: short hairpin ribonucleic acid to knockdown NOGGIN gene, COL: collagen; BV-BMP2/TGF β3: baculovirus expression vector carrying either the BMP2 or the TGF β3 gene, MAP: mussel adhesive proteins; Gal-KP: galactosyl-knock-out; a-CaP: amorphous calcium phosphate; *these studies were based on uncultured SVF instead of culture-amplified ASCs.

induction) [93, 111, 114–116, 125, 128, 131, 136, 138, 140, 145–147] or uncultured SVF [112, 123] paving the way to an easier translation of preclinical evidence to the clinical setting.

10. Clinical Use of ASCs for Bone Regeneration/Reconstruction

When attempting to translate preclinical evidence to the clinical field, the manipulation of human tissues, for the production of clinical-grade human SVF cells and ASCs to be employed as therapeutic devices, must be carried out according to the current good manufacturing practices (GMP). The national regulatory agencies (i.e., the Food and Drug Administration in USA and the European Medicines Agency in EU) provide the official rules and guidelines that guarantee safe and controlled procedures [148]. In particular, the SVF should be classified as a minimally manipulated tissue, whose isolation does not require seeding and culturing. Conversely, all procedures involving culture-expanded MSC configure advanced cell therapies and must comply with institutional GMP rules for cell manipulation, which must be carried out into a cell factory of a certified facility.

In recent years, ASCs attracted the overwhelming interest of clinicians and industry, being multipotent stem cells endowed with trophic and immune-modulatory properties, residing into a widely available and relatively accessible adult tissue. This has been generating a confusing scenario that often risks to configure clinical misconduct, when putative innovative cell therapies are provided within uncontrolled trials to incorrectly informed patients, in a wide range of clinical applications. On this regard, a useful lesson for "naive" clinicians may be provided by the controversial debate, recently brought by Italian media, around the "Stamina Foundation," which promoted the use of bone marrow-derived MSCs as a "compassionate, as yet-unapproved" treatment of neurodegenerative diseases (including spinal muscular atrophy) in terminally ill children [149]. After all, the proposed protocol for MSC processing, the so called "Vannoni's method," was carried out in inappropriate facilities (according to the Italian Medicines Agency, AIFA) and was based on flawed and plagiarized data [150].

In bone reconstructive surgery, autologous or allogeneic bone graft still represents the gold standard treatment although hampered by local morbidity and largely relying on donor availability, expecially in the case of large segments to be harvested. Therefore, the need for alternative procedure has rapidly lead to experimental procedures based on ASCs. Despite the increasing amount of scientific data on ASCs and an extremely wide number of preclinical studies confirming their bone regenerative potential *in vivo*, only few controlled clinical trials, aimed at assessing the efficacy and safety of ASCs in patients with bone-related disorders, have been concluded and published (for review see [148] and [151]) and few others are being currently carried out (http://www.clinicaltrials.gov/). In particular, successful results have been obtained in distinct trials using autologous ASC for craniofacial bone reconstruction [149, 152–154].

Lendeckel and colleagues employed ASC for the reconstruction of a large pediatric posttraumatic calvarial defect, which is always challenging and troublesome. In this case, an interdisciplinary surgical equipe implanted a resorbable macroporous sheet as a scaffold for ASC and milled autograft cancellous bone. The complex procedures yielded a stable osteointegrated graft that showed marked ossification at the 3 months followup [153]. Also Thesleff and collaborators used ASCs for calvarial reconstruction, testing alternative biomaterials (β-TCP and resorbable mesh bilaminate scaffold), and obtaining successful results in adult patients [152]. Mesimäki and colleagues used autologous ASCs seeded on a beta-tricalcium phosphate (β-TCP) scaffold doped with recombinant human BMP2 to treat a large maxillary defect resulting from a benign tumor resection in an adult patient. They achieved satisfactory outcomes, obtaining new, mature, vital, and vascularized bone eight months after surgery, with good osteointegration and stability [154]. More recently, Sandor and colleagues published the successful reconstruction of large anterior mandibular bone defects using ASC seeded on a β-TCP premolded scaffold based on patient's computed tomography data [149].

The partial drawbacks of experimental ASC-based bone reconstructive procedures are represented by the need to expand cells *ex vivo* for two-to-three weeks to achieve the appropriate cellular yield prior to the implantation, which implies multiple surgical interventions. Moreover, extended *in vitro* ASC expansion may be associated to genomic instability leading to either structural or numeric chromosomal aberrations [155], though it is still unclear whether this may represent a real risk for the recipient patient. Recent research efforts have been spent to develop *ad hoc* devices for the rapid one-step isolation of the SVF from liposuctioned adipose tissue to be grafted without prior *ex vivo* culture amplification manipulation [156]. Further development of such devices may allow overcoming and implementing fat harvesting for ASC isolation aimed at reconstructive surgery.

11. Conclusions

Around 3000 publication surveyed in the scientific databases point towards the definition of ASCs as the most effective and safe cell type for regenerative medicine approaches. Bone regeneration is currently the most promising field for clinical translation of experimental ASCs protocols. Nonetheless, the rapidly growing development of research in the field of biocompatible scaffolds is widening the field of ASCs applications in multidisciplinary scenarios, allowing cells to grow, differentiate, and be exposed to cytokines and growth factors.

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Review Article

Human Induced Pluripotent Stem Cells from Basic Research to Potential Clinical Applications in Cancer

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The human induced pluripotent stem cells (hiPSCs) are derived from a direct reprogramming of human somatic cells to a pluripotent stage through ectopic expression of specific transcription factors. These cells have two important properties, which are the self-renewal capacity and the ability to differentiate into any cell type of the human body. So, the discovery of hiPSCs opens new opportunities in biomedical sciences, since these cells may be useful for understanding the mechanisms of diseases in the production of new diseases models, in drug development/drug toxicity tests, gene therapies, and cell replacement therapies. However, the hiPSCs technology has limitations including the potential for the development of genetic and epigenetic abnormalities leading to tumorigenicity. Nowadays, basic research in the hiPSCs field has made progress in the application of new strategies with the aim to enable an efficient production of high-quality of hiPSCs for safety and efficacy, necessary to the future application for clinical practice. In this review, we show the recent advances in hiPSCs' basic research and some potential clinical applications focusing on cancer. We also present the importance of the use of statistical methods to evaluate the possible validation for the hiPSCs for future therapeutic use toward personalized cell therapies.

1. Introduction

Cancer is a major cause of mortality through the world. This disease evolves by a process of clonal expansion, genetic diversification, and clonal selection. The dynamics are complex and with highly variable patterns of genetic diversity and resultant clonal architecture [1]. Cancer cells have diverse biological capabilities that are conferred by numerous genetic and epigenetic modifications [2]. Several studies have been done with the aim of identifying biomarkers involving cancer for the development of new molecular target therapies. In recent years, different high-throughput platforms have been used for the genomic, transcriptomic, proteomic, and epigenomic analyses to search for new biomarkers involved in cancer and to bring new insights into the several aspects of cancer pathophysiology including angiogenesis, immune evasion, metastasis, altered cell growth, death, and metabolism [2–7].

There are several pioneering examples of genomic aberrations being discovered in cancer cells and the findings being successfully translated into therapeutic agents with considerable effects on the practice of cancer medicine. The first genomic alteration found to be consistently associated with a human malignancy, the chronic myeloid leukemia (CML), was the Philadelphia chromosome, discovery by Nowell and Hungerford in 1960 [8]. The cytogenetic and molecular studies showed that this chromosomal alteration involves a reciprocal translocation between chromosomes 9 and 22, resulting in a fusion gene, the BCR-ABL. The BCR-ABL fusion gene encodes a constitutively active leukemogenic protein tyrosine kinase [9]. More than 30 years after the discovery of the Philadelphia chromosome, a small molecule inhibitor of this CML biomarker was developed, the imatinib mesylate. BCR-ABL kinase activity is inhibited

by the selective activity of imatinib, a target agent that has demonstrated remarkable efficacy and tolerability. This is the first example of a target molecular therapeutic agent in cancer [10, 11]. It has been shown that imatinib blocks the cells proliferation and induces apoptosis in BCR-ABL expressing hematopoietic cells. Imatinib has been used as a first line therapy for CML patients. Different patterns of response to imatinib treatment have been recognized, ranging from best-case scenarios of rapid and unwavering response to difficult situations of intolerance and resistance, with the appearance of clonal cytogenetic abnormalities in Philadelphia chromosome-negative cells [12–14]. The resistant cancer cells emerged in different kinds of tumors, and research groups are studying these molecular mechanisms, especially in cancer stem cells (CSC) because of their dual role, as a tumor-initiating cell and as a source of treatment resistance cells [15–18].

Several approaches have been used to understand cancer pathogenesis, as animal models and cell cultures, using mainly the cell lines. Much of our understanding of cancer cell biology, including the aspects of gene regulation and signaling pathways, has come from studies of cancer cells in culture. But, theoretically, the best model to study cancer is the primary patient samples, but the amount of obtained cells may be inadequate for various analyses [2, 19, 20]. So, the recent discovery of the human induced pluripotent stem cells, hiPSCs, opens a new perspective to study the biology of different diseases, including cancer [19–21]. The hiPSCs are being used to make disease models, to develop new drugs, to test toxicity, and in regenerative medicine. The reprogramming technology offers the potential to treat many diseases, including neurodegenerative diseases, cardiovascular diseases, and diabetes. In theory, easily accessible cell types (such as skin fibroblasts) could be obtained from a patient and reprogrammed, effectively recapitulating the patients' disease in a culture system. Such cells could then serve as the basis for autologous cell replacement. However, depending on the methods used, reprogramming adult cells to obtain hiPSCs may pose significant risks that could limit their use in clinical practice. For example, if viruses are used to genomically alter the cells, the expression of cancer-causing genes "oncogenes" may potentially be triggered. So, many different groups have successfully generated iPSCs, but due to different techniques, their methods of calculating efficiency of conversion are varied [22–24]. In this review, we show the recent advances in hiPSCs basic research and some potential clinical applications focusing on cancer. We also present the importance of the use of statistical methods to evaluate the possible validation for the hiPSCs for future therapeutic use toward personalized cell therapies.

2. Human Induced Pluripotent Stem Cells: Discovery and the Development of Different Methods to Generate hiPSCs

The first generated induced pluripotent stem cells (iPSCs) were in mice by the Yamanaka's group at Kyoto University, Japan, in 2006. It is a recent discovery that iPSCs are derived from somatic cells through ectopic expression of specific transcription factors [25]. In 2007, human iPSCs were first generated by the same group by transducing adult human dermal fibroblasts with viral vectors carrying the key pluripotency genes, Oct3/4, Sox2, Klf4, and c-Myc (Yamanaka factors), using a retroviral system [26]. In 2007, Thomson's group at the University of Wisconsin-Madison, EUA, also generated human iPSCs. They used the factors Oct4, Sox2, Nanog, and LIN28 and a lentiviral system to reprogram human somatic cells to pluripotent stem cells that exhibit the essential characteristics of embryonic stem cells (ESCs) [27]. The ESCs are pluripotent cells derived from the inner cell mass of the preimplantation blastocyst. These cells are potential renewable sources of all human tissues for regenerative medicine, and, for this reason, they are very valuable to understand the early events of human development, in gene therapy and for new drug discovery. However, the usage of ESCs is a highly controversial issue on moral, social, and ethical grounds. This is because the process involves the destruction of a blastocyst, which is considered a human embryo with the potential of developing into a person. The research using the ESCs is prohibited in some countries, while in other countries the research using the ESCs is allowed under legislation but remains tightly restricted [22]. So, the research using hiPSCs, which are derived from human somatic cells, does not present the ethical dilemmas as the research using the ESCs. In Table 1, we show the advantages and disadvantages in using the iPSCs.

In the experiment of Dr. Yamanaka, the ectopic expression of "embryonic factors" was cloned and promoted in the differentiated human cells. Initially, 24 genes were analyzed and selected. Among these genes, there were genes involved in the maintenance of pluripotency like Oct3/4, Nanog, and Sox2; there were genes overexpressed in the tumors related with fast proliferation and maintenance of undifferentiated stage like STAT-3, Ras, c-Myc, Klf4, and Beta-catenin and genes expressed in the early stages of development such as FGF4, Zfp296, Utf1, and others. For the expression of these genes, they were selectively amplified from cDNA template by PCR, cloned into plasmid and *in vitro* introduced in fibroblast cells through retroviral transduction. After infections and confirmation of expression of the introduced genes, the fibroblast cells were analyzed to observe cell phenotype. Dr. Yamanaka performed a series of evaluations in a single or combined gene to verify which ones were essential or able to induce alterations in the differentiated fibroblast cell morphology, growth, and gene expression profile similar to ESCs. Among the initial genes studied, only the Oct3/4, Sox2, c-Myc, and Klf4 appeared to be important, generating the iPSCs. This study established a new concept in the science scenario: the *in vitro* induced pluripotent stem cells. The hiPSCs technology represents an important platform with the potential to advance in medical therapy by personalizing regenerative medicine and by creating new human disease models for research and therapeutic tests. The discovery that adult somatic stem cells can be reprogrammed into pluripotent cells is so important that, in 2012, Dr. Yamanaka

Table 1: Advantages and disadvantages in iPSCs utilization.

Advantages	Disadvantages
Avoid human embryos' use	Oncogene use for induction iPSCs phenotype
Capacity to induce stem cell like phenotype	Use of integrative DNA methodology
New promises to cellular therapy	Genomic instability and aberrations
Possibility of studying several diseases, including cancer	Increase risk of the development of cancer

was awarded with the Nobel Prize in Physiology or Medicine [25, 26, 28].

Basically, the methodology used to generate hiPSCs implies in the specific gene amplification by PCR, insertion of this product in a DNA vector, and introduction of this cloned gene in the host cell. The foreign DNA vector can be inserted in the receptor by several different ways, like the viral transduction. The method using viral transduction has efficiency to introduce the DNA vector inside cell and successful integrating of the DNA cloned in the host cell's genome, and this is the main advantage of viral method. The DNA vector viral integrates in host genome cell particularly due to long terminal repeats (LTR) present in both extremities of virus genome. These LTRs are compound by hundreds of nucleotides repetitions that, by recombination, attach the DNA inner contained in genomes [29].

Many approaches have used viral particles carrying DNA constructions that can be integrated in the genome's cell randomly. In fact, it is the main counterpart of iPSCs utilization. Therefore, reprogramming by cloning with the usage of viral strategies and long-term culture can also induce abnormalities in these pluripotent cells. *In vitro* cultures, sometimes iPSCs have demonstrated genomic instability. Unlike other stem cell cultures, the genomic instability is more common in early passages [30]. It is believed that this phenomenon is due to genetic reprogramming [30, 31]. This enhanced genomic instability in iPSCs can involve p53 protein inactivation, which is important to proliferation and DNA repair machinery activation in response to DNA damage [32].

Additionally, the viral DNA that carries cloned gene of interest can integrate in any *loci* in genome host cells. This implies many consequences, such as (1) integration into DNA sequence that encodes essential gene, disrupting its function which can cause loss of cell viability; (2) disrupt regions that coordinate expression of important genes, like promoter or enhancers regions, mainly if these genes contain "tumor suppression functions"; (3) the viral DNA may integrate in DNA regions that are responsible for negative regulation of "oncogenes," allowing their constitutive expressions [33, 34]. Chromosomal instability, mutational possibilities, and use of known oncogenes, c-Myc and Klf4, to produce iPSCs, have implicated in the high incidence of cancer development in preclinical tests induced by iPSCs [35]. These observations have increased the discussion about the possibility of the usage of iPSCs in cellular therapies.

Another point is that the stimulation of loss of differentiation state to generate iPSCs also involves epigenetics reprogramming process and differential expression of noncoding functional RNA (ncRNA). A recent study discovered that there are more miRNA upregulated in the iPSCs than in the ESCs. These miRNAs have been frequently found related in the cancer development [36].

Most strategies currently under use to generate iPSCs are based on gene delivery via retroviral or lentiviral vectors [26, 27, 37–39]. However, most experiments involved integration in the host cell genome with an identified risk for insertional mutagenesis and oncogenic transformation. To avoid such risks, which are incompatible with therapeutic prospects, significant progress has been made with transgene-free reprogramming methods based, for example, on Sendai virus, direct mRNA, or protein delivery to achieve conversion of adult cells into hiPSCs [40–45]. So, there have been several improvements of the gene transduction method for making safe iPSCs. Due to an intense discussion about the use of hiPSCs in cellular therapies, since they are not completely safe, a lot of works, trying to establish *in vitro* stem cells derived from a variety of sources, has emerged. For example, bone marrow derived hematopoietic stem cells, multipotent mesenchymal stromal cells derived from bone marrow, umbilical cord blood, and adipose tissue. The ideal source of the cell to be isolated from the patients and used for reprogramming must have easy accessibility. This means that it is not necessary to have surgery to get the cells, it is possible to obtain them from a skin biopsy, for example, with minimal risk procedures, availability in large quantities, relatively high reprogramming efficiency, and fast iPSCs derivation speed [45]. Thus, new sources to obtain stem cell has also emerged; new strategies to induce cell reprogramming without the use of viral particles have been used aiming for safety and efficiency to generate hiPSCs with the purpose of their use in clinical practice [46–51]. For detection of high-quality hiPSCs, specialized cell tests may be conducted for making efficient differentiation protocols [52]. Now, basic research should be focused on characterizing the hiPSCs at cytogenetic and molecular levels to observe if these cells retain the genetic stability. It is necessary to understand how the cellular reprogramming works at molecular level, generating new knowledge in cell signaling pathways, comparing the different cell sources and the different methods used to generate the hiPSCs with the basic requirements of high quality and safety for their use in patients. In Table 2, a summary of the main methods used to generate iPSCs is shown.

3. Human iPSCs: Potential Clinical Applications in Cancer

This is the first review study focusing on the potential use of hiPSCs in clinical applications for cancer. We ask the following question: how can the hiPSCs, which may cause malignant transformation, be used for study and for possible application in the treatment of cancer?

The hiPSCs can lead to clinical applications as the study of the disease biology, making disease models, developing

Table 2: Summary of the methods used to generate iPSCs.

Methodology	Cell type	Genome integration	Efficiency of iPSC induction	References
Retroviral transduction	Fibroblast, neuronal, keratinocyte, blood cells, adipose, and liver cells	Yes	High	Takahashi et al., 2007 [26]/Lowry et al., 2008 [37]
Lentiviral transduction	Fibroblast and keratinocyte	Yes	High	Yu et al., 2007 [27]/Moore, 2013 [38]
Inducible lentiviral transduction	Fibroblast, melanocytes, beta-cells, blood cells, and keratinocyte	Yes	High	Maherali et al., 2008 [39]
Adenoviral transduction	Fibroblast	No	Low	Stadtfeld et al., 2008 [40]
Plasmid vector	Fibroblast	No	Low	Si-Tayeb et al., 2010 [41]
Cell-free lysate or protein extract	Fibroblast and adipose stromal cells	No	Low	Kim et al., 2009 [42]
Cellfusion	Fibroblasts and adult thymocytes	No	Low	Cowan et al., 2005 [43]
Sendai viral transduction	Fibroblast and $CD34^+$ cord blood cells/$CD34^+$ cells from CML patient/ Peripheral blood mononuclear cells	No	High	Ban et al., 2011 [49]/Kumano et al., 2012 [19]/Churko et al., 2013 [50]
Minicircle DNA	Adipose stem cells	No	High	Narsinh et al., 2011 [51]
Episomal vectors	Mononuclear bone marrow and cord blood cells	No	High	Hu and Slukvin, 2013 [48]

new drugs, and testing toxicity. Recent progress in the reprogramming field has demonstrated important disease models using iPSCs in both gene target therapies, for example, the sickle cell anemia and augmentation therapy, for example, for Hemophilia A. The gene therapy refers to the introduction of genetic material into particular cells or tissues for therapeutic purposes especially in gene corrections for mutations in monogenic genetic diseases [22, 53].

Cancer is a complex disease, characterized by genetic and epigenetic alterations. Many researchers are trying to identify biomarkers involved in tumor initiation as well as the steps involved during the evolution of disease. The main purpose of using biomarkers is to develop new drugs for cancer therapy. Furthermore, the identification of biomarkers can be used for early diagnosis and for therapeutic stratification groups aiding the medical staff to choose the appropriate treatment for that patient [2, 54].

Theoretically, the best model to study cancer pathogenesis is the primary patient samples, but the amount of obtained cells may be inadequate for various analyses. Recently, it was reported that iPSCs can be generated not only from normal tissue cells but also from malignant cells [19, 55–58]. So, the hiPSCs are highly relevant to study the multiple stages of oncogenesis, from the initial cellular transformation to the hierarchical organization of established malignancies providing a human cell model to study the stages of disease [59, 60]. In this sense, there are some examples. Kim and colleagues (2013) used the hiPSCs as a model to study the pancreatic ductal adenocarcinoma (PDAC). This cancer carries a dismal prognosis and lack a human cell model of early disease progression. In this study, the authors made the following hypothesis: if human PADC cells were converted to pluripotency and then allowed to differentiate back into pancreatic tissue, they might undergo early stages of cancer [59]. So, the iPSCs technology can provide a live human cell model of early pancreatic cancer and disease progression.

Another example for the potential clinical applications of hiPSCs in disease modeling for studying cancer is in hematological malignancies. Primary samples of hematologic malignancy are usually difficult to be expanded in cultures. However, after they are reprogrammed to iPSCs, they can expand unlimitedly. The iPSCs technology has been used to study myeloproliferative diseases as chronic myeloid leukemia (CML) [19] and juvenile myelomonocytic leukemia (JMML) [61]. Many studies are being performed to elucidate the mechanisms of tyrosine kinase inhibitor (TKI) resistance in CML stem cells and to overcome the resistance in these patients. Kumano and colleagues (2012) established the CML-iPSCs by Sendai virus system and confirmed the resistance of these cells to imatinib [19]. So, they developed a model to study the CML disease and the TKI resistance. Another example for the use of iPSCs is the JMML. JMML is an aggressive myeloproliferative neoplasm of young children initiated by mutations that deregulated cytokine receptor signaling. Children with this disease have a poor prognosis. Gandre-Babbe [61] generated iPSCs from two JMML patients. In this study, the authors suggested the relevance of this method to explore the pathophysiology and treatment of JMML [61]. Emerging developments of iPSCs research can be used as a tool in modeling hematopoietic disorders and could lead to new clinical applications in gene and cell therapies [20]. The advantage of using disease modeling with iPSCs technology is that it allows the generation of pluripotent cells from any individual in the context of his/her own particular genetic identity including individuals with simple forms of disease and those with complex multifactorial diseases of unknown genetic identity [45]. In drug screening, the use of hiPSCs would be used to verify the response to a specific target gene and to research the single nucleotide polymorphism related to each individual that influences the ability of an individual to effectively metabolize and clear drugs and toxins. In particular, hepatotoxicity and cardiotoxicity are two

Table 3: Summary of cancer-derived hiPSCs.

Type of cancer (hematologic malignancies and solid tumors)	Aim of study	Method of generation of the cancer hiPSCs	References
Myeloproliferative disorder (MPD) with JAK2-V617F somatic mutation	To generate iPS cells to provide a renewable cell source and a prospective hematopoiesis model for investigating MPD pathogenesis	Frozen peripheral blood $CD34^+$ cells from 2 patients with MPD/retroviral transduction	Ye et al., 2009 [55]
Chronic myeloid leukemia (CML)	To address whether human cancer cells can be reprogrammed into iPSCs	Cell line, KBM7, derived from blast crisis stage of CML/retroviral transduction	Carette et al., 2010 [56]
Chronic myeloid leukemia (CML)	To eliminate the genomic integration and background transgene expression, toward advancing iPSCs technology for the modeling of blood diseases	Bone marrow mononuclear cells from a patient with CML (chronic phase)/episomal vectors	Hu et al., 2011 [57]
Chronic myeloid leukemia (CML)	To investigate CML pathogenesis on the basis of patient-derived samples	Two patients samples of CML (chronic phase) bone marrow cells, retrovirus and Sendai virus system	Kumano et al., 2012 [19]
Juvenile myelomonocytic leukemia (JMML)	To explore the pathophysiology and treatment of JMML	Two pediatric patient's samples from bone marrow or peripheral blood/lentivirus	Gandre-Babbe et al., 2013 [61]
Gastrointestinal cancer	To study new cancer therapies via reprogramming approaches in cancer cells	Gastrointestinal cell lines of cancers from esophageal, stomach, colorectal, pancreas, and liver and bile ducts/lentiviral and retroviral	Miyoshi et al., 2010 [58]
Gastrointestinal cancer	Generate a human cell model of early pancreatic cancer and disease progression for biomarkers detection for useful diagnosis	Tissue from the center of pancreatic ductal adenocarcinoma (PDAC) sample of patient/lentivirus system	Kim et al., 2013 [59]

principal causes of drug failure during preclinical testing. The variability in individual responses to potential therapeutic agents is also a major problem in effective drug development. The advantage of iPSCs technology is that it allows the generation of various cell lines that may represent the genetic and potentially epigenetic variation of a broad spectrum of the population. This approach used the *in vitro* model of disease to identify new drugs to treat disease [45].

Although some studies showed that cancer-derived hiPSCs is possible (Table 3), it is necessary a continuous progress in the iPSCs technology. Reprogramming cancer cells has been demonstrated to be harder than generation of normal iPSCs because of the genetic and epigenetic status of these cells. To try to overcome this difficulty, some researchers are testing other possibilities to generate cancer-derived hiPSCs by the application of other factors in addition to the Yamanaka factors, such as exogenous expression of miRNA302 and chemical compounds, as azacitidine (DNA methyltransferase inhibitor) and knockdown of p53, p21, and Ink4/Arf [19, 62]. Another point, here, for the normal and cancer cells, it is the genes delivery systems for the iPSCs generation. The integration site of retrovirus in the iPSCs may affect the gene expression and change the disease phenotype after redifferentiating them into the original lineages. So, efficient induction of transgene-free iPSCs such as using Sendai virus and episomal systems has been reported [19, 48, 57]. But, we can have in mind, as mentioned by Ramos-Mejia and collaborators (2012), that the difficulties in reprogramming cancer cells do not seem exclusively due to technical barriers or the need for improved reprogramming technologies. But, it seems that the biological barriers such as cancer-specific genetic mutations, epigenetic remodeling, or accumulation of DNA damage may influence the reprogramming of human cancer cells [63].

The cancer-derived hiPSCs represents important systems for modeling cancer pathogenesis, aiding in the discovery of new diagnostic and prognostic biomarkers, and for the development of new therapies for cancer. For example, Yang and collaborators (2012) demonstrated a tumor tropism of intravenously injected human iPSC-derived neural stem cells and their gene therapy application in a metastatic breast cancer mouse model. In this study, the authors used a lentiviral transduction method to derive hiPSCs from primary human fibroblasts and then generated neural stem cells (NSCs) from the iPSCs. The NSCs are able to home not only on brain tumors but also on solid tumors of a nonneural origin. This intrinsic tropism occurs because the presence of cytokines, chemokines, and growth factors released from the tumor cells. Yang and collaborators investigated whether the iPSCs derived NCS can be used as a cellular delivery vehicle for cancer gene therapy. For this propose, the cells were transduced with a baculoviral vector containing the herpes simplex virus thymidine kinase suicide gene and injected through tail vein into tumor-bearing mice. The transduced NCSs were effective in inhibiting the growth of the breast tumor and the metastatic spread of the cancer cells in the presence of ganciclovir, leading to the prolonged survival of

the tumor-bearing mice. This study demonstrated the use of iPSC-derived NSCs for cancer gene therapy [64].

A potential clinical application of hiPSCs in cancer is in the field of immunotherapy [66–69]. Traditional treatment modalities are all based on destroying cancer cell by irradiation, chemotherapy, or surgery. Although, they can effectively kill or remove cancer cells, the use of these treatments often is limited because a number of health cells also tend to be destroyed and, in some cases, may occur the recidive of cancer. In the case of cancer, the immune system alone often fails to effectively fight the tumor for the following reasons: (1) the normal immune system is "blind" to tumor cells because the tumor cells are derived from the body's own cells. The body "thinks" about the tumor as "self," a phenomenon known as tumor tolerance; (2) the immune system may recognize certain cancer cells, but the response may not be strong enough to destroy cancer; (3) the tumor has the ability to defend itself secreting some substances that allow its survival and expansion. In the case of cancer, the immune system needs a boost to enhance its response to become more effective. So, the immunotherapy strategies include antitumor monoclonal antibodies, cancer vaccines, adoptive transfer of *ex vivo* activated T or natural killer cells, and administration of antibodies that either stimulate immune cells or block immune inhibitory pathways. The impact of immunotherapy was initially demonstrated in patients with advanced cancer and then translated to the adjuvant setting of patients with operable disease at high risk for postoperative recurrence [70].

Therapies based on the use of autologous immune cells are among the best candidates for cancer immunotherapy. The dendritic cell vaccines have demonstrated very encouraging responses for some solid tumors, while in melanoma T-cell therapies have exceeded 70% objective response rates in selected Phase I trial [71]. However, it is difficult to obtain a sufficient number of functional dendritic cells (DCs) in DC-based immunotherapy. In this sense, some studies are being performed using the iPSCs. Iwamoto and colleagues (2013) used the iPS cell-derived DCs (iPSDCs) and compared the therapeutic efficacy of iPSDCs and the equivalent to that of bone marrow-derived DCs (BMDCs). In this study, the authors examined the capacity for maturation of iPSDCs compared with that of BMDCs in addition to the capacity for migration of iPSDCs to regional lymph nodes. The therapeutic efficacy of the vaccination was examined in a subcutaneous tumor model. The vaccination with genetically modified iPSDCs achieved a level of therapeutic efficacy as high as vaccination with BMDCs. This study showed experimentally that genetically modified iPSDCs have an equal capacity of BMDCs in terms of tumor-associated antigen-specific therapeutic antitumor immunity. Therefore, vaccination strategy may be useful for future clinical application as a cancer vaccine [67].

The immunotherapy based on the adoptive transfer or gene-engineered T cells can mediate tumor regression in patients with metastatic cancer [72]. Adoptive T-cell immune therapy is based on the isolation of tumor-specific T cells from a cancer patient, *in vitro* activation, expansion of these T cells, and reinfusion of the T cells to the patient [73]. The adoptive immunotherapy with T cells is an effective therapeutic strategy for combating many types of cancer. However, the limitations associated with the number of antigen-specific T cells represent a major challenge to this approach [74]. The recent iPSCs technology and the development of an *in vitro* system for gene delivery are able to generate iPSCs from patients. The iPSCs have a great potential to be used in adoptive cell transfer of antigen-specific CD8(+) cytotoxic T lymphocytes [75, 76]. Some research groups are studying methods to generate T lymphocytes from iPSCs *in vitro* and *in vivo* programming antigen-specific T cells from iPSCs for promoting cancer immune surveillance [76].

Natural killer (NK) cells play a critical role in host immunity against cancer. In response, cancer develops mechanisms to escape NK cell attack or induce defective NK cells. Current NK cell-based cancer immunotherapy aims to overcome NK cell paralysis using several approaches. One approach is the genetic modification of fresh NK cells or NK cell lines to highly express cytokines, Fc receptors, and/or chimeric tumor-antigen receptors. Therapeutic NK cells can be derived from various sources, including peripheral or cord blood cells, stem cells, or even induced pluripotent stem cells (iPSCs), and a variety of stimulators can be used for large scale production in laboratories or good manufacturing practice [77].

Adult stem cell therapies have provided success for more than 50 years, through reconstitution of the hematopoietic system using bone marrow, umbilical cord blood, and mobilized peripheral blood transplantation. Mesenchymal stem cell (MSC) mediated therapy is a fast-growing field that has shown to be safe and effective in the treatment of various degenerative diseases and tissues injuries. The expansion and manipulation of human MSCs are important approaches to immune regulatory and regenerative cell therapies. MSCs are fibroblast-like cells of the BM microenvironment called "marrow stromal cells," which were able to support hematopoiesis. These cells have adult stem cell properties as they could differentiate into cartilage, bone, adipocytes, and muscle cells. MSCs are a promising tool for cell therapies because they are easily accessible from various tissue sources as bone marrow (BM-MSC), fat, and umbilical cord [78]. These cells have been widely tested and showed efficacious in preclinical and clinical studies for cardiovascular and neurodegenerative diseases, orthopedic injuries, graft-versus-host disease (GvHD) following bone marrow transplantation, autoimmune diseases, and liver diseases [78, 79].

Because BM-MSC can be easily harvested from adult sources and cultured *in vitro*, many preclinical and clinical studies have used BM-MSC. Although these cells show great potential for clinical use, there are some problems. The need for extensive cell number for use poses a risk of accumulating genetic and epigenetic abnormalities that could lead to malignant cell transformation. Binato and colleagues (2013) studied the stability of human MSCs during *in vitro* culture in several passages using cytogenetic, cellular, and molecular methods, and it was observed that these cells demonstrated chromosomal instability and molecular changes during passage 5 [80]. Although easy access to BM-MSC is recognized as a great advantage, extended *in vitro*

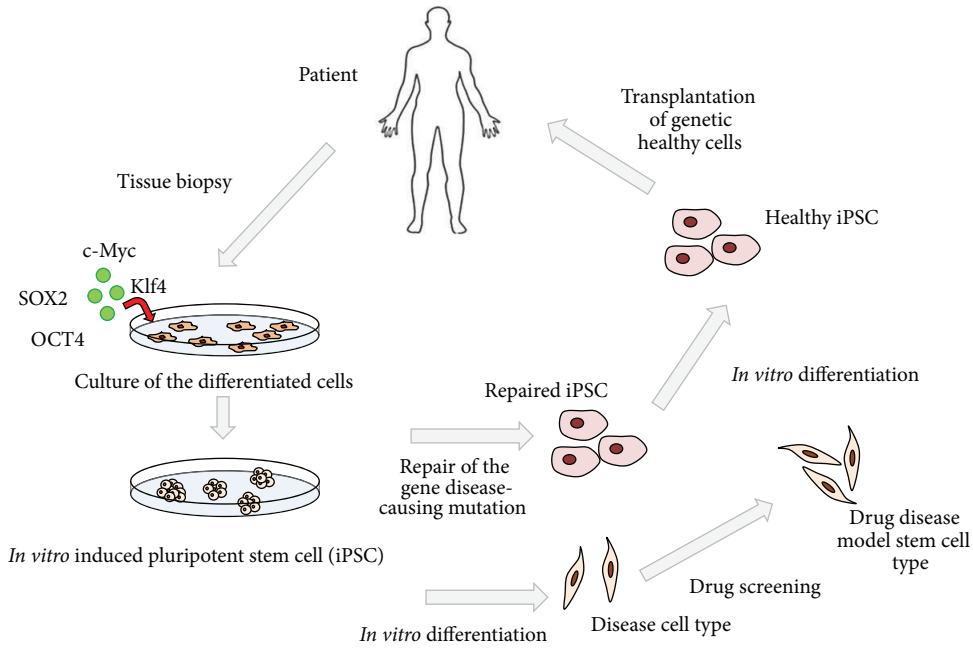

Figure 1: Potential applications of human iPSCs. The iPSCs technology can be potentially used in disease modeling, drug discovery, gene therapy, and cell replacement therapy. Differentiated cells are acquired by biopsies from human tissues and *in vitro* cultured under stem cell transcription factors, such as SOX2 (*SRY-box containing gene 2*), c-Myc (*v-myc avian myelocytomatosis viral oncogene homolog*), OCT4 (*octamer-binding transcription factor 3*), and KlF4 (*Kruppel-like factor 4*). After induction of pluripotency phenotype, the cells, known as iPSCs, can be utilized to redifferentiation in specific disease, to drug screening, or to have the genomic defect corrected, and then the iPSCs become able to be reutilized as health cells in the regenerative therapies.

cultures reduce the differentiation potential of MSC, which limits their therapeutic efficacy [78]. So, to overcome this problem, MSCs derived from iPSC may be considered for human cell and gene therapy applications as iPSCs have the potential to be expanded indefinitely without senescence. A greater regenerative potential of MSCs is observed derived from iPSCs which may be attributed to superior survival and engraftment after transplantation, because of higher telomerase activity and less senescence as compared to BM-MSC. Genetically manipulated MSCs may also serve as cellular therapeutics since MSCs can be used as a target drug delivery vehicles [78]. In Figure 1, we can see the generating of hiPSCs and the potential applications of these cells.

4. Statistical Methods to Evaluate the Possible Validation for the hiPSCs for Future Therapeutic Use

Medicine is full of mysteries. For centuries, people are trying to understand how the human body works. Many advances were made. As a consequence, human being has been living more and better.

The human body is a complex structure, influenced by many factors. So, it is difficult to answer medical questions. A tool that can help to ask such questions is based on a mathematical concept: the concept of probability. In fact, the tool we are talking about is the theory of mathematical statistics, which is the study of how to deal with data by means of probability models.

Clinical research relies on quantitative measurements. Impressions, intuitions, and beliefs are important in medicine, but only when they are together with a solid base of numerical information. This base allows more precise communication between clinicians and between clinicians and patients, as well as an error estimate. Clinical outcomes such as the occurrence of disease, death, symptoms, or functional impairment can be counted and expressed as numbers. In most clinical situations, diagnosis, prognosis, and treatment results are uncertain for an individual patient. A person will experiment a clinical outcome or not: the prediction is rarely accurate. Therefore, the prediction needs to be expressed as a probability. This can explain why probability models are important to clinical research.

Probability models lead to *statistical hypothesis tests* and *estimates*. They are used to draw inferences and reach conclusions about data, when only a part of a population, a sample, has been studied. When we consider a sample, we need to have in mind what variables we are considering under study. Also, the number of its elements is very important. For example, if we are interested in estimating *one mean*, the Central Limit Theorem establishes that the sampling distribution of means will be approximately normal even when its population is not distributed normally, provided that the sample size is large. If n denotes the number of elements of a sample, $n \geq 30$ is our definition of "large" [81].

If we decide to approximate clinical measurements by a normal curve, we are deciding to use a parametric test. Because normal distribution has nice mathematical properties (bell-shaped, symmetric, etc.), using a parametric test leads to better results compared with a nonparametric one. In other words, we say that nonparametric tests are less powerful, in the sense that they lead to a small probability to reject the null hypothesis, when it is false.

The iPSCs are undifferentiated cells that have the capacity to proliferate in undifferentiated cells both *in vitro* and *in vivo* (self-renewal) and to differentiate into mature specialized cells. Because this is a new discovery, there are open questions regarding, mainly, the safe application of stem cell therapy using the iPSCs. As we have presented in this work, many different groups have successfully generated iPSCs, but due to different techniques, until now, there is no standard information about the safety and effectiveness of the use of iPSCs in the clinical practice.

All scientific studies aim to answer a question that arises by observations of the researcher or the results of previous studies. Structuring a study helps answering questions in a systematic way (Figure 2). We note that a question well formulated is of great importance to the success of a study.

In order to have a better understanding of how we can minimize the problems, which occurs with the use of iPSCs, we think it is important to consider the following questions.

(1) How does the cellular reprogramming work at cytogenetic level?

(2) How does the cellular reprogramming work at molecular level?

(3) Is there an association between cell sources (fibroblasts from skin, stem cells from bone marrow, umbilical cord blood, and adipose tissue) and the self-renewal capacity?

(4) Is there an association between sex of the patients and self-renewal capacity?

(5) Do pediatric patients have more success than adult patients in the reprogramming therapy?

(6) What kind of tissues can make the introduction of the hiPSCs easier?

(7) Which methods used to generate the hiPSCs are better related with safety?

(8) How can we compare different diseases and the use of the iPSCs?

There are several tests commonly used in the medical literature; they are presented in Table 4. When we use such tests, we compute a P value. The P value is the probability of obtaining a result as extreme as or more extreme than the sample value, assuming that the null hypothesis is true. The sample value is calculated. Depending on the test we use, there is a specific formula to calculate the sample value. Appropriate computer software can do such a calculation.

In many situations, populations are so large that it is impossible to describe their central tendency and dispersion by studying 100% of their members, or by studying a sufficiently large portion of population to justify treating sample statistics as population parameters. In other situations, clinicians may study a new phenomenon with little basis to determine a population parameter. In these cases, we use estimates. Two types of estimates of a population parameter can be used: a point estimate and an interval estimate. A point estimate is a single numerical value of a sample statistic used to estimate the corresponding population parameter. Point estimates are not used widely because, in general, values of some statistic can vary from sample to sample. So, an interval estimate is typically used. Interval estimates are also called confidence intervals. Confidence intervals provide more information on, for example, the mean of a variable in the considered population than just the sample mean. When the sample mean is calculated, we know that there is a sample to sample variation, that is, if another sample was selected (i.e., if other patients were selected), the sample mean would rarely be the same. Thus, the confidence interval provides a set built in such a way that if a large number of different samples were selected and we built confidence intervals for all of them, the value of the population mean would be contained in 95% of the intervals. In this case, we have a 95% confidence interval. In general, researchers use 95% confidence intervals, but 99% is also a very used.

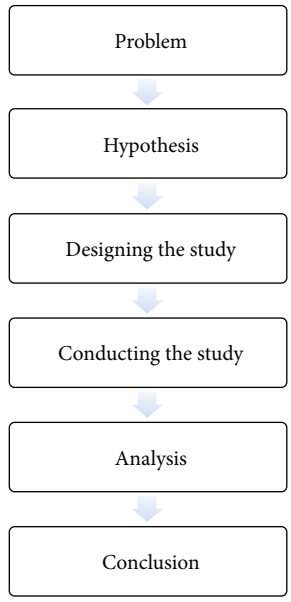

Figure 2: Structure of a clinical study.

We finish by saying that probability models are important tools that *can help* making decisions and must be used if the numerical outcomes are clinically meaningful. Accumulated experience and specific knowledge must be combined with numerical results to assess the usefulness of a medical decision.

5. Conclusions

An important point in the research using hiPSCs is that these cells do not present the ethical dilemmas as the research using

Table 4: Statistical tests usually used in the medical literature.

To test the statistical significance of the difference between...		
Two or more proportions	Chi-square	Nonparametric
Two proportions	Fisher's exact	Parametric
Two medians	Mann-Whitney	Nonparametric
Two means	*t*-Student	Parametric
More than two means	Kruskal-Wallis (one-factor)	Nonparametric
Two or more than two variances	Bartlett	Parametric
More than two means	ANOVA (one-factor)	Parametric
More than two means	ANOVA (more-factors)	Parametric
To test the correlation between two variables	Spearman's rank correlation test	Nonparametric
To test the correlation between two variables	Pearson's correlation test	Parametric

Adapted from: Fernandez et al. 2012 [65].

the ESCs. Since the first description of iPSCs generation, there has been a great improvement in the methods to generate these cells. The main problem with using these cells is the possibility of developing tumors. However, basic research should aim at the improvement of methods to generate the iPSCs. It is also very important to obtain a characterization of these cells at cytogenetic and molecular levels, in order to understand how reprogramming works in signaling pathways. The different sources of cells to generate iPSCs may be compared. Many technical and basic knowledge are necessary before the use of iPSCs in the clinical practice. The possibility to induce pluripotency in somatic cells or even further to induce cell transdifferentiation through the forced expression of reprogramming factors has offered a new field for cancer research and future possible applications in the clinical practice. The recent findings regarding the use of iPSCs for modeling different types of cancer like solid tumors and hematological malignancies represent an ideal tool to study the multiple stages of cancer, for the discovery of new drugs designed for specific biomarkers and for testing drugs' toxicity. Another important point is the possibility to use the iPSCs for immunotherapy in cancer. So, the use of hiPSCs may contribute to the development of future personalized cell therapies and open new possibilities for the treatment of cancer patients.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Biomedical and Clinical Promises of Human Pluripotent Stem Cells for Neurological Disorders

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Neurological disorders are characterized by the chronic and progressive loss of neuronal structures and functions. There is a variability of the onsets and causes of clinical manifestations. Cell therapy has brought a new concept to overcome brain diseases, but the advancement of this therapy is limited by the demands of specialized neurons. Human pluripotent stem cells (hPSCs) have been promised as a renewable resource for generating human neurons for both laboratory and clinical purposes. By the modulations of appropriate signalling pathways, desired neuron subtypes can be obtained, and induced pluripotent stem cells (iPSCs) provide genetically matched neurons for treating patients. These hPSC-derived neurons can also be used for disease modeling and drug screening. Since the most urgent problem today in transplantation is the lack of suitable donor organs and tissues, the derivation of neural progenitor cells from hPSCs has opened a new avenue for regenerative medicine. In this review, we summarize the recent reports that show how to generate neural derivatives from hPSCs, and discuss the current evidence of using these cells in animal studies. We also highlight the possibilities and concerns of translating these hPSC-derived neurons for biomedical and clinical uses in order to fight against neurological disorders.

1. Introduction

Neurological disorders include a variety of hereditary and sporadic diseases that involve the chronic and progressive loss of neuronal structures and functions. We can divide these into two major groups depending on the onset of disease, which are (1) early onset neurodevelopment diseases and (2) late onset neurodegenerative diseases. Since aging is the most consistent risk factor for neurodegenerative diseases and we are now experiencing an increase in the numbers of the elderly population, it is of great importance to develop the treatments for these diseases. Although the rapid development of novel diagnostic methods and therapeutic approaches are in the stages of development, there is limited evidence of effective systems that can prevent and cure the diseases. Cell replacement therapy by using stem cells is a promising strategy to treat these diseases because certain pathological conditions are affected by neuronal loss. The possibility of generating abundant differentiated cells from human stem cells for cell replacement therapies provides a plausible avenue to treat such diseases. Neural stem cells that are isolated from adult donor or from fetal brain tissues have been considered as reasonable resources for this purpose; however, adult stem cells are of a very limited quantity, and histocompatibility is a major drawback.

Human pluripotent stem cell (hPSC) technologies, including human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs), are potentially prominent processes for manipulating human illnesses in terms of disease modeling, tissue engineering, drug discovery, and cell therapy. The pluripotent developmental potential of hPSCs and the success of transplanting their differentiated derivatives in animal disease models demonstrated the principle of using hPSC-derived cells as a regenerative source for transplantation therapies for human diseases. However, before hPSCs can be translated into clinical use, a thorough

understanding of the basis of hPSCs is mandatory. One of the major concerns that hinders the application of hPSCs for cell and tissue therapy in human is histocompatibility. Notably, recent data support the concept that hESCs and their differentiated derivatives possess immune-privileged properties [1], suggesting that cells derived from hESCs may provide a potential tool for the induction of immunotolerance [2]. In another scenario, for which the term "personalized pluripotent cells" has been coined, people could use their own somatic cells to be reprogrammed back to the pluripotent cell state [3]. The feasibility of reprogramming was first demonstrated by somatic cell nuclear transfer (SCNT) or cloning [4]. Somatic cells of patients are fused with enucleated oocytes; thereafter, hESCs could be established in culture and be induced to *in vitro* differentiation to provide patient-specific cells and tissues [5]. However, the reprogramming of the somatic nucleus in an oocyte is still inefficient. In addition, accessing a source of human oocytes is not only a rare opportunity but it is also the ethical concern of the moment [6]. As an alternative to reprogramming by SCNT, adult human fibroblasts can be directly reprogrammed into a state that is similar to that of hESCs by the expression of only four factors: OCT4, KLF4, SOX2, and c-Myc [7] and such reprogrammed cells are termed as "induced pluripotent stem cells" or iPSCs.

Since hPSCs have great potential to differentiate into all cell types, the most common strategy in salvaging the neural function after injuries is not the direct transplantation of these pluripotent cells but applying their various differentiated cells instead. Specification of hPSCs *in vitro* along neural progenitor pathways would also allow the investigation of early human brain development, including regulatory signals for cell commitment and neurogenesis. Additionally, the cells could be used for the screening for new drugs and carcinogenic or toxic compounds that cannot be analyzed *in vivo* due to limited samples and ethical constraints. Ultimately, the potential utility of hPSC-derived neurons for treating neurological diseases is just at the beginning because there are several issues that need to be taken into account, that is, efficiency, safety, and functionality. In this paper, we review the promising benefits of hPSCs for the analysis and treatment of neurological disorders.

2. Approaches for the Derivation of Neural Derivatives from Human Pluripotent Stem Cells

The transplantation of stem cells which are committed to a specific lineage can avoid *in vivo* teratoma formation which is caused by the rapid growth and uncontrolled spontaneous differentiation of hPSCs [8]. Nevertheless, an efficient differentiation of hPSCs into clinically appropriate progenitors or specific targeting cell types remains a key challenge. The differentiation studies of hPSCs have intensively focused on exploring the roles of growth factors and small molecules [9–12]. Several approaches have been used to achieve *in vitro* neural differentiation from hPSCs, aimed at generating regionally specified neural progenitors or differentiated neurons/glial subtypes [13]. Reportedly, hPSCs have been differentiated successfully into several types of neural derivatives, including neural progenitor cells [11, 12, 14–20], neural crest progenitors [21], motor neurons [22–25], sensory neurons [9], dopaminergic neurons [26, 27], and specific glial subtypes [28]. The differentiation systems were initially achieved through cell aggregation or embryoid body (EB) formation in the presence of retinoic acid (RA) as a starting point for the isolation and culture of highly purified populations of neural progenitor cells [16]. These progenitors were able to be cultivated for about 25 population doublings as neurospheres in suspension culture, and they express markers of the early neuroectoderm, such as Nestin, polysialylated (PSA) NCAM, Musashi1, and PAX6 [16]. The neural progenitor cells were able to be differentiated into neurons, astrocytes, and to a minor degree of cells expressing oligodendrocyte markers. However, as hPSCs are pluripotent, the efficiency of neural conversion is limited and lineage selection is usually needed to ensure the enrichment of a specific differentiated population. Most of the lineage induction protocols employed the addition of morphogens or growth factors to the hESC aggregates in suspension cultures. For this reason, EB formation has some drawbacks. Because a high concentration of morphogens or growth factors is required in order for the factors to reach cells inside the aggregates [29], cells on the surface and those inside the aggregates will present a varied degree of exposure to morphogens, which create a wide range of cell lineages or cells at various developmental stages. In addition, it is impossible to visualize the continual change of cell morphology in response to treatment because of the cluster nature.

To overcome these drawbacks, a simpler way to reconstitute neural commitment *in vitro* and achieve efficient neural production relies upon monolayer differentiation of hPSCs. However, when applying a similar monolayer differentiation system used for directing mouse embryonic stem cells (mESCs) to neural lineage, hPSCs generated a large proportion of nonneural lineage cells. This is mainly due to the active BMP signaling pathway in hPSCs [30]. Therefore, the only approach that has been shown to induce efficient hESC neural differentiation is by directly inhibiting the BMP and/or SMAD signaling pathways [12, 31, 32]. Treatment of hPSCs with noggin, a BMP antagonist, generated a homogeneous, morphologically distinct population of cells that expressed neuroectodermal markers, including PAX6, Musashi1, and SOX2 and with no detection of mesoderm and endoderm lineage markers [12]. Noggin alone appears to, at least, initiate hPSCs differentiation toward neural lineage. The formation of "neural rosettes" is another morphological marker of *in vitro* differentiation of hESCs to neural cells which is reminiscent of the *in vivo* structural formation of a developing neural tube [33]. The culture of hPSCs in chemically defined medium with noggin resulted in $PAX6^+/SOX1^-$ neural rosettes, and the additional supplementation of fibroblast growth factor 2 (FGF2) induced $PAX6^+/SOX1^+$ neural rosettes [34]. Rosette-forming neural stem cells expressing anterior markers of the nervous system, such as Forse1, have shown the broadest differentiation potential [35]. These cells propagated in the presence of FGF2 and retained Forse1 expression, even though

FGF2 was considered to caudalize the cell fate of neural stem cells [36]. Moreover, the cells in neural rosettes are capable of multiplying by symmetrical division over a period of time and are able to differentiate cell types of both anterior-posterior and central-peripheral types of the nervous system and could be maintained in a long-term culture by stimulating Sonic hedgehog (Shh) and Notch pathways [35]. Neural progenitor cells derived from hPSCs are also capable of producing astrocytes and oligodendrocytes, either under basal conditions or with the medium supplemented with ciliary neurotrophic factor or platelet-derived growth factor (PDGF) [37]. Mature astrocytes express specific astroglial markers such as glial fibrillary acidic protein (GFAP) and S100β. However, the proportion of oligodendrocytes differentiating from hPSCs among the differentiated progeny is generally very low [17]. It has been shown that Olig2$^+$ neural progenitors can be readily differentiated from hESCs in response to SHH and RA [38]. These Olig2$^+$ progenitors generate mostly motor neurons during the neurogenic period. Moreover, Olig2$^+$ progenitors persist after neurogenesis and become oligodendrocytes. This suggests that the Olig2$^+$ progenitors may differentiate into oligodendrocytes, and highlights the importance of Olig2 in oligodendrocyte development [39]. It is noted that during embryonic neurodevelopment, glial cells, for example, astrocytes and oligodendrocytes are generated after the birth of major neuronal types [40]. The same neurogenesis to gliogenesis transition is preserved when neuroectodermal cells are cultured [41] or hPSCs are differentiated along the neural lineage [42]. The noteworthy temporal sequence of neuronal and glial differentiation corresponds to the timeline observed from limited samples of fetal tissues [42].

It is suggested that the intrinsic program governing neuronal and glial lineage development is retrained *in vitro* and highlights the feasibility of obtaining specific neuronal subtypes from hPSCs when appropriate inducers are applied at an optimal time point. In addition to extrinsic factors, forced expression of specific transcription factors, for example, *NGN2* and PAX6, could speed and enrich the generation of neural lineage from hPSCs [43–45]. However, this transcription factor-based approach is needed for the development of an exogenous DNA-free system prior to translating it to patients.

3. Human Pluripotent Stem Cells Accelerate Biomedical Research for Neurological Disorders

Understanding the molecular interactions underlying developmental disorders of the brain is hindered by limited accessibility to early embryos and an inadequate amount of stage- and cell type-specific materials. Although these developmental diseases are unlikely to be cured by cell replacement therapies, a complete picture of disease pathology by disease modeling will facilitate the discovery of effective compounds that can improve patient conditions [46]. Recent results indicate that the differentiation of hPSCs in culture follows the hierarchical set of signals that regulate embryonic development in the generation of the germ layers and specific cell types [47]. Establishment of *in vitro* differentiation models that recapitulate brain development will form the foundation for dissecting molecular interactions. The ability to access and manipulate populations representing early neural developmental stages in the hPSC differentiation cultures provides a new approach for addressing the questions of lineage commitment.

The investigation of neural induction paradigms in hPSCs has significant implications for the insights into early human brain development. In recent years, more sophisticated and chemically defined culture systems have been developed. Anti-BMP signaling is thought to play a crucial role in neural induction [12, 31]. Further studies found that the high efficiency of neural induction with BMP antagonist treatment is consistent with its role in the default model of neural induction [48]. In addition to the shared signaling pathways, temporal consequences are similar between *in vivo* and *in vitro* systems. For example, during *in vivo* development, the neural tube formation completes when the human embryo is approximately 3 weeks old. On the other hand, *in vitro* differentiation of hPSCs toward neural lineage is characterized by the formation of a neural rosette observed at about days 15–17 of differentiation [12, 17], reminiscent of the transverse-section of the neural tube. It can be speculated that *in vivo* development events in terms of spatial and temporal changes are grossly recapitulated during the *in vitro* formation of neural rosettes. One of the characteristic features of neural progenitor cells is the positional identity they acquire during neural induction and patterning, which plays a key role in the fate specification of neuronal subtypes. The positional information is imparted upon neural progenitor cells via morphogenetic gradients secreted by surrounding tissues. Partially, to mimic the positional information in a culture petri dish, morphogens that affect rostrocaudal and dorsoventral fate choices are applied together or in sequence. Applying FGF8, which influences midhindbrain fate, and Shh, a ventralizing molecule, further induces hPSC-derived neuroepithelial cells into midbrain dopaminergic neurons [49]. On the other hand, the inhibition of Wnt signaling, together with the activation of SHH signaling, enhances forebrain induction from hPSCs [50]. Absence or presence of these positional morphogens in the *in vitro* differentiation system leads to the production of a variety of neuronal subtypes. This would mean that the addition of morphogens or small molecules at a specific time and space is needed to pattern neural progenitor cells into a particular neuronal subtype, and the foundation of this knowledge is originally based on the current understanding of neurodevelopment. The refinement of signaling pathways that control specific neuronal subtype specification *in vitro* will lay the foundation for studying affected neuron pathology in human neurological diseases.

In addition to modeling early brain development, iPSCs are a powerful tool for modeling diseases [46]. The idea of disease modeling is to derive iPSCs from the patient's own cells and then induce them into the pathogenic cell types *in vitro*. Several laboratories have already established hiPSCs from patients suffering from Parkinson's disease, amyotrophic lateral sclerosis, and spinal muscular dystrophy

[51, 52]. Upcoming sophisticated differentiation and purification protocols would be necessary to generate cells that show comparable physiological conditions to disease stages. Moreover, hPSCs and their differentiated derivatives could be applied to chemical compound screening assays for the development of new potential pharmaceuticals and toxic or mutagenic reagents [53]. While primary cell cultures or established cell lines are commonly used for both purposes, hPSCs offer several advantages. The developmental equivalence of hPSC-derived and embryonic populations provide a more rigorous system for evaluating the teratogenic and embryotoxic effects of a substance, in addition to general mutagenic and cytotoxic effects [54]. A protocol based on the differentiation of hPSCs has been established and validated for use in toxicity testing [55]. Additionally, genetic modification enables the tailoring of hPSC lines for specific purposes. For example, specific genes can be altered to increase sensitivity to mutagens or drugs [56, 57], or tissue-specific reporter genes can be introduced to detect changes in gene expression induced by toxic chemicals or therapeutic agents [58].

4. Therapeutic Promises of Human Pluripotent Stem Cells for Neurological Disorders: Perspectives of Animal Studies

Neurological disorders are the complex disintegration of neurons as well as many types of neuroglia in the brain and/or spinal cord. The early phase therapy, such as applying trypsin plasminogen activators (tPAs) in stroke patients during the first few hours, can be used only in early diagnosed patients [59]. Nevertheless, there is still no effective treatment, which can ameliorate that the functional deficit exists in the human subjects. Several researchers established the protocols to generate neural progenitor/stem cells, motor neurons, oligodendrocyte progenitor cells *in vitro* and then transplanted these cells into various animal models in order to verify the ability to restore neuronal functions *in vivo*. There are promising evidences of differentiation, maturation, and integration of the grafted cells into the endogenous neural circuitry in animal models [60, 61]. The hPSC-derived cells, which were introduced into the animal models, were restricted to one specific cell lineage in order to reduce the risk of tumorigenesis when compared with the direct transplantation of hPSCs [62]. Experimental studies in animal models are necessary to extrapolate the therapeutic effects of transplanted cells. Currently, there are two main strategies for assessing the efficacy of hPSCs for treating neurological disorders in animal models. First, hPSC-derived neurons and/or neuroglia were substituted into mice with injured neural circuitry by using intracerebral transplantation. Second, hPSCs were delivered systemically or locally into the brain where they might act through some other mechanisms to promote the differentiation, such as immunomodulation, neuroprotection, and stimulation of angiogenesis.

The neural derivatives of hPSCs are plausible sources for cell replacement therapies. Several diseases were already experimented in an animal setting, such as stroke and brain ischemia [63–66], spinal cord injury [23, 67, 68], Parkinson's disease [69], spinal muscular atrophy, amyotrophic lateral sclerosis [24, 70], and demyelinating diseases [71]. These provisional studies were performed in specific diseases environment in order to endow a prospect of pathological conditions, in which the results could be implemented for clinical interpretations. In this section, we discuss some prominent examples of neurological disorders that have been conducted in animal transplantation studies using hPSCs.

4.1. Stroke. The vast majority of neurological disorders falls into the group of devastating pathology and takes place in the cerebral arteries which are called *cerebrovascular accident* (CVA) or *stroke* [72]. Stroke is an overwhelming condition with lifelong functional deficits in patients due to tremendous loss of neuronal circuitry in the brain. The recovery of stroke patients is often incomplete even when they have received physical therapy training to promote functional recovery. Consequently, there is great enthusiasm for using cell therapy to restore and replenish dead cells and tissues after brain injury an expectation of functional improvements. Stroke is typically a consequence of a thrombotic or embolic occlusion in a major cerebral artery, most often the middle cerebral artery (MCA). Experimental focal cerebral ischemia models have been established to imitate human stroke and serve as an indispensable tool in the field of stroke research [73]. Models of cerebral ischemia can either be artery or vein occlusion via mechanics or thromboembolisms. An ischemic model is categorized into global and focal ischemia. Global ischemia is the restriction of blood flow, affecting the entire brain area, whereas focal ischemia is characterized by a reduction of cerebral blood flow in a distinct region of the brain. The global ischemia can be further divided into complete and incomplete types, while focal ischemia can be performed in both focal and multifocal cerebral ischemia [74]. Moreover, cerebral vessel occlusion can occur either in the proximal middle cerebral artery (pMCAo) (large vessel occlusion) or distal MCA (dMCAo) (small vessel occlusion). Thrombotic occlusion can be induced either via the injection of blood clots or thrombin into the MCA or by photothrombosis after intravenous injection of Rose Bengal [75]. Recently, there has been evidence in hPSCs transplantation, which focused on cortical injury. The studies were performed by dMCAo rather than transient MCAo models, which turned to both cortical and striatal injury [63, 64]. These studies showed a substantially decreased infarct volume after the transplantation of neural progenitor cells derived from hPSCs. Nevertheless, until now, experimental stroke studies in transgenic animal models have had limited success. This highlights the significant contribution of vascular risk factors found in certain clinical situations, which is also viable in the human systems [75, 76].

4.2. Spinal Cord Injury. Spinal cord injury (SCI) is another important neurological disorder which can be used to reveal the therapeutic effects of transplanted cells in animal studies. SCI causes permanent paralysis in patients due to the low rate of regeneration in the central nervous system (CNS). Robust cell death in the injured region happens from seconds to weeks after SCI, which results in the formation of the

cavities or cysts that block the ascending and descending neurotransmission. This phenomenon occurs immediately after SCI, including neuronal fiber damage, mass ischemic neural cell necrosis and apoptosis, and glial scar formation, and leads to extensive secondary tissue injuries. SCI is a devastating condition, characterized by the disruption of axonal connections, failure of axonal regeneration, and loss of motor and sensory function. The therapeutic promise of stem cells has been focused on cell replacement, but many obstacles remain, in particular neuronal integration following transplantation into the injured CNS. Various cell types have been selected based on their ability to form myelin protein, promote and guide axonal growth, and bridge the site of injury. In addition, transplanted cells also secrete trophic factors, which may have neuroprotective effects and/or promote plasticity in the spared spinal cord. Therefore, the advantageous effects of these cellular therapies are multifactorial and often difficult to attribute to one single mechanism. Most cell transplantations are delivered directly into the site of injury or adjacent area by injecting a few microliters of cell suspension via fine needles or glass capillaries. Attempts have been made to deliver cell substrates to the injured cord via intrathecal injection [77–79] or even systemically via intravenous infusions. Rodent models of SCI are used, and the transplantation is typically performed 1-2 weeks after the injury and referred to "subacute" treatment, since transplantations performed immediately after "acute" injury generally yield poor results due to the robust inflammatory response initiated at the time of injury [80]. In order to promote functional recovery, stem cell transplantation must be done after inflammatory responses. The optimal time-point for cell therapy contains several benefits, for instance, the inhibition of neuronal apoptosis and necrosis, the enhancement of neuronal regeneration, and the promotion of axon regeneration and remyelination; therefore, understanding the timeline of secondary damage cascades is definitely critical [81]. Reportedly, hPSCs have been investigated and their therapeutic efficacy and safety for SCI *in vivo* have been verified [23, 68]. hESCs were differentiated into motor neuron progenitors (MPs) and oligodendrocyte progenitor cells (OPCs). The functional recovery was compared after transplanted either MPs or OPC alone, or the combination of MPs and OPCs into the SCI mice. The functional locomotor recovery of transplanted animals with MPs and OPCs showed significant improvement of the hind limb, better than the groups that were treated with a single cell type [23]. A protocol was recently developed for the creation of long-term self-renewing neuroepithelial-like stem (lt-NES) cells from hPSCs [68]. These hPSC-lt-NES cells exhibit reliable characteristics, including homogenous population, continuous expandability, stable neuronal/glial differentiation ability, and the capacity to generate functional mature neurons in a monolayer platform [68]. Promisingly, when transplanted into the SCI model (NOD-SCID mice), these transplanted cells have a comparable therapeutic potential, similar to neural stem cells (NSCs) derived from human fetal spinal cord (hsp-NSCs).

4.3. Parkinson's Disease. The key pathology of Parkinson's disease (PD) is motor symptoms. This is due to the progressive degeneration of mesencephalic dopaminergic (DA) neurons that project to the striatum and subsequent reductions in striatal dopamine levels. Initial pharmacological treatment with L-dihydroxyphenylalanine (L-DOPA) can ameliorate symptoms, but effectiveness of this compound gradually decreases overtime. The progression of motor deficits then requires additional treatments, including deep brain stimulation. The breakthrough lines of evidence of promising stem cell research are appealing as an alternative choice to fight against the disease. In the laboratory, PD animals can be induced by systemic injection of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [69] or the central administration of the neurotoxin, 6-hydroxydopamine injection [82]. MPTP treatment is currently a gold standard for generating PD animal models. Ingestion of this compound results in the fabrication of motor deficits that are similar to PD. MPTP subsequently converts to MPP^+ in the brain, which is later reuptaken into DA neurons by the dopamine transporter (DAT). MPP^+ exerts a blockage on the electron transport chain in the mitochondria of DA neurons and generates reactive oxygen species (ROS), which effectively kill the neurons [83]. There is still controversy as to whether MPTP-treated mice contained Lewy body-like inclusions, a hallmark of PD. Only a few inclusions were found after 3 weeks of chronic MPTP treatment. By 24 weeks, several of the remaining tyrosine hydroxylase (TH) positive neurons contained α-synuclein and ubiquitin-immunoreactive inclusions [84]. On the other hand, 6-hydroxydopamine (6-OHDA) is preferentially transported into DA neurons by the DAT, where it gets accumulated and produces ROS. This toxin is needed for the administration via intraparenchymal injection because it does not cross the blood-brain barrier. Unilateral administration results in asymmetric circling behaviour, which is suitable for the evaluation of therapeutic interventions [85]. The preclinical study using MPTP-treated monkey models showed the therapeutic effects of transplanted cells at different stages, undifferentiated hESCs, and hESC-derived neural progenitor cells. As expected, the transplantation of undifferentiated hESCs at day 14 (before SHH and FGF8 induction) showed tumor formation. To induce neural progenitor cells for the transplantation, hESCs were induced with SHH for either 35 or 42 days prior to the addition of FGF8, BDNF, and GDNF for another 1-2 weeks. The grafts were well demarcated and showed no malignancy or even teratoma. The transplanted cells were mainly contributed to immature neural cells and DA neurons. The evaluation of functional neurological scores revealed that monkeys implanted with day 42 neurospheres had significant behavioural improvement over another group. The key statement of this finding was to support a prolonged differentiation maturation of neural progenitor cells that led to a favourable result regarding reduced tumor growth and functional grafts [69].

4.4. Motor Neuron Diseases. Recent progress in cell-based modeling using hPSC-derived motor neurons (MNs) has opened a new window to understanding the pathological

development of motor neuron diseases. MNs exclusively reside within the ventral horn of the spinal cord and project axons to muscles to control their activity in organized and discrete patterns as the lowest unit in the hierarchy of the motor pathway. The most remarkable MN diseases are spinal muscular atrophy (SMA) and amyotrophic lateral sclerosis (ALS). SMA is characterized by severe muscle weakness, symmetrical proximal muscle weakness, lack of motor development, and hypotonia [86]. SMA is the cascade of genetic deficits, resulting in the decrease of survival of motor neuron (SMN) protein levels. The transgenic model of SMN gene deletion has yielded important insights into the pathogenesis of the disease. ALS, also known as Lou Gehrig's disease, is a devastating adult-onset neurodegenerative disorder, characterized by a progressive loss of both cortical and spinal motor neurons. The clinical manifestations of ALS are progressive myasthenia and general amyotrophy, eventually resulting in paralysis and death [87]. Transgenic mice that overexpress mutant human superoxide dismutase 1 (*SOD1*) gene reproduce clinical and histopathological features of human ALS. This animal model is of interest for the investigation of ALS pathogenesis and for the testing of therapeutic approaches [88]. Stem cell-based therapies have demonstrated therapeutic potential in SMA and ALS. For example, MNs derived from hiPSCs, which were obtained from an SMA patient, exhibited shortened neurite extensions and diminished survival in culture compared to healthy MNs [89]. Conversely, ALS patient-derived MNs did not present any defect [52]. MNs derived from hESCs proved their therapeutic capacity via *in ovo* and *in vivo* transplantation of the spinal cord [24]. The results revealed that MNs can survive for at least 6 weeks in the rat spinal cord and also outgrow the axons toward peripheral targets. This study provided strong promise for future applications in preclinical models and translational applications of hESC-derived MNs. It will be essential to develop animal models for specific conditions of MN diseases to address the question of whether hESC-derived MNs can survive and function in a particular MN disease environment.

4.5. Multiple Sclerosis. Multiple sclerosis (MS) is an autoimmune-mediated inflammatory disease. It is characterized by multifocal regions of inflammation and myelin devastation, which leads to demyelination and neuronal loss. MS is presented by multiple signs and symptoms, with relapses and remissions of the disease stages. Even though there are several approved treatments for MS, many patients do not optimally respond to those approaches. A number of laboratory animals are described as demyelinating disease models. Experimental autoimmune encephalomyelitis (EAE) is one of the most commonly characterized disease and is employed as an animal model for MS [90]. EAE mice are a model of CNS autoimmune disease that follows immunization with certain CNS antigens and subsequent administration of heat-inactivated Mycobacterium tuberculosis and pertussis toxin. Induced animals will develop a strong immune response with signs of inflammation, demyelination, axonal loss, and gliosis, which is similar to MS pathology in humans. Besides EAE, it is previously reported that an MS model could be induced by viral infection [71]. A number of human viral pathogens have been considered to be involved in eliciting myelin-reactive lymphocytes and/or antibodies that subsequently infiltrate the CNS and damage the myelin sheath [91, 92]. Mouse hepatitis virus (MHV) infection results in an acute encephalomyelitis, followed by chronic demyelination in animals. This observation is similar to clinical and histologic profiles of MS patients. Human oligodendrocyte progenitor cells (OPCs) derived from hESCs transplanted in the MS model have been shown to promote remyelination in mice that are persistently infected with MHV [71]. The ability of preclinically applicable cells to facilitate remyelination in an animal model of MS would be a crucial step towards developing novel therapies.

5. Safety Considerations of Using hPSC-Derived Neurons

As mentioned, hPSCs offer a possible unlimited supply of disease-specific progenitor cells for regenerative medicine. The selected cell types can be variable, according to material sources, culture conditions, and differentiation protocols. These issues are important and need to be considered prior to translating preclinical outcomes into clinical studies. Undefined biological supplements, which are used for cell sustenance in the processes of isolation, expansion, and differentiation of hPSCs, may cause undesired problems in patients. For example, the maintenance of the hPSCs in growth-arrested mouse embryonic fibroblasts (MEFs) may worsen therapeutic potential due to the transmission of xenopathogens, altering the genetic background, and promoting the expression of immunogenic proteins in hPSCs [93]. Recently, there has been evidence of using xenofree iPSC-derived neural progenitor cells transplanted into ischemic stroke models [65]. This study represented the success of the derivation iPSCs in feeder- and serum-free systems. The cells could still be differentiated into functional neurons, which are transplantable into stroke animals.

Another challenge for the clinical considerations of hPSCs is how to efficiently induce and enrich pluripotent cells for a desired phenotype. In order to select desired cell types, certain approaches have been applied, such as antibody-based selection for specific surface antigens by FACS sorting. However, these approaches need to be improved in order to produce a large and viable population. To avoid tumor formation in the engrafted tissues, the pluripotent state of differentiated hPSCs needs to be verified to confirm no existing contamination of pluripotent cells among differentiated cells. Directing the pluripotent cells into the multipotent NSCs appears to be necessary for safety considerations and effectiveness in clinical translation. The precise stages of differentiation for transplantation remain unclear. The gene and epigenetic profiles are needed to validate reliable cell types before transplantation [94, 95]. Therefore, safe and effective clinical translation of hPSCs for neurological disorders is required for thoroughly understanding of both inherent and noninherent cellular mechanisms that maintain pluripotency and differentiation programs.

6. Future Challenges of Neural Derivatives for Biomedical Research and Clinical Applications

Based on the principle of developmental biology, a set of neurons and glia has been successfully differentiated from hPSCs. Modeling neurological diseases using hPSCs has the potential to provide a valuable impact on biomedical research and regenerative medicine. The possible risks are that the high variability in the protocols generates specific neuron subtypes and the ability to mimic disease-specific phenotypes. Manipulation of culture environment, such as oxidative stress, may enhance pathological phenotypes of neural derivatives derived from diseased iPSCs [96]. Although hPSC-derived neural derivatives were proved to be functional *in vitro* and were able to correct phenotypes of diseased mice, there are still several issues remaining to be solved prior to realizing clinical translation, for example, the purity of transplanting cells, sites of transplantation, graft versus host diseases, tumorigenesis, and integration of transplanted cells. The development of safer iPSCs is necessary to avoid exogenous DNA integration that can subsequently affect genomic alterations and cause tumor. Finding specific biomarkers for cell sorting may provide a solution to the selection of desired cell types for transplantation. In principle, patient-specific iPSCs should provide immunogenically matched tissues; however, further validation is still needed for safety issues in order to avoid any possible tissue rejections [97, 98].

Another key advantage of hiPSCs over the current transplantation approaches is the possibility of correcting mutations by homologous recombination technology. The generation of genetically corrected iPSCs by genome-editing technology is a mainstay for the advancement of iPSC technology to generate healthy iPSC lines for individual patients [99, 100]. The differentiation of specific neural derivatives from genetically corrected iPSCs could provide a source of neurons for therapeutic transplantation. Besides, several neurological disorders are noncell autonomous, and neuronal death is driven by factors in the cellular environment, such as oxidative stress and inflammatory cytokines. The transplantation of nonneuronal cells, for example, astrocytes and oligodendrocytes, to refine the microenvironment conditions is thus a practical strategy. Another interesting concept in stem cell therapy is the dual effects of stem cell transplantation together with noninherent effects, such as rehabilitation or exercise. Since the majority of neurological disorder patients receive physiotherapy training, the combination of two approaches could enhance the therapeutic outcomes. To this end, the concerted efforts on hPSC research have made great progress toward cell replacement therapies; however, it is important to support the most carefully designed clinical studies for the best safety for patients in the future.

Conflict of Interests

The authors declare that they have no conflicts of interests.

Authors' Contribution

Nopporn Jongkamonwiwat and Parinya Noisa equally contributed to this work.

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Review Article

Stem Cell Therapy in Bladder Dysfunction: Where Are We? And Where Do We Have to Go?

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To date, stem cell therapy for the bladder has been conducted mainly on an experimental basis in the areas of bladder dysfunction. The therapeutic efficacy of stem cells was originally thought to be derived from their ability to differentiate into various cell types. Studies about stem cell therapy for bladder dysfunction have been limited to an experimental basis and have been less focused than bladder regeneration. Bladder dysfunction was listed in MESH as "urinary bladder neck obstruction", "urinary bladder, overactive", and "urinary bladder, neurogenic". Using those keywords, several articles were searched and studied. The bladder dysfunction model includes bladder outlet obstruction, cryoinjured, diabetes, ischemia, and spinal cord injury. Adipose derived stem cells (ADSCs), bone marrow stem cells (BMSCs), and skeletal muscle derived stem cells (SkMSCs) are used for transplantation to treat bladder dysfunction. The main mechanisms of stem cells to reconstitute or restore bladder dysfunction are migration, differentiation, and paracrine effects. The aim of this study is to review the stem cell therapy for bladder dysfunction and to provide the status of stem cell therapy for bladder dysfunction.

1. Introduction

Although numerous treatments for bladder dysfunction including bladder overactivity or underactivity have previously been developed, the improvement in voiding dysfunction is not been fully achieved. Stem cells are defined as cells with an ability to propagate themselves through self-renewal and generate mature cells of multiple lineages through differentiation [1]. Given their unique abilities of site, specific migration, plasticity and potential for tissue repair or regeneration, stem cells and their relationship to repair injury or damage in various organ systems have recent interest.

For the bladder dysfunction, bladder outlet obstruction (BOO) is a well-known and well-established bladder dysfunction model. Other bladder dysfunction models are still at an immature state. Medical and surgical efforts to treat and prevent BOO are ongoing, as are studies to better understand the effects and clear mechanisms of BOO at a cellular level.

Mesenchymal stem cells (MSCs) augment healing through cell replacement and stimulation of cell proliferation and angiogenesis. While numerous reports have shown the ability of MSCs to engraft tissues such as lung, liver, heart, and brain, data is still scarce about the repair of bladder dysfunction [2–4].

The aim of this review is to provide the current status of stem cell therapy for bladder dysfunction and also to discuss future prospective on this issue.

2. Stem Cells for Treatment of Bladder Dysfunction

MSCs are self-renewing cells with pluripotent capacity to differentiate into various cell types including osteoblasts, chondrocytes, myocytes, adipocytes, and neurons [5].

While all MSCs including bone marrow-derived stem cells (BM-MSCs), skeletal muscle-derived stem cells (SkMSCs), and adipose tissue-derived stem cells (ADSCs) exhibit similar biological properties and therapeutic capabilities, their availability and scalability differ greatly according to therapeutic purpose. For example, while SkMSCs require a

long expansion time with complicated isolation procedure, ADSCs can be prepared within hours.

ADSCs are mesenchymal stromal cells found in the perivascular space of adipose tissue. ADSCs have the advantage of abundance and easy access when compared with other stem cell types [6]. ADSCs express common stem cell surface markers, genes, and differentiation potentials as MSCs [7]. ADSCs have demonstrated efficacy in experimental studies of urologic conditions [8, 9].

SkMSCs are used mainly in artificial injured model including pelvic nerve injury [10, 11]. As a stem cell source of autologous transplantation, SkMSCs have several advantages because skeletal muscle is the largest organ in the body and can be obtained relatively easily and safely. One of other advantages of SkMSCs is that they can be harvested easily during surgery.

Cells in the $CD34^+/CD45^-$ fraction (Sk-34 cells) and $CD3^-/CD45^-$ fraction (Sk-DN cells) are able to synchronously reconstitute nerve-muscle blood vessel units after transplantation. Transplantation of SkMSCs causes significant functional recovery through cellular differentiation into skeletal muscle cells, vascular cells (vascular smooth muscle cells, pericytes, and endothelial cells), and peripheral nervous cells (Schwann cells and perineurium) [12, 13].

3. Mechanisms of Stem Cell in Recovery of Bladder Dysfunction

Stem cells (SCs) are self-renewing adult stem cells with multipotent differentiation potential. SCs can become many types of tissues either via transdifferentiation or via cell fusion and allow the regeneration and functional restoration [14] and are an important source for cell replacement [15]. They can serve as vehicles for gene transfer, proliferate, and differentiate into bladder smooth muscle cells to repopulate damaged bladder.

3.1. Migration. Recruitment of SCs to the bladder in BOO appears to be associated with increased blood flow and decreased tissue hypoxia, which contributes to improvement in histopathological and functional parameters [16].

MSCs are recruited to inflammation, ischemia, or damaged sites in response to specific chemokines expressed by damaged tissue [17]. A large body of literature exists in the roles of by recruited stem cells in a number of different organ systems and also it is demonstrated in injured bladder, too [18–21].

3.2. Differentiation. The ideal mechanism of stem cell therapy is differentiation, but only a few studies have demonstrated real differentiation into bladder smooth muscle (Table 1). Bladder regeneration by differentiation has been frequently reported in nonpathogenic bladder model. Differentiation pathway plays at most a minor role in the therapeutic effect by SCs transplantation. One explanation is the immortal strand hypothesis [22]. Labeled DNA in dividing cells will be quickly diluted by cell divisions but will be retained for much longer periods in slowly dividing stem cells. If the segregation of sister chromatids into stem cell daughters is not random, and if the stem cell retains the older unlabeled template strands, then the stem cell will lose all labels by the second division after administration of the label as a pulse.

Successful differentiation of stem cells into smooth muscle for bladder repair and replacement was reported by several studies in non-pathogenic model for tissue regeneration [23, 24].

3.3. Paracrine Effect. While differentiation has long been considered the main mechanism, it is logical to hypothesize that paracrine release of cytokines and growth factors by transplanted MSCs or their neighboring cells is responsible for the observed effects.

In this regard, SCs secretory factors have been shown to exert therapeutic effects by the modulation of local and systematic inflammatory responses, the stimulation of local tissue regeneration, and/or recruitment of host cells. MSCs themselves could not substitute the damaged cells directly but secrete a growth factor and contribute to reducing fibrosis through paracrine mechanisms [25].

BM-MSCs or ADSCs could secrete many growth factors including hepatic growth factor (HGF), nerve growth factor (NGF), brain-derived growth factor (BDNF), glial-derived growth factor (GDNF), insulin-like growth factor (IGF), vascular endothelial growth factor (VEGF), and ciliary neurotrophic growth factor (CNTF) [26] and play an essential part in the antifibrosis effects in injured organ, which implies that reducing fibrosis is managed by paracrine mechanisms rather than by cell incorporation [27–29]. Among the growth factors, HGF is a potent mitogen for hepatocytes, is secreted by MSCs, plays an essential part in the angiogenesis and regeneration of the tissue, and acts as a potent antifibrotic agent [30, 31].

In addition to antifibrotic mechanisms, BM-MSCs or ADSCs may provide antioxidant chemicals, free radical scavengers and heat shock proteins in ischemic tissue [32].

4. Stem Cell Therapy in Pathologic Model of Bladder Dysfunction

To date, the BOO model is the prominent model for bladder dysfunction, and other several pathologic models are in challenging state. The BOO model and cryo-injured model have a similar mechanism to induce bladder dysfunction. The BOO model also has ischemia mechanism which is similar to ischemia model. To date, for spinal cord injuries, there have been no studies published describing the grafting of stem cell into the injured bladder. Most studies have dealt with directly grafting of stem cells or bone marrow derived cells into injured spinal cord directly.

4.1. Bladder Outlet Model. Bladder outlet obstruction (BOO) caused by collagen deposit is one of the most common problems in elderly males. The collagen deposition in bladder occurs frequently during development of various pathological processes and eventually cumulates in bladder fibrosis, and finally induces a flaccid bladder. This bladder fibrosis adversely affects the smooth muscle function and the bladder compliance [33]. Bladder dysfunction after BOO is related

Table 1: Studies about stem cell therapy for bladder dysfunction.

Source	Mechanism of bladder dysfunction	Stem cell	Animal	Transplantation route	Tracking of stem cell	Bladder activity	Functional study	Smooth muscle differentiation	Remarks
Lee et al. [19]	BOO	Human BM-MSC	Rat	Direct transplantation into bladder	Iron oxide nanoparticle	Overactivity	UDS done: improvement of ICI, MVP, and RU	No	In vivo MRI examination for tracking MSC
Song et al. [20]	BOO	Human BM-MSC	Rat	Direct transplantation into bladder	None	Overactivity	UDS done: improvement of ICI and MVP	Yes	Transfection of human HGF gene into MSC
Woo et al. [21]	BOO	Mice BM-MSC	Mice	Intravenous injection	None	Overactivity	UDS done: improvement of capacity and compliance	No	Expression of chemokine CCL2
Tanaka et al. [36]	BOO	BMD cell	Mice	Direct transplantation into BM	GFP labeling	Overactivity	UDS done: improvement of compliance and capacity	No	BOO was made by periurethral collagen injection, radiation was induced, and CCL2 induction increased.
Nishijima et al. [39]	BOO	BM cell	Rat	Direct transplantation into bladder	GFP labeling	Underactivity	UDS done: improvement of ICI and RU	Yes	Underactive model was created
Chen et al. [40]	Chronic ischemia by bilateral iliac artery ligation	BM-MSC	Rat	Intra-arterial injection	BrdU labeling	Underactivity	UDS done: improvement of ICI	No	Doxazosin mesylate was intragastrically administrated
Huang et al. [41]	Chronic ischemia by hyperlipidemia	ADSC	Rat	Direct transplantation into bladder/intravenous injection	EdU labeling	Overactivity	UDS done: improvement of ICI and VV	Yes	Direct transplanted group showed better differentiation and better functional improvement
Zhang et al. [46]	Diabetes	ADSC	Rat	Direct transplantation into bladder/intravenous injection	EdU labeling	Overactivity. underactivity	UDS done: improvement of ICI and VV	No	Direct transplanted group showed better differentiation and better functional improvement
De Coppi et al. [54]	Cryoinjured	BM-MSC and AF-MSC	Rat	Direct transplantation into bladder	GFP labeling	N/A	N/A	Yes	Cell fusion in vitro
Huard et al. [56]	Cryoinjured	Muscle-derived cell	Mice	Direct transplantation into bladder	LacZ staining	Underactivity	Contractility test using muscle strip done: improvement of contractility	Yes	Ex vivo gene transfer using β-galactosidase
Sakuma et al. [57]	Cryoinjured	Adipocyte derived fat cell	Mice	Direct transplantation into bladder	GFP labeling	Underactivity	N/A	Yes	TGF-β signaling for smooth muscle differentiation
Nitta et al. [10]	Pelvic nerve injured	Sk-MSC	Rat	Damaged nerve lesion	GFP labeling	Underactivity	UDS done under electrical stimulation: improvement of IVP	Yes	Autologous and heterologous models were experimented together
Kwon et al. [11]	Pelvic nerve injured	Muscle-derived cell	Rat	Damaged nerve lesion	Enkephalin staining	Underactivity	UDS done under electrical stimulation: improvement of IVP	No	Autograft

Table 1: Continued.

Source	Mechanism of bladder dysfunction	Stem cell	Animal	Transplantation route	Tracking of stem cell	Bladder activity	Functional study	Smooth muscle differentiation	Remarks
Mitsui et al. [49]	SCI	Neural stem cell	Rat	Damaged cord lesion	BrdU labeling	Overactivity	UDS done: improvement of VV and VP	No	No difference in the incidence of detrusor overactivity
Temeltas et al. [52]	SCI	Bone marrow stromal cell	Rat	Damaged cord lesion	E-NACM staining	Overactivity	UDS done: improvement of VV, RU and VP	N/A	Glial restricted precursor was also treated
Hu et al. [53]	SCI	Bone marrow stromal cell	Rat	Intravenous injection	BrdU labeling	Overactivity	UDS done: improvement of compliance and capacity	N/A	External urethral sphincter activity was checked

BOO: bladder outlet obstruction; SCI: spinal cord injury; MSC: mesenchymal stem cell; BMD: bone marrow derived cell; ADSC: adipose derived stem cell; AF-MSC: amniotic fluid derived mesenchymal stem cell; Sk-MSC: Skeletal muscle derived mesenchymal stem cell; UDS: urodynamic study; ICI: inter-contraction interval; MVP: maximal voiding pressure; RU: residual urine; VV: voided volume; VP: voiding pressure; IVP: intravesical pressure; GFP: green fluorescent protein.

to alterations in ultrastructure properties of the smooth muscle and collagen. In bladder outlet obstruction, bladder instability was found [34]. Compensated bladder dysfunction with overactive bladder is expected after 6 weeks [33].

Recently, Lee et al. have reported that transplantation of primary human MSCs labeled with nanoparticles containing superparamagnetic iron oxide into the bladder wall of a rat BOO model inhibited bladder fibrosis and induced improvement of bladder dysfunction [19].

Growth factors have been reported in the bladder development and the remodeling of the bladder wall after outlet obstruction [35]. This finding was also demonstrated by Song et al. [20] that displayed human MSCs overexpressing HGF by pairing clonal human MSCS with HGF inhibited collagen deposition and improved cystometric parameters in BOO of rats.

Woo et al. [21] reported that MSCs engraftment had improved compliance compared to those without engraftment. Polymerase chain reaction revealed a 2-fold increase in CCL2 expression, but there were no significant changes in other chemokine. Additionally, CCL2 in the BOO model was identified by Tanaka et al. [36]. In their study, bone marrow derived cells were present in the urothelial and stromal layers after BOO. An activated epidermal growth factor receptor was found in cells associated with bone marrow derived cells [36].

A possible explanation for bladder dysfunction is decreased local blood flow, which means significant tissue ischemia. Increased intraluminal pressure is thought to cause vessel compression, which is further aggravated by fibrosis and hypertrophy [37, 38].

Not all the BOO model results reveal overactivity. Nishijima et al. [39] showed that transplanted bone marrow cells may improve bladder contractility by differentiating into smooth muscle-like cells in underactive bladder by BOO.

The applicability of MSCs to the treatment and/or prevention of bladder dysfunction by BOO would provide a new, potentially powerful addition to the limited armamentarium of existing therapies.

4.2. Bladder Ischemia Model. The bladder ischemia model is established using bilateral iliac artery ligation [40] or hyperlipidemia [41]. Several studies [42] showed that artery stenosis and blood insufficiency can cause significant changes of the bladder's structure and functionality. The mechanism of ischemia-induced bladder dysfunction is complicated, which may be related to ischemic denervation. This causes M-cholinergic receptor hypersensitivity to acetylcholine [43] and results in detrusor overactivity which leads to a more ischemic state of bladder wall. Considering the high rate of ischemic changes in the elderly, it is possible that the ischemia rat model could also be used for bladder dysfunction which is caused by aging detrusor.

Chen et al. [40] reported that pathological and functional changes of the bladder ischemic model are similar to the human aging detrusor. Chen et al. [40] also reported that the injection of stem cell suspension into the common iliac artery in rats with ischemic bladder, followed by intragastric administration of doxazosin mesylate, which makes transplanted stem cells regenerate in the bladder tissue, increases the percentage of smooth muscle content and nerve cells, and improves bladder detrusor function. Huang et al. [41] showed that direct injection to the bladder or intravenous injection of ADSCs improved urodynamics and tissue parameters in the rat model of hyperlipidemia associated overactive bladder.

Azadzoi et al. [44] found that hyperlipidemia induced chronic ischemia increases transforming growth factor-β1 in the bladder which leads to fibrosis and noncompliance.

4.3. Diabetes Model. Daneshgari et al. [45] proposed that diabetic bladder dysfunction (DBD) typically evolves in a time-dependent progression of both storage and voiding problems. The early phase of DBD manifests as detrusor overactivity, which represents leading to urinary frequency and urgency. However, over time, progressive oxidative stress and neuropathy lead to decompensation of the detrusor musculature, thereby leading to the underactive or atonic bladder.

Zhang et al. [46] reported that improved voiding function was noted in ADSCs-treated rats as compared with phosphate-buffered saline-treated rats. DBD pattern was hypocontractile bladders in their experimental model. Though some ADSCs differentiated into smooth muscle cells, paracrine pathway seems to play a main role in this process as well. This means that transplantation of ADSCs could result in reduction of apoptosis and preservation of "suburothelial capillaries network."

4.4. Spinal Cord Injured Model. Spinal cord injury (SCI) induces complete deterioration of bladder compliance, function, infection, and other lower urinary tract complications [47]. SCI rat exhibited increased bladder wall thickness that contained a larger percentage of collagen [48]. The goal of bladder treatment in patients with SCI is to reduce infections, preserve renal function, and to improve patient' quality of life. Transplantation of neural stem cells into the injured spinal cord has been reported to improve bladder function in animal models [49]. However, no study has shown whether MSCs grafting into the bladder wall can influence bladder function following SCI.

As the mature central nervous system cannot generate new neurons and glial cells, bladder functional recovery is limited following SCI. However, recent studies suggest that transplanted neural progenitor cells promote recovery of the bladder function through regeneration of the injured site [49–52]. In most of these studies, stem cells have been injected into the injured lesion directly with a needle. Hu et al. [53] showed that intravenously transplanted bone marrow stromal cells (BMSCs) survived in the L3-4 and had beneficial effects on the recovery of bladder function in the rats after spinal cord injury.

4.5. Cryo-Injured Model. The cyro-injured model induces bladder hypertrophy with loss of smooth muscle and increase of collagen which represent a similar mechanism with the BOO model [54]. The major outcome of the stem cell transplantation in the cryo-injured bladder seems to be that of preventing the increase in size of surviving smooth muscle

cells (SMCs) along with a poor differentiation drive to SMC lineage. This can exert an important effect on the remodeling process in the injured bladder, which is characterized by the development of a compensatory SMC hypertrophy. As reported by Somogyi et al. [55], functional (and structural) impairing of innervation (and reinnervation) in the cryo-injured bladder can induce long-lasting tonic contractions to more effectively empty the bladder.

De Coppi et al. [54] showed protective effect of MSCs transplantation using AF-MSC and BM-MSC. They concluded that stem cell transplantation has a limited effect on smooth muscle cell regeneration. Instead, it can regulate postinjury bladder remodeling, possibly via a paracrine mechanism.

Huard et al. [56] showed that injected muscle-derived cells (MDCs) are capable of not only surviving in the lower urinary tract, but also improving the contractility of the bladder in cryo-injured model. They used modified MDCs which were genetically engineered to express the gene encoding β-galactosidase.

Sakuma et al. [57] showed that dedifferentiated fat cells can differentiate into smooth muscle cell lineages and contribute to the regeneration of bladder smooth muscle tissue using human adipocyte derived dedifferentiated fat cells in cryo-injured model.

4.6. Other Bladder Dysfunction Model. Nitta et al. [10] showed that significantly higher functional recovery was noted by transplantation of skeletal muscle-derived multipotent stem cells in bladder branch of the pelvic plexus (BBBP) injured model. The transplanted cells showed incorporation into the damaged peripheral nerves and blood vessels after differentiation into Schwann cells, perineurial cells, vascular smooth muscle cells, pericytes, and fibroblasts around the bladder.

Kwon et al. [11] reported that similar cell transplantation of muscle-derived cells isolated was achieved using the preplate technique, in the unilaterally transected pelvic nerve model in rats. They performed functional measurement of intravesical pressures by electrical stimulation of the transected pelvic nerve and obtained significant functional recovery through crosssectional group comparison analysis 2 weeks after transplantation.

4.7. Bladder Regeneration in Nonpathogenic Model. For bladder tissue engineering, three pioneering studies have demonstrated that embryoid body derived stem cells or BMSCs seeded on small intestinal submucosa (SIS) facilitated the regeneration of partially cystectomized bladder [58–60]. Recently hair stem cells and ADSCs seeded on bladder acellular matrix (BAM) have also shown bladder regeneration potential [61, 62]. In studies which deal with the use of synthetic scaffolds instead of SIS and BAM, Sharma et al. reported that BMSCs seeded on poly (1,8-octanediol-co-citrate) thin film supported partial bladder regeneration [63], and Tian et al. showed that myogenic differentiated BMSCs seeded on poly-l-lactic acid scaffold exhibited bladder engineering potential [64, 65]. Likewise, poly-lactic-glycolic acid seeded with myogenically differentiated human ADSCs maintained bladder capacity and compliance when grafted in hemicystectomized rats [66].

Bladder tissue engineering using MSCs might show better results than by using differentiated cells. MSCs were shown to migrate to the bladder grafts and differentiate into SMC [67]. These cells achieved fast replacement of the grafts with appropriate neural function and less fibrosis [57].

4.8. Human Study. The human bladder is one organ to which stem cell technology could be applied. But human studies are scarce and there were no studies regarding pathologic bladder dysfunction. Studies could only be found in regards to the neobladder and urethral sphincter. Urologists need an appropriate substitute for the traditional conduits and neobladders, given their complications of adhesions, mucus formation, incomplete emptying, and metabolic and malignant transformations. Pioneering research has created artificially engineered bladder tissues using autotansplantation [68]. Urothelial and muscle cells obtained by bladder biopsy were grown in a culture for 7 weeks and transplanted in layers on a biodegradable bladder-shaped scaffold made of collagen and polyglycolic acid. During a mean follow-up of just under 4 years, all patients had improved bladder function with no major surgical or metabolic complications.

5. Discussion

5.1. Stem Cell Selection. Classification into embryonic or adult stem cells is useful to distinguish ethical issues associated with the destruction of an embryo to yield cells for research purposes. Whereas embryonic stem cells are considered to be pluripotent, adult stem cells are thought to be restricted in their potency by their tissue of origin. This concept remains controversial, as adult stem cells can also produce mature cells not normally seen in their tissue of origin under certain conditions. The exact molecular mechanism of this phenomenon, known as *plasticity* or *transdifferentiation,* is yet to be discovered [69]. However, the recent discovery of induced pluripotent stem cells (IPSs) has shown some light into the molecular basis of stem cells [70]. These IPSs were mostly derived from skin, but recently urinary tract derived IPSs were shown to be more efficient than skin-derived iPSCs in bladder differentiation which was demonstrated by expression of urothelial-specific markers including uroplakins, claudins, and cytokeratin and stromal smooth muscle markers including α-smooth-muscle actin, calponin, and desmin. These disparities highlight the epigenetic differences between individual IPS lines and represent the importance of organ-specific IPSCs for tissue-specific studies [71].

Although immature (embryonic or fetal) stem cells may be more efficient than adult counterparts in points of providing the supporting acellular matrix with multipotent progenitor cells which could differentiate into distinct cell lineages, fetal-type MSCs were not superior to adult-type MSCs in terms of contributing to the formation of new differentiated SMCs or vascular cells despite the nominal higher plasticity of immature stem cells in real experiments [54, 72].

5.2. Tumorigenesis. Regardless of their tissue origin, all stem cell types could not avoid the main issue of tumorigenesis and if they themselves become tumors or whether they encourage the growth/metastasis of existing tumors. While several studies on the former aspect have generated results due to tumor cell line contamination, an ever increasing number of publications are sounding the alarm on the latter issue. However, these studies relied on the use of animal models transplanted with tumor cell lines, an approach whose clinical relevance has long been questioned. This is also the main factor as to the main reason why human study is not easy.

Clinical human studies of muscle-derived cell transplantation have recently been performed in only urethral sphincter dysfunction, and no studies have been performed yet in bladder dysfunction. Eight women with stress urinary incontinence (SUI) were treated with muscle-derived stem cells [73–75].

Although, autologous transplantation of bladder stem cells could be possible and could avoid the limitation of theoretical and ethical standpoints, the exact culture conditions to direct autologous nonbladder stem cells to transdifferentiate into urothelial cells are yet to be established.

5.3. Route of Transplantation. In contrast to local injection of MSCs, intravenously administered MSCs are distributed throughout the whole animal. Furthermore, there is the concern of causing capillary clogging when larger cell types such as MSCs are infused, a complication that could result in hemodynamic compromise, interference with pulmonary gas exchange, and respiratory distress [76]. Intravenously injected MSCs are localized mainly to the pulmonary capillary bed [77]. Local injection may have a better effect than intravenous injection does. A recent study by Huang et al. [41] also showed better improvement of bladder function in the direct injection group than in the systemic injection group.

5.4. Metabolic Memory. For administration of SC, "metabolic memory" has to be considered first. It suggests that SCs derived from pathologic animals such as diabetes behave differently than those derived from healthy animals [78]. Be that as it may, it seems most likely that only individuals with a metabolic derangement leading to tissue damage will seek out this form of treatment. Hence, the diabetic or other systemically ill animal models are appropriate research subjects for preclinical research.

5.5. Gene Therapy. Although gene therapy to be efficacious and one of the promising therapeutic options, effective gene transfer into stem cells must be achieved without inducing detrimental effects on their biological properties. Although modification of MSCs to overexpress HGF has an effective means to maintain or enhance the capacity of MSCs and to be efficacious for bladder fibrosis therapy [20], the choice of vector for cell transduction should be carefully considered. The selected vector should have high transduction efficiency and should ensure stable and long-term transgene expression from the cell vehicle and be devoid of any damaging effect on cell viability.

5.6. Limitation of Experimental Study. Without intermediary examination, such as survival urodynamic or bladder biopsy at a midpoint in the experiment, also, whether stem cells may serve a more preventative or ameliorative role are too early to determine. It is hard to know whether obstructed bladders appear to undergo early signs of obstruction that are then later reversed or the pathological process is avoided altogether.

6. Conclusions

There are interesting results with experimental use of stem cells to treat bladder dysfunction. The use of MSCs has shown great promise in several animal studies.

Although significant challenges are still need to overcome challenges for human application, this novel technology has the potential to become a major source of cells for treatment of bladder dysfunction. In order to determine the exact role of stem cells in treatment of bladder dysfunction, more trials are needed.

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Research Article

Sympathetic Innervation Induced in Engrafted Engineered Cardiomyocyte Sheets by Glial Cell Line Derived Neurotrophic Factor *In Vivo*

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The aim of myocardial tissue engineering is to repair or regenerate damaged myocardium with engineered cardiac tissue. However, this strategy has been hampered by lack of functional integration of grafts with native myocardium. Autonomic innervation may be crucial for grafts to function properly with host myocardium. In this study, we explored the feasibility of *in vivo* induction of autonomic innervation to engineered myocardial tissue using genetic modulation by adenovirus encoding glial cell line derived neurotrophic factor (GDNF). GFP-transgene (control group) or GDNF overexpressing (GDNF group) engineered cardiomyocyte sheets were transplanted on cryoinjured hearts in rats. Nerve fibers in the grafts were examined by immunohistochemistry at 1, 2, and 4 weeks postoperatively. Growth associated protein-43 positive growing nerves and tyrosine hydroxylase positive sympathetic nerves were first detected in the grafts at 2 weeks postoperatively in control group and 1 week in GDNF group. The densities of growing nerve and sympathetic nerve in grafts were significantly increased in GDNF group. No choline acetyltransferase immunopositive parasympathetic nerves were observed in grafts. In conclusion, sympathetic innervation could be effectively induced into engrafted engineered cardiomyocyte sheets using GDNF.

1. Introduction

Recently, myocardial regeneration has been expected as a new therapeutic strategy for severe heart failure. To date, numerous studies have been reported demonstrating improvement of heart function in support of this goal [1, 2]. However, functional integration of graft with native myocardium is still an unsolved issue.

In order to conduct myocardial regeneration therapy successfully, appropriate integration with host myocardial

tissue will be crucial. Transplanted engineered myocardial tissue without innervation may not function appropriately in accordance with host, since normal cardiac tissue is properly innervated, and its function is precisely regulated by the systemic autonomic nervous system [3]. Therefore, to achieve functional integration with host myocardium, autonomic innervation of transplanted myocardial engineered tissue should be important.

It is well known that neuronal function and innervation are regulated by target organ-derived neurotrophic factors [4]. Therefore, neurotrophic factors have been extensively investigated in animal models of nerve injury to further enhance and accelerate the process of nerve regeneration and functional recovery [5]. A member of the transforming growth factor superfamily, GDNF, has been shown to promote the survival and function of several neuronal populations in the peripheral nervous system [6, 7]. Furthermore, we demonstrated that GDNF effectively promoted the sympathetic neuron outgrowth to cocultured cardiomyocytes and played an important role in inducing cardiac sympathetic innervation [8, 9]. In the present study, we explored the feasibility of induction of autonomic innervation into transplanted engineered cardiomyocyte sheets by adenoviral overexpression of GDNF in the graft tissue.

2. Materials and Methods

All animal experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996) and approved by the Animal Care and Use Committee of Nagoya University (Protocol no. 24061).

2.1. Isolation of Neonatal Rat Ventricular Cardiomyocyte and Construction of Engineered Cardiomyocyte Sheets. Primary cultures of neonatal cardiomyocytes were prepared as reported previously [10]. Briefly, ventricles from 1- to 3-day-old GFP-positive Wistar neonatal rats were digested at 37°C in Hank's balanced salt solution containing collagenase (Worthington Biochemical Corporation, Lakewood, NJ, USA). Isolated cells were suspended in culture medium M199 (Gibco BRL, Carlsbad, CA, USA) containing 10% fetal bovine serum, 0.2% penicillin-streptomycin, and 2.7 mmol/L glucose. Cells were seeded at a cell density of $3.0 \times 10^5/\text{cm}^2$ onto temperature-responsive culture dishes (CellSeed, Tokyo, Japan) and incubated at 37°C in a humidified atmosphere with 5% CO_2. On the next day, 2 μL phosphate buffered saline (PBS) containing adenovirus encoding GDNF (AdGDNF) (5 to 15 m.o.i) or PBS only (used as control) was added to the medium of culture dishes, respectively, and cardiomyocytes were continuously cultured for another 3 days. To release confluent cells as a cell sheet from the bottom of culture dishes, cells were incubated at 20°C. Engineered cardiomyocyte sheets were detached spontaneously within 1 hour and floated into the aqueous media. Immediately after detachment, the cell sheets were gently aspirated into the tip of a 10 mL pipet and transferred onto appropriate culture surfaces. Once placed, the medium was dropped onto the center of the sheet to spread folded parts of the transferred engineered cardiomyocyte sheets. After spreading cell sheets, the medium was then aspirated to adhere the cell sheet to the culture surface. To layer cell sheets, another cardiomyocyte sheet was transferred into the first dish in the same way. The second sheet was positioned just above the first sheet. Identical procedures were repeated to layer the third sheets.

2.2. Animal Model and Engineered Cardiomyocyte Sheets Transplantation. Male Wistar rats (8–10 weeks old, weight 222–325 g) were used to create heart cryoinjury model. Rats were anaesthetized with intraperitoneal injection of pentobarbital sodium (50 mg/kg), then intubated and mechanically ventilated with room air. Under aseptic condition, thoracotomy was performed through the left fifth intercostal space, and the heart was exposed. To create heart cryoinjury, a steel cryoprobe (3.5 mm in diameter), soaked in liquid nitrogen, was applied to the beating heart in the region of the anterior wall of the left ventricle for 10 seconds. Then the cryoprobe was removed, and the frozen tissue was allowed to thaw for 10 minutes. The freeze-thaw procedure was repeated twice. Triple-layered engineered cardiomyocyte sheets were then transplanted onto the injured anterior wall of the left ventricles. After 15 minutes, air was evacuated from the cavity, and the chest was closed, and then spontaneous normal respiration was restored. The rats were maintained under postoperative care and were given tacrolimus (Astellas Pharma Inc, Tokyo, Japan) at 10 mg/kg/d on the day before surgery transplantation, then on every day after surgery. They were euthanized at three time points (1, 2, and 4 weeks postoperatively).

2.3. Histology Examination. Engineered cardiomyocyte sheets, harvested 4 days after culture, were processed for GDNF immunostaining to confirm adenoviral transfection. Rat hearts were obtained at 1, 2, and 4 weeks postoperatively and rapidly placed in PBS containing paraformaldehyde (4%, adjusted to pH 7.4) at 4°C for 2 hours, washed in PBS, and sequentially transferred to graded (10, 20, and 30%) solutions of sucrose in PBS for 4 hours in each concentration. Hearts were embedded in Tissue-Tek-II OCT compound (Sakura Finetek Japan, Tokyo, Japan) and frozen on dry ice and then were cryosectioned and stained with primary antibodies for α-actinin (AA) (rabbit monoclonal, Sigma-Aldrich), growth associated protein-43 (GAP43, mouse monoclonal, Sigma-Aldrich), tyrosine hydroxylase (TH, mouse monoclonal, Sigma-Aldrich), neurofilament-M (NFM-M, rabbit polyclonal, Chemicon International), choline acetyltransferase (ChAT, mouse monoclonal, Sigma-Aldrich), and GDNF (goat polyclonal, R&D system, Minneapolis, MN). The sections were incubated with secondary antibodies conjugated with Alexa 568, 633 (Molecular Probes, Carlsbad, CA, USA). All confocal microscopic images were obtained using LSM 510 microscope (Carl Zeiss, Jena, Germany). The densities of GDNF or AA positive cardiomyocytes, GAP43 or TH positive nerve fibers were evaluated using Image-Pro Plus software (Media Cybernetics, Inc., Bethesda, MD, USA).

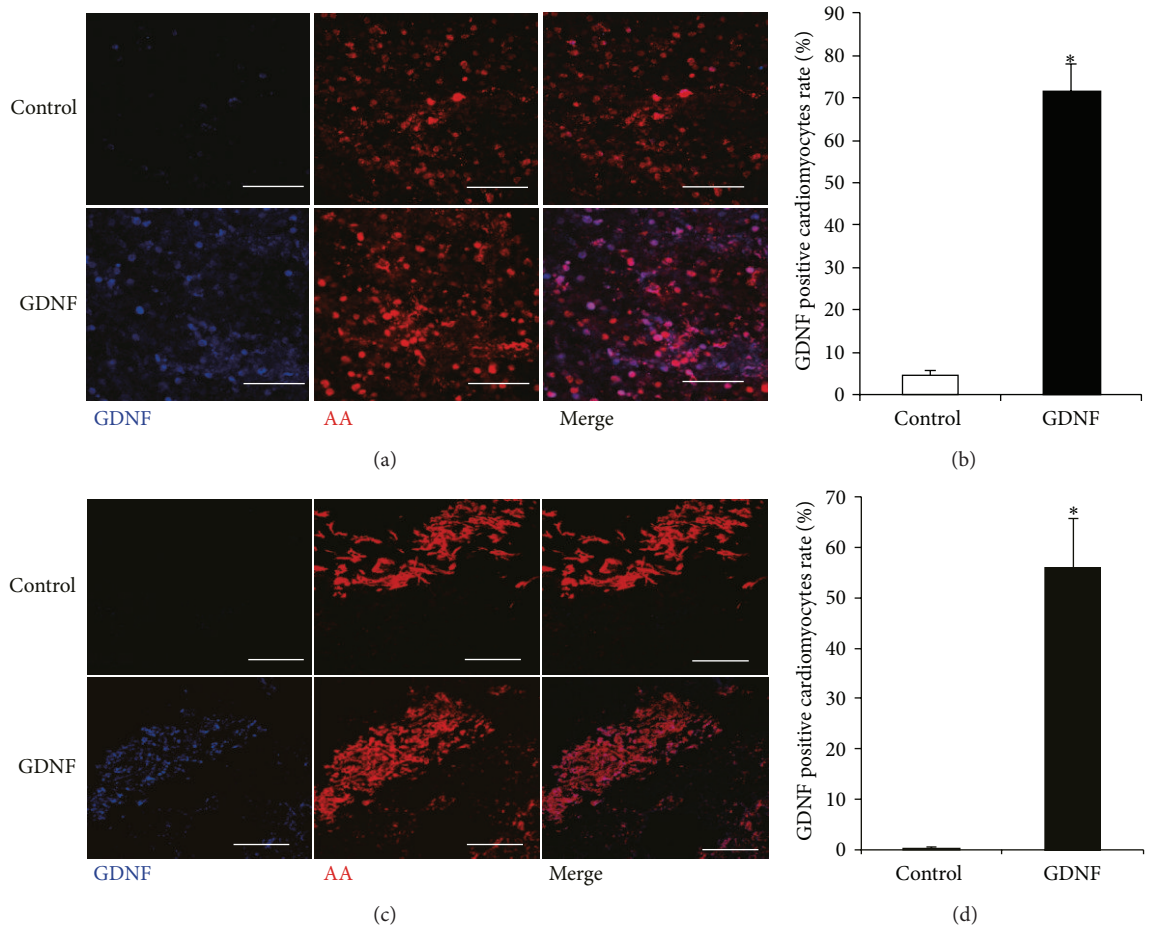

Figure 1: Overexpression of GDNF *in vitro* and *in vivo*. (a) Representative images of immunofluorescent staining for α-actinin (AA) and glial cell line derived neurotrophic factor (GDNF) in the control and GDNF cardiomyocytes after culture for 4 days. (b) Quantitative analysis of GDNF positive cells area in the control and GDNF cardiomyocytes ($^*P < 0.05$, versus control, $n = 4$). (c) Representative images of immunofluorescent staining for α-actinin (AA) and GDNF in the control and GDNF grafts at 4 weeks after transplantation. (d) Quantitative analysis of GDNF positive cells area in the control and GDNF grafts ($^*P < 0.05$, versus control, $n = 4$). Scale bars indicate 100 μm. AA: α-actinin; GDNF: glial cell line derived neurotrophic factor.

2.4. Quantitative Analysis of Sympathetic Innervation. We measured innervation in cardiac grafts at 3 time points, in 6 sections per animal ($n = 5$ animals per data). In each section, the six fields that contained the most nerve fibers were analyzed. We defined that the nerve density was the ratio between the total area of nerves and the total engrafted cardiomyocytes area by ImageJ software, as described previously [11].

2.5. Statistical Analysis. Data analyses were performed with SPSS for Windows (version 16.0). All data were described as mean ± standard deviation (SD). Comparison between two groups was analyzed using Student's t-test. A value of $P < 0.05$ was considered as statistically significant.

3. Results

3.1. GDNF Overexpression In Vitro and In Vivo. To confirm adenoviral transfection and gene expression, AdGDNF transfected cardiomyocytes or control cardiomyocytes were harvested to perform immunofluorescence staining after 4 days of culture. GDNF protein was abundantly expressed in GDNF group cardiomyocytes, while GDNF was faintly detected in the control cardiomyocytes (Figure 1(a)). The ratio of GDNF-positive cells area was 4.6 ± 1.2% in control group and 71.7±6.3% in GDNF group (Figure 1(b)) ($P < 0.05$, versus control, $n = 4$). In addition, to examine the long-term GDNF overexpression by adenoviral gene transfer, we also conducted immunostaining for cardiomyocyte sheets 4 weeks after transplantation. Abundant GDNF protein was observed in GDNF grafts, while no obvious GDNF was detected in control grafts (Figure 1(c)). Quantitative analyses of the relative GDNF positive cells area in control and GDNF grafts were 0.1 ± 0.2% and 56.0 ± 9.8% (Figure 1(d)) ($P < 0.05$, versus control, $n = 4$). These results suggested that stable GDNF overexpression of cardiomyocytes could be achieved *in vitro* and *in vivo* by genetically modifying cardiomyocytes.

3.2. Morphology of Cardiac Cryoinjury Model and Engrafted Engineered Cardiomyocyte Sheets. In order to avoid variation

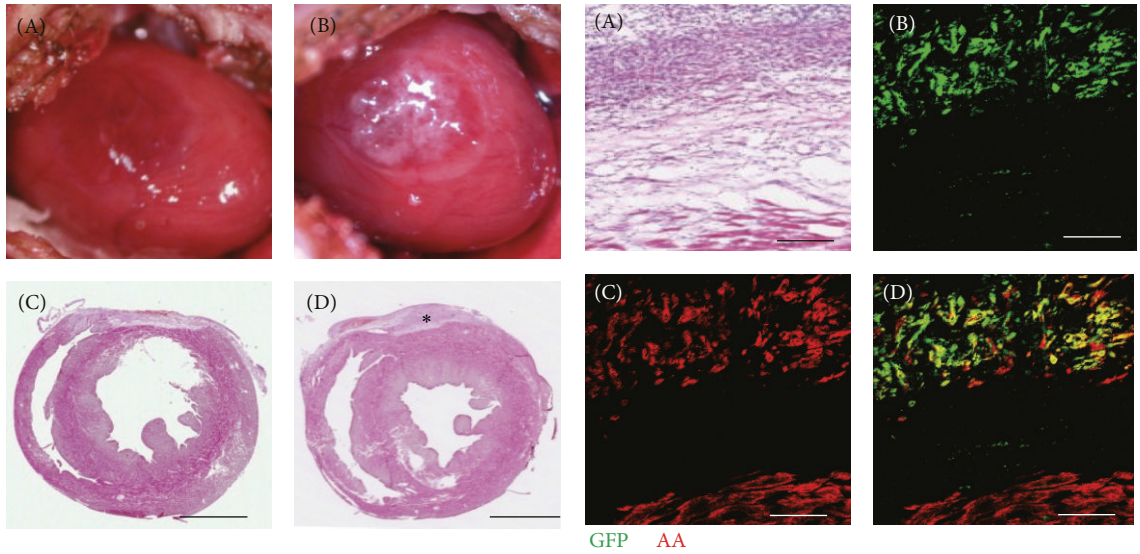

Figure 2: Morphology of cardiac cryoinjury model and engrafted engineered cardiomyocyte sheets. (a): (A) and (B) show cryoinjury on the epicardial surface of the left ventricle and transplantation with engineered cardiomyocyte sheets. (C) and (D) show representative hematoxylin/eosin staining of cross section of cryoinjured hearts without or with cardiomyocyte sheets transplantation 4 weeks after operation. Asterisk shows cardiomyocyte sheets transplanted on the epicardial surface of cryoinjured heart. (b): (A) shows representative hematoxylin/eosin staining of cardiomyocyte sheets 4 weeks after transplantation; (B)–(D) show the serial sections immunolabeling with α-actinin (AA, red), which marks cardiomyocytes, and GFP (green),which marks grafts. The grafted cardiomyocyte sheets can be identified by double positive for AA and GFP. Scale bars: 2 mm in Figures 2(a)C and 2(a)D; 200 μm in Figure 2(b). AA: α-actinin.

of size, depth, and location of myocardial injury, we used a cardiac cryoinjury model as previously reported [12]. In the cryoinjured hearts, a round myocardial injury was observed on the epicardial surface of the left ventricle and covered with cardiomyocyte sheets (Figures 2(a)(A) and 2(a)(B)). Fibrotic tissues with 0.21 to 0.36 mm depth were observed on the epicardial surface at 4 weeks after operation (Figure 2(a)(C)). On the other hand, the transplanted engineered cardiomyocyte sheets were observed on the surface of cryoinjured myocardium and surrounded with fibrotic tissue (Figure 2(a)(D)). In order to track the transplanted cardiomyocyte sheets, GFP-transgenic rat neonatal ventricular cardiomyocytes were used. Four weeks after operation, engrafted cardiomyocyte sheets could be detected on the surface of cryoinjured heart by immunofluorescence staining, which show double positive for α-actinin and GFP (Figure 2(b)). These results indicated that the procedure used in the present study could create heart cryoinjury and transplanted cardiomyocytes could survive 4 weeks after transplantation.

3.3. Neural Growth in Engrafted Engineered Cardiomyocyte Sheets. To examine the autonomic innervation in engrafted engineered cardiomyocyte sheets, we first investigated the growing nerves in the grafts at 1, 2, and 4 weeks after operation by immunostaining for GAP43, a maker for neural growth. At peri-injured areas, abundant GAP43 immunopositive nerves were detected in both group grafts at three time points. No GAP43 positive nerve fibers were observed in the control grafts until 2 weeks after transplantation, but some were observed 1 week in GDNF grafts (Figure 3(a)). GAP43 positive nerves were increased in the both group grafts over time. However, much more nerves were observed in GDNF group compared with control group at the same time point. The observation was confirmed by the measurement of the density of growing nerve fibers in the grafted cardiomyocyte sheets (Figure 3(b)) ($P < 0.05$, versus control, $n = 5$). These results indicated that GDNF effectively promoted neural growth in the grafts.

3.4. Sympathetic Innervation in Engrafted Engineered Cardiomyocyte Sheets. To further investigate the type of nerve fibers in the engrafted engineered cardiomyocyte sheets, we did immunostaining for TH and ChAT to identify sympathetic and parasympathetic nerve in the grafts at 1, 2, and 4 weeks postoperatively. TH positive sympathetic nerve fibers were only subtly observed in the grafts until 2 weeks after transplantation in the control group, while the nerve fibers were clearly detected as early as 1 week after transplantation in GDNF group. More nerve fibers were observed over time in both group grafts (Figure 4(a)). Quantitative analysis of TH positive nerves area in the grafts shows that much more sympathetic nerves were observed in the GDNF group grafts compared with the control at the same time points (Figure 4(b)) ($P < 0.05$, versus control, $n = 5$).

On the other hand, no ChAT immunoreactive parasympathetic nerves were observed either at peri-infarct area or in the engrafted cardiomyocyte sheets (samples were obtained from GDNF cardiomyocyte sheets transplanted rats), while the pattern of GAP43 positive nerves and TH positive nerves was similar with that of NF-M positive nerves in the same

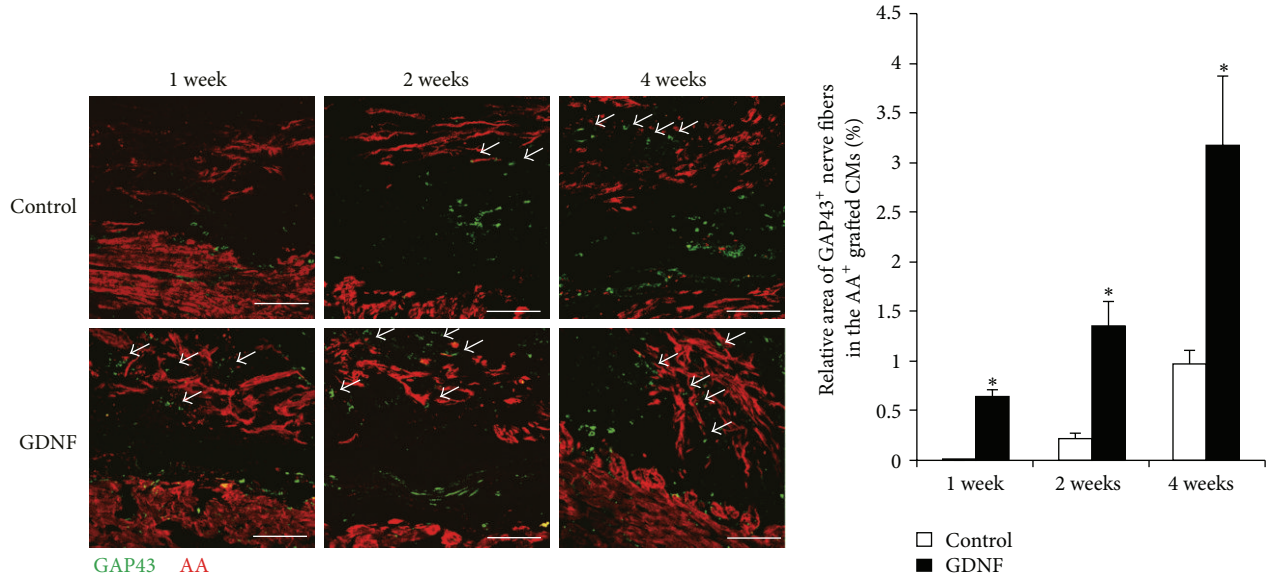

FIGURE 3: Neural growth in engrafted engineered cardiomyocyte sheets. (a) Representative images of immunofluorescent staining for α-actinin (AA) and growth associated protein 43 (GAP43) in the control and GDNF grafts at 1, 2, and 4 weeks after cardiomyocyte sheets transplantation. Arrows indicate growing nerves in the grafts. (b) Quantitative analysis of GAP43 positive nerve area in the control and GDNF cardiomyocyte sheet grafts ($^{*}P < 0.05$, versus control, $n = 5$). Scale bars indicate 200 μm. AA: α-actinin; GAP43: growth associated protein 43.

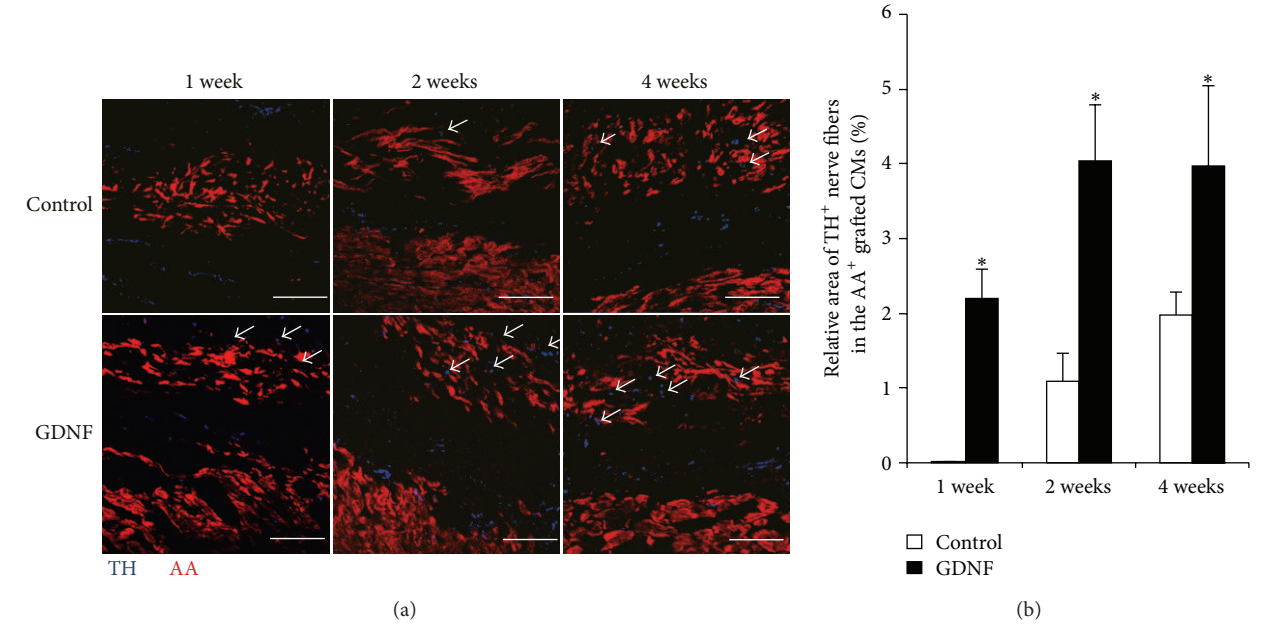

FIGURE 4: Sympathetic innervation in engrafted engineered cardiomyocyte sheets. (a) Representative images of immunofluorescent staining for α-actinin (AA) and tyrosine hydroxylase (TH) in the control and GDNF grafts at 1, 2, and 4 weeks after cardiomyocyte sheets transplantation. Arrows indicate sympathetic nerves in the grafts. (b) Quantitative analysis of TH positive nerve area in the control and GDNF cardiomyocyte sheet grafts ($^{*}P < 0.05$, versus control, $n = 5$). Scale bars indicate 200 μm. AA: α-actinin; TH: tyrosine hydroxylase.

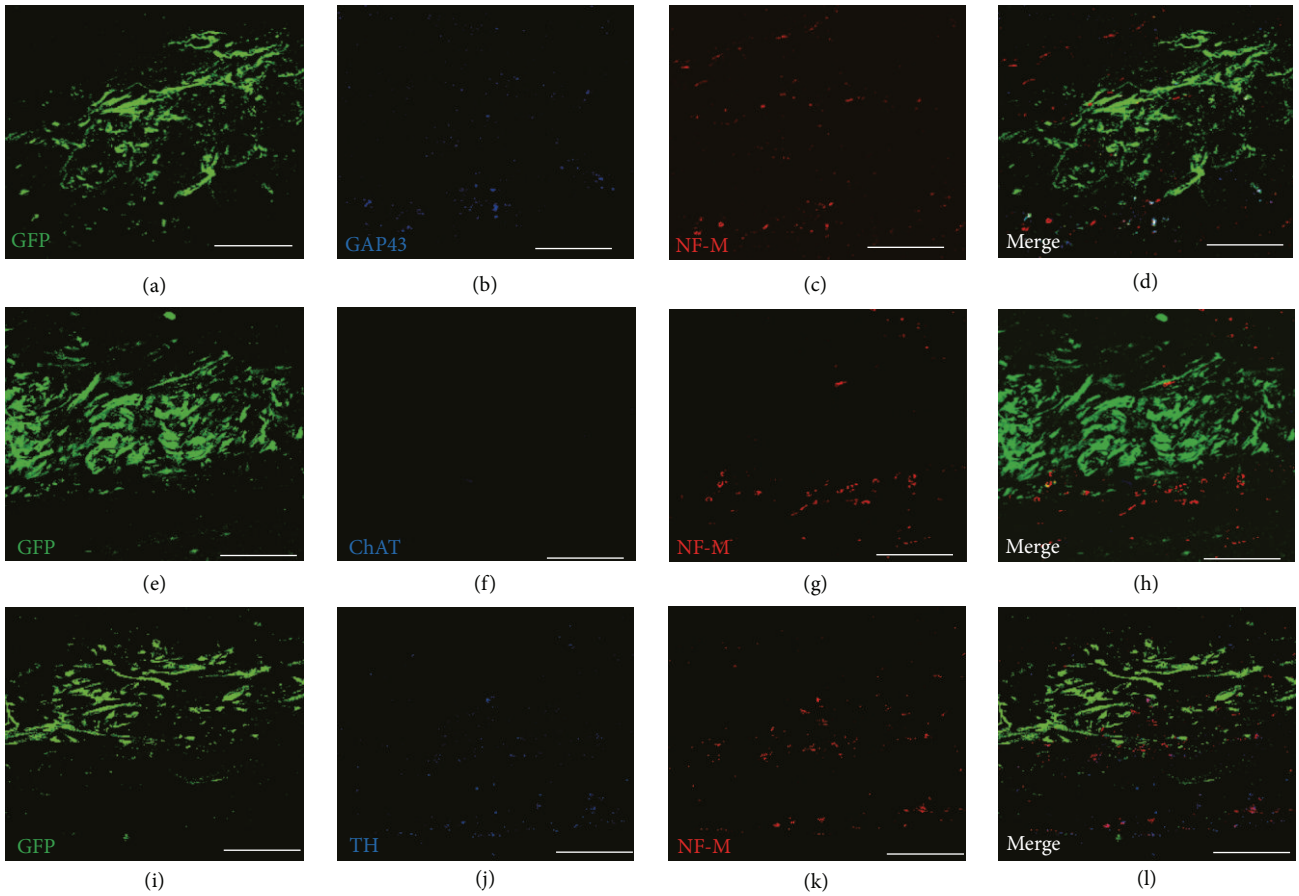

FIGURE 5: Patterns of growing nerves (GAP43), sympathetic nerves (TH), parasympathetic nerves (ChAT), and autonomic nerves (NF-M) in the grafts. (a)–(d), (e)–(h), and (i)–(l) show representative images of triple immunostaining for NF-M/GAP43/GFP, NF-M/ChAT/GFP, and NF-M/TH/GFP in the GDNF grafts at 4 weeks after transplantation. Scale bar indicates 200 μm. GAP43: growth associated protein 43; TH: tyrosine hydroxylase; ChAT: choline acetyltransferase; NF-M: neurofilament-M.

sample (Figure 5). These results were confirmed in four independent experiments. These findings indicated that sympathetic, but not parasympathetic, innervation in engrafted engineered cardiomyocyte could be efficiently enhanced by GDNF.

4. Discussion

The present study showed a new technology to induce sympathetic innervation in engrafted engineered cardiomyocyte sheets. With the use of *in vitro* gene transfer strategy, overexpression of GDNF, the sympathetic innervation in grafts was significantly improved.

Recently, myocardial regeneration therapy is considered as a promising treatment for the patients with heart failure. To conduct cell therapy more safely, scaffold-free cardiac cell sheets techniques are engineered using temperature-responsive culture dishes. With this procedure, cells can be harvested as intact sheets, and the three-dimension tissues are constructed by layering these cell sheets [13]. Transplantation of cell sheets onto damaged hearts improved heart function in several animal models [14, 15]. However, long-term survival and functional integration of grafts with native myocardium are still two crucial issues for clinical application in future. Vascularization of graft is widely investigated and has been shown benefit for survival [16, 17]; however, little information is available for autonomic innervation in engrafted engineered myocardial tissue. In the present study, we used cell sheet technique to construct engineered triple-layered cardiomyocyte sheets and transplanted them onto the cryoinjured hearts. We demonstrated that sympathetic but not parasympathetic innervation in the transplanted engineered cardiomyocyte sheets at 2 weeks after transplantation and provided evidence that this process could be significantly promoted by genetically modifying cardiomyocytes to overexpress GDNF.

It is a promising strategy to combine gene therapy and tissue engineering or cell therapy for treatment of diseases. Previously, vascular growth has been successfully induced into tissue-engineered scaffolds by combination of VEGF overexpressing adipose-derived stromal cells and endothelial cells transplantation [18, 19]. In addition, transplantation of GDNF overexpressing Schwann cells has been

reported to enhance regeneration of bilaterally transected erectile nerves in rats [20]. By similar strategy, in the present study, overexpression of GDNF in the graft tissue by adenovirus transfection effectively induced sympathetic innervation into the transplanted engineered myocardiocyte sheets.

GDNF has been shown to promote the survival and function of several neuronal populations in the peripheral nervous system [6, 7]. Recent findings have shown that GDNF was expressed in the hearts of murine embryos and neonates by quantitative RT-PCR [21] and upregulated after chemical sympathectomy in rats, suggesting a role in sympathetic nerve regeneration [22]. In addition, artemin, a neurotrophic factor of GDNF family, was shown to express along blood vessels in the early developmental stages and promote the development of sympathetic innervation of blood vessels [23, 24]. Previously, we have reported that GDNF enhanced sympathetic axon growth toward cardiomyocytes [8]. In this study, our results showed that GDNF also effectively promoted sympathetic innervation into transplanted engineered cardiomyocyte sheets *in vivo*.

It should be noted that the type of nerve innervating transplanted cardiomyocytes seems to be dependent upon the transplant site, as they are derived from the surrounding tissues. In the rat heart, the sympathetic nerves are distributed in the subepicardial layer throughout most surfaces and penetrate into myocardium along coronary arterial pathways, while parasympathetic nerves are mainly located around conducting system. In this study, engrafted engineered cardiomyocytes were innervated by TH positive sympathetic fibers but not parasympathetic nerves. Similar findings have been reported in previous studies about transplanted islets [25].

There are several limitations to the present study. First, it is worth to note that sympathetic hyperinnervation may cause life-threatening arrhythmias. In this study, we used GDNF to increase sympathetic innervation in transplanted cardiomyocyte sheets, and it may induce arrhythmias. To assess the potential risk of sympathetic innervation in engrafted cardiomyocyte sheets, we performed *in vivo* programmed electric stimulation at 4 weeks after transplantation; however, no induction of arrhythmia was detected (data not shown). Maybe our model would not be suitable for discussing arrhythmogenesis because the size of the cryoinjury is apparently too small to induce arrhythmias. Further examination is needed.

Second, in this study, we showed that sympathetic innervation occurs in engrafted cardiomyocytes and demonstrated this could be promoted by GDNF, although we did not present the evidence showing that sympathetic innervation can promote engrafted cardiomyocytes functionally integrated with host myocardium. Further functional assessment is needed.

In conclusion, our work has demonstrated that sympathetic innervation could be effectively induced into engrafted engineered cardiomyocyte sheets by GDNF. This study may be an important step to engineer functional myocardium in myocardial regeneration therapy.

Conflict of Interests

The authors declare no conflict of interests that might prejudice the impartiality of this research except that one of authors (Tatsuya Shimizu) is a consultant of CellSeed (Tokyo, Japan).

Acknowledgments

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Research Article

Promotion of Hepatic Differentiation of Bone Marrow Mesenchymal Stem Cells on Decellularized Cell-Deposited Extracellular Matrix

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Interactions between stem cells and extracellular matrix (ECM) are requisite for inducing lineage-specific differentiation and maintaining biological functions of mesenchymal stem cells by providing a composite set of chemical and structural signals. Here we investigated if cell-deposited ECM mimicked *in vivo* liver's stem cell microenvironment and facilitated hepatogenic maturation. Decellularization process preserved the fibrillar microstructure and a mix of matrix proteins in cell-deposited ECM, such as type I collagen, type III collagen, fibronectin, and laminin that were identical to those found in native liver. Compared with the cells on tissue culture polystyrene (TCPS), bone marrow mesenchymal stem cells (BM-MSCs) cultured on cell-deposited ECM showed a spindle-like shape, a robust proliferative capacity, and a suppressed level of intracellular reactive oxygen species, accompanied with upregulation of two superoxide dismutases. Hepatocyte-like cells differentiated from BM-MSCs on ECM were determined with a more intensive staining of glycogen storage, an elevated level of urea biosynthesis, and higher expressions of hepatocyte-specific genes in contrast to those on TCPS. These results demonstrate that cell-deposited ECM can be an effective method to facilitate hepatic maturation of BM-MSCs and promote stem-cell-based liver regenerative medicine.

1. Introduction

Liver failure as a serious health problem currently only relies on clinical transplantation surgery [1]. Due to the high cost of surgical procedures, shortage of donors' liver grafts, and major immune rejections, cell-based liver tissue engineering instead sparked immense attraction in the treatment of end-stage liver cirrhosis and infections [2]. An amount of bioartificial liver support devices has been developed to prolong patients' lives that are mostly based on cell therapy using human [3] or animal hepatocytes [4]. Animal studies have shown that these devices temporarily improved or replaced liver functions such as urea, bile acids, and lipid metabolism [5]. However, this technology is limited because of the scarcity of human autologous hepatocytes and the risk of rejection to xenogenic cells [6].

Mesenchymal stem cells (MSCs) as a promising source for liver regenerative medicine, compared with mature

hepatocytes, have advantages in various tissue sources, robust self-renewal potential, multilineage differentiation capacity, and immunological tolerance [7]. There is increasing evidence that MSCs have the potential to develop into hepatocyte-like cells *in vitro*, not only expressing hepatocyte-specific genes and proteins but also metabolizing urea and synthesizing albumin [8]. A previous clinical trial demonstrated that transplantation of bone marrow mesenchymal stem cells (BM-MSCs) improved short-term efficacy and long-term prognosis of liver failure patients [9]. However, cell transplantation therapy toward clinical applications remains challenging due to the poor efficiency of stem cell transdifferentiation and relatively lower biological functions in contrast to mature hepatocytes [10].

Extracellular matrix (ECM), providing biophysical and chemical signals, plays a pivotal role in stem cell adhesion, migration, proliferation, differentiation, and matrix remodeling [11]. Ouchi et al. demonstrated that coating of type I collagen and fibronectin enhanced the expression of liver-specific genes in primary hepatocytes [12]. ECM proteins, such as collagens and laminin, mixed with growth factors were potent to facilitate stem cells differentiating to hepatic lineage [13]. In addition, threedimensional (3D) bioscaffolds were developed to mimic *in vivo* extracellular matrix microenvironment to support cell survival and hepatic differentiation of MSCs and embryonic stem cells [14]. A recent report showed that decellularized biomatrix from liver organ largely preserved the structural and componential characteristics of the original tissue network and improved functions of adult hepatocytes [15]. Moreover, from the view of the interactions between cells and environment, cell-deposited ECM membrane preserved topographical structures and composition of various proteins to facilitate cells rapidly forming *in vivo* fibrillar adhesions, evidenced by links between $\alpha_5\beta_1$ integrin, paxillin, and fibronectin [16]. Numerous studies have been reported that ECM is essential to maintain differentiated phenotypes and liver-specific functions in primary hepatocytes [17]. Therefore, ECM is essential to construct *in vivo* stem cell microenvironment [18] and has potential to be utilized in stem cell *in vitro* expansion and differentiation [19].

In the current study, we obtain decellularized ECM deposited by BM-MSCs and hypothesize that cell-derived ECM provides natural stem cell extracellular microenvironment, improves MSC proliferation, and facilitates MSC differentiating to hepatocyte-like cells. Our long-term goal is to develop a suitable therapeutic strategy by utilizing decellularized ECM to produce sufficient functional hepatocytes for liver tissue engineering and treatment of chronic liver diseases.

2. Materials and Methods

2.1. Decellularization of Cell-Deposited ECM. Tissue culture polystyrene (TCPS) plates (Corning, Tewksbury, MA, USA) were firstly pretreated with 0.2% gelatin solution (Sigma-Aldrich, St. Louis, MO, USA) for 1 h at 37°C, followed by 1% glutaraldehyde (Sigma) and 1 M ethanolamine (Sigma) for 30 min separately at room temperature. BM-MSCs (Lonza Group Ltd., Basel, Switzerland) were seeded on pretreated plates in α-MEM medium (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone (Invitrogen, Carlsbad, CA, USA). After reaching 90% confluence, 100 μM of L-ascorbic acid phosphate (Sigma) was added, and cells were cultured for additional 8 days. To decellularize cell-deposited ECM, cells were removed by PBS supplemented with 0.5% Triton X-100 (Sigma) and 20 mM NH_4OH (Sigma) for 5 min at 37°C, rinsed with PBS, and stored at 4°C for future use.

2.2. Scanning Electron Microscopy (SEM) of Cell-Deposited ECM. Decellularized cell-deposited ECM were fixed in 4% paraformaldehyde (Sigma) and dehydrated in a series of alcohol at increasing concentrations (50%, 75%, 80%, 95%, and 100% solution). The morphology of decellularized ECM was examined by a scanning electron microscope (SEM S-520; Hitachi High-Technologies, Tokyo, Japan).

2.3. Immunofluorescence Staining. ECM was fixed in ice cold methanol for 10 min, blocked in 1% BSA, and incubated in appropriately diluted primary antibodies: antitype I collagen, antitype III collagen, antifibronectin, antilaminin (Abcam, Cambridge, MA, USA) and antidecorin (Santa Cruz Biotechnology, Dallas, TX, USA). After three rinses with PBS, ECM was incubated with a secondary antibody (Alexa Fluor 488 donkey anti-mouse IgG [H + L] or Alexa Fluor 488 donkey anti-rabbit IgG [H + L]) (Invitrogen). The fluorescence images were obtained by an IX71 fluorescence microscope (Olympus Corporation, Tokyo, Japan) and processed with Image-ProPlus software (Media Cybernetics Inc, Rockville, MD, USA).

2.4. Cell Culture and Fluorescein Diacetate (FDA) Staining. BM-MSCs were seeded in 24-well plates at a density of 1,000 cells/well at 37°C with 5% CO_2 under two different conditions: TCPS and ECM. The medium was changed every other day. Cells were washed with PBS and then incubated in FDA (5 μg/mL; Sigma) solution at 37°C for 10 min. After rinsing with PBS, fluorescent images were captured by an Olympus IX71 microscope and processed with Image-ProPlus software.

2.5. Cell Proliferation Assay. As described previously [20], BM-MSCs ($n = 5$) were lysed, and the amount of DNA was measured with Quant-iT PicoGreen dsDNA assay kit (Invitrogen) using a SynergyMx Multimode Reader (BioTek, Winooski, VT, USA) as described by the manufacturer.

2.6. Measurement of Intracellular Reactive Oxygen Species. Intracellular reactive oxygen species (ROS) generation was measured with 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma). In brief, 2×10^5 cells ($n = 4$) were incubated in 10 μM of DCFH-DA for 20 min at 37°C. DCF fluorescence was measured by a BD dual laser FACS Calibur (BD Biosciences, San Jose, CA, USA) with 10,000 events collected

Table 1: Primers used for real-time RT-PCR.

Gene	Primer sequence (5′-3′)	GeneBank accession
GAPDH	F: AGAAAAACCTGCCAAATATGATGAC R: TGGGTGTCGCTGTTGAAGTC	NM_002046
CuZn-SOD	F: GGTGGGCCAAAGGATGAAGAG R: CCACAAGCCAAACGACTTCC	NM_000454.4
Mn-SOD	F: GGGGATTGATGTGTGGGAGCACG R: AGACAGGACGTTATCTTGCTGGGA	BC012423.1
ALB	F: TGCTTGAATGTGCTGATGACAGGG R: AAGGCAAGTCAGCAGGCATCTCATC	NM_000477.5
TDO2	F: TCCTCAGGCTATCACTACCTGC R: ATCTTCGGTATCCAGTGTCGG	NM_005651.3
CYP3A4	F: AAGTCGCCTCGAAGATACACA R: AAGGAGAGAACACTGCTCGTG	NM_017460.5
CYP7A1	F: AGAAGCATTGACCCGATGGAT R: AGCGGTCTTTGAGTTAGAGGA	NM_000780.3
CK18	F: AATGGGAGGCATCCAGAACGAGAA R: GGGCATTGTCCACAGTATTTGCGA	NM_199187.1
HNF-4A	F: GGAACATATGGGAACCAACG R: AACTTCCTGCTTGGTGATGG	NM_178849.2

for each sample, and data were analyzed with WinMDI (Windows Multiple Document Interface for Flow Cytometry) 2.9 software.

2.7. Surface Markers Characterized by Flow Cytometry. Samples ($n = 3$) of each 3×10^5 BM-MSCs were firstly incubated in PBS containing 0.1% ChromPure Human IgG whole molecule (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) and 1% NaN_3 then in appropriately diluted mouse monoclonal antibodies of CD34, CD45, CD90, and CD105 (Abcam). After washing with cold PBS, BM-MSCs were incubated with the secondary antibody (Alexa Fluor 488 donkey anti-mouse IgG [H + L]). Negative controls received equivalent amounts of isotype-matched antibodies (Abcam). Cells were analyzed on a BD dual laser FACS Calibur (BD Biosciences, San Jose, CA, USA) with 10,000 events collected for each sample, and data were analyzed with WinMDI 2.9 software.

2.8. Hepatic Differentiation of BM-MSCs. To induce hepatic differentiation, BM-MSCs cultured on TCPS and ECM were incubated in DMEM/F12 medium (Thermo Fisher Scientific) supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL fungizone, 20 ng/mL of HGF (PeproTech Asia, Rehovot, Israel), and 10 ng/mL FGF-4 (PeproTech Asia) for 2 weeks. Thereafter, differentiation medium was changed to maturation medium (DMEM/F12 supplemented with 10% FBS, 20 ng/mL oncostatin M [sigma], 100 μM dexamethasone [sigma], ITS Premix (BD Biosciences, San Jose, CA, USA), 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL fungizone) and maintained for another 2 weeks. Medium was collected and stored at −80°C for the measurement of urea concentration.

2.9. Periodic-Acid-Schiff (PAS) Staining for Glycogen. Differentiated cells on day 21 and day 28 were fixed in 4% paraformaldehyde and then incubated in 1% periodic acid solution (Sigma) for 5 min at room temperature. Followed by rinsing with PBS, cells were incubated in Schiff's reagent (Sigma) for 15 min. Images were captured by an Olympus IX71 microscope.

2.10. Evaluation of Urea Synthesis. The concentration of urea in culture medium was measured by a commercially available QuantiChrom urea assay kit (BioAssay Systems, Hayward, CA, USA) according to the manufacturer's instructions. The absorbance was measured by a SynergyMx Multimode Reader at 520 nm.

2.11. Real-Time Reverse Transcription-Polymerase Chain Reaction (Real-Time RT-PCR). Total RNA was extracted from samples ($n = 4$) by TRIzol reagent (Invitrogen). For each sample, 1 μg of total RNA was reverse transcribed by PrimeScript RT reagent kit as described by the manufacturer (TaKaRa, Mountain View, CA, USA). To quantify the mRNA, cDNA equivalent to 20 ng of total RNA was used for real-time PCR analysis with GoTaq qPCR Master Mix (Promega, Madison, WI, USA). Genes including CuZn superoxide dismutase (CuZn-SOD), Mn superoxide dismutase (Mn-SOD), albumin (ALB), tryptophan 2,3-dioxygenase (TDO2), cytochrome P450 7A1 (CYP7A1), cytochrome P450 3A4 (CYP3A4), cytokeratin 18 (CK18), and hepatocyte nuclear factor 4 alpha (HNF-4A) were detected. GAPDH was as an internal standard. The primer sequences are listed in Table 1. real-time PCR was performed by an ABI7500 Realtime PCR Detection (Applied Biosystems, Foster City, CA, USA) and calculated with computer software (Perkin-Elmer, Wellesley, MA, USA).

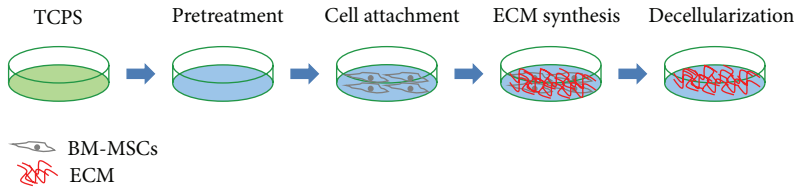
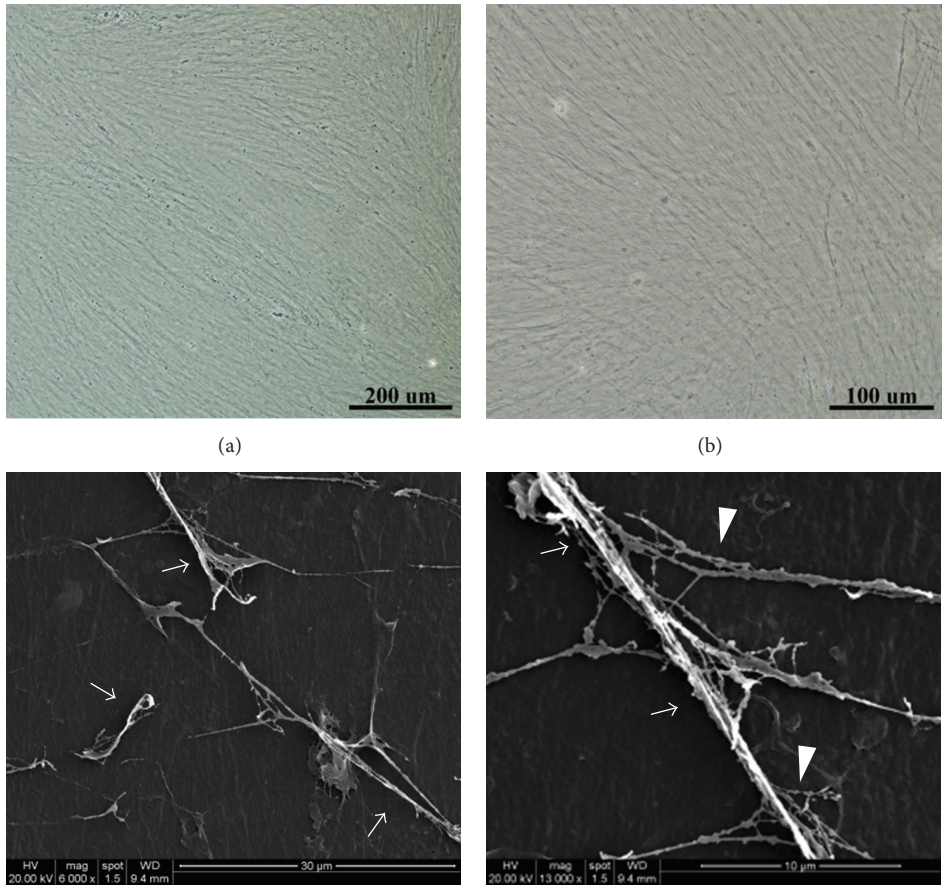


Figure 1: The protocol of preparing decellularized ECM. Conventional TCPS flasks were pretreated with gelatin, glutaraldehyde, and ethanolamine. L-ascorbic acid phosphate was supplemented to increase ECM production by BM-MSCs. ECM was decellularized by treating with Triton X-100 and NH_4OH.



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

Figure 2: Characterization of decellularized cell-deposited ECM. The morphology of cell-deposited ECM showed fibrous structure under a light microscope ((a) scale bar = 200 μm; (b) scale bar = 100 μm), and SEM revealed fibrillar microstructure of ECM ((c) scale bar = 30 μm; (d) scale bar = 10 μm). A bundle of fibrillar collagen fibers (arrow) and beaded fibers (arrowhead) are observed.

2.12. Statistical Analysis. All data are expressed as mean ± standard error (S.E.). Statistical differences between two groups were determined by one-way analysis of variance (ANOVA) followed by Student's unpaired t-test with SPSS software package (SPSS Inc, Chicago, IL, USA). Significance is indicated by a P value of <0.05.

3. Results

3.1. Characterization of Decellularized ECM. The preparation process of decellularized ECM deposited by BM-MSCs was described in Figure 1. To optimize decellularized ECM for cell culture and differentiation, pretreatments with gelatin, glutaraldehyde, and ethanolamine were used to increase the adhesive strength between culture surface and ECM. L-ascorbic acid phosphate was added in culture medium to increase the generation of ECM and the treatment of Triton X-100 and NH_4OH was used to remove original cells and cellular residues.

Cell-deposited ECM after decellularization showed a fibrous structure (Figures 2(a)-2(b)) and the microstructure of fibrillar network was further observed via SEM

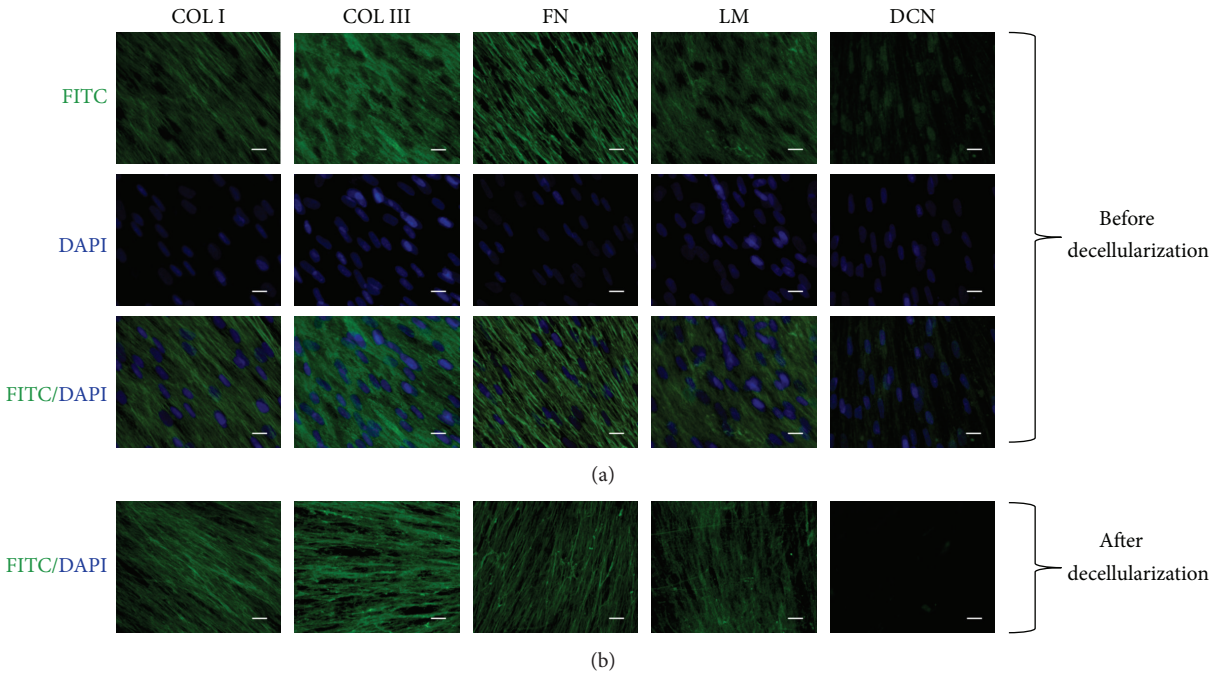


Figure 3: Detecting of multiply matrix proteins and cell nuclei in decellularized ECM before (a) and after (b) decellularization. The procedure of decellularization results in complete removal of original BM-MSCs in ECM deposition. Immunofluorescence staining of ECM retained type I collagen, type III collagen, fibronectin, and laminin except decorin. Scale bar = 20 μm.

(Figures 2(c)-2(d)). We found bundles of fibers (825.4 ± 114.3 nm in diameter), collagen fibrils (320.6 ± 49.5 nm in diameter), and beaded filaments that were possibly attached glycosaminoglycans. The gaps between fibers were left by decellularization of deposited cells, evidenced by a similar diameter of fibroblasts (4.7–11.7 μm).

Immunofluorescence staining revealed that decellularization preserved most matrix proteins that were identical to native liver such as type I collagen, type III collagen, fibronectin, and laminin. However, decorin as a small proteoglycan that was expressed in cytoplasm or pericellular matrix was undetectable after decellularization. DAPI staining of ECM before and after decellularization confirmed the success of removing cellular residues (Figure 3).

3.2. Cell Culture on Decellularized ECM. When cultured on ECM, BM-MSCs formed a small and spindle-like shape and maintained uniformly morphological appearance. In contrast, cells on TCPS showed a large and flattened morphology (Figure 4(a)). DNA content of BM-MSCs in 24-well plates was measured to evaluate proliferative activity. For cells cultured on ECM, DNA content is 5.4-fold as that of TCPS group (91.8 ± 6.1 ng/well versus 17.1 ± 3.9 ng/well) after 5-day culture (Figure 4(b)). With regard to intracellular ROS, cells on ECM showed a dramatically suppressed level in contrast to TCPS group (mean fluorescence intensity 263.2 ± 25.9 versus 823.4 ± 45.2), indicating that cell-deposited ECM was an effective culture system to reduce oxidative stress (Figure 4(c)). Moreover, mRNA expressions of CuZn-SOD (Figure 4(d)) and Mn-SOD (Figure 4(e)) were elevated by 81.2% ± 6.7% and 59.1% ± 10.1%, respectively, in the cells cultured on ECM than cells on TCPS. These results indicate that cell-deposited ECM abolishes redundant free radicals in BM-MSCs through superoxide dismutases pathway.

3.3. Immunophenotypes of BM-MSCs on ECM. Flow cytometry analysis was performed to characterize the immunophenotypes of BM-MSCs cultured on TCPS or ECM. For standard MSC surface markers, the cells were strongly positive for CD90 (99.9% in TCPS versus 99.7% in ECM) and CD105 (96.5% in TCPS versus 81.5% in ECM), whereas the cells were negative for CD34 (0.5% in TCPS versus 0.8% in ECM) and CD45 (1.6% in TCPS versus 1.4% in ECM) (Figure 5). These data suggested that BM-MSCs expanded on decellularized ECM exhibited the same surface phenotypes as those cultured on TCPS.

3.4. Effect of ECM on Liver-Specific Functions. BM-MSCs were induced to hepatogenesis in differentiation medium for 2 weeks and incubated in maturation medium for another 2 weeks. The morphology of BM-MSCs was changed from a spindly to round shape when cells were induced to differentiate on ECM. The results of PAS staining were positive in both TCPS and ECM groups on day 21, but the staining was significantly more intensive in ECM group compared with TCPS group on day 28 (Figure 6(a)). The result indicated that the ability of glycogen storage was enhanced in the differentiated cells on cell-deposited ECM.

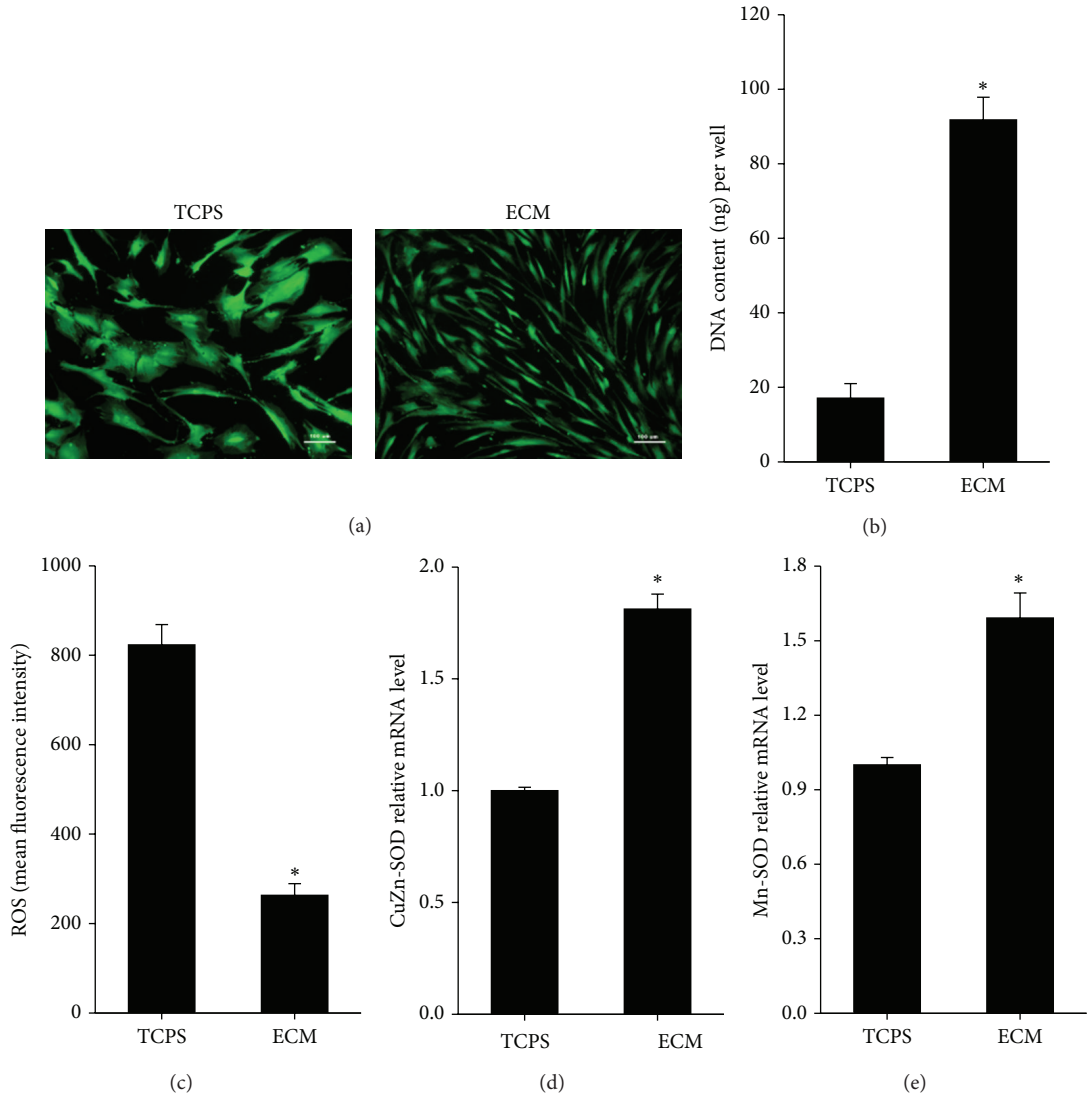


Figure 4: Cell culture of BMSCs on TCPS and cell-deposited ECM. Morphological changes and density of BMSCs were detected by FDA staining (a). ECM improved proliferation of BMSCs in 5-day culture (b). BMSCs on ECM showed a lower level of ROS than the cells on TCPS (c). mRNA levels of CuZn-SOD (d) and Mn-SOD (e) in the cultured cells were determined by real-time RT-PCR. Scale bar = 100 μm. All values are mean ± S.E. of independent 4~5 experiments performed (proliferation assay $n = 5$; ROS assay $n = 4$; PCR $n = 4$); $^{*}P < 0.05$.

The results of urea synthesis in differentiated BM-MSCs showed no significant difference on day 7 (8.8 ± 0.1 μg/mL/24 h versus 9.0 ± 0.2 μg/mL/24 h) or on day 14 (9.3 ± 0.4 μg/mL/24 h versus 9.1 ± 0.3 μg/mL/24 h) in TCPS and ECM groups. However, the urea concentration of differentiated BM-MSCs cultured on ECM was 8.7% higher than that of TCPS group on day 21 (10.5 ± 0.2 μg/mL/24h versus 9.7 ± 0.1 μg/mL/24 h, $P < 0.05$) and 7.3% higher on day 28 (10.9 ± 0.2 μg/mL/24 h versus 10.2 ± 0.2 μg/mL/24 h, $P < 0.05$) (Figure 6(b)). The data suggested that ECM improved the biological function of urea secretion in hepatocyte-like cells from BM-MSCs.

3.5. Expressions of Hepatocyte-Specific Genes in Differentiated BM-MSCs. Figure 7 shows relative mRNA expression of hepatocyte-specific genes, such as ALB, TDO2, CYP7A1, CYP3A4, CK18, and HNF-4A. Cell-deposited ECM significantly increased the expression of ALB compared with TCPS group by 89.9% on day 14 and by 114.9% on day 28 (Figure 7(a)). BM-MSCs cultured on ECM expressed a higher level of TDO2 than cells on TCPS (by 25.1% on day 14 and by 109.4% on day 28) (Figure 7(b)). mRNA of CYP7A1 was higher in the cells on ECM than TCPS group (by 123.6% on day 14 and by 33.5% on day 28) (Figure 7(c)). Similarly, cell-deposited ECM increased CYP3A4 mRNA by 54.8% on day 14 and by 57.0% on day 28 higher than TCPS (Figure 7(d)). With regard to genes of CK18 (Figure 7(e)) and HNF-4A (Figure 7(f)), ECM significantly upregulated mRNA levels in differentiated BM-MSCs compared with TCPS group (by 21.2% on day 28 of CK18 and by 84.1% on day 28 of HNF-4A).

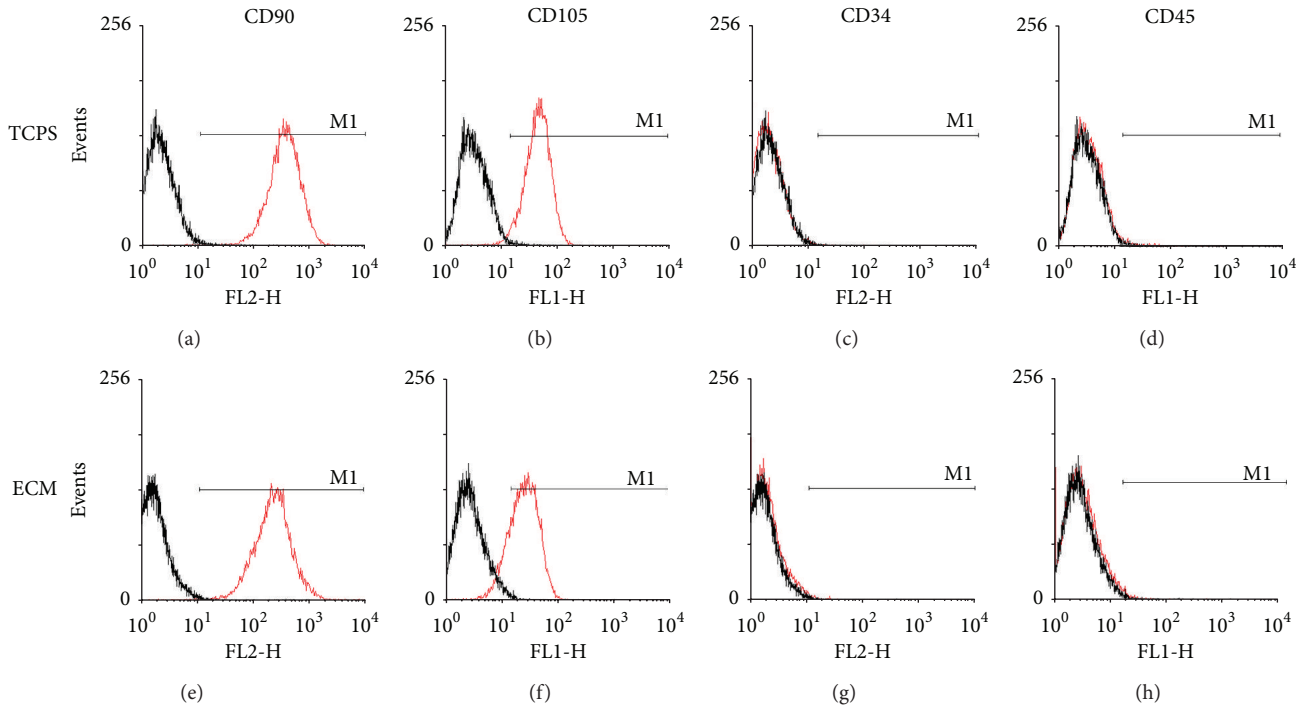


Figure 5: Immunophenotype analysis revealed that BMSCs cultured on TCPS and ECM were positive for CD90 and CD105 but negative for CD34 and CD45.

4. Discussion

In the current study, we used decellularized ECM deposited by BM-MSCs as cell culture substrate to mimic *in vivo* stem cell microenvironment and examined the effects of cell-deposited ECM on cell proliferation, expressions of surface markers, stress of intracellular ROS, and hepatic lineage differentiation. Our data showed that the process of decellularization preserved the structure and most matrix components of ECM. In contrast to conventional TCPS monolayer culture system, BM-MSCs expanded on ECM showed similar expressions of stem cell surface markers, significantly increased cell proliferation, and attenuated intracellular ROS. Moreover, decellularized ECM promoted the lineage-specific differentiation of BM-MSCs into hepatocyte-like cells, indicated by stronger staining of glycogen, enhanced urea synthesis, and higher expressions of hepatocyte-specific genes.

MSCs have been investigated for an alternative source of cell-based liver regenerative medicine because they have abilities of self-renewal and multilineage differentiation, and there are no ethical issues compared with embryonic stem cells. Sufficient cell number is the primary requirement for cell transplantation because mature hepatocytes have less self-renewal ability. Conventional TCPS monolayer culture system is hard to mimic tissue-specific extracellular microenvironment and results in cell senescence and loss of multipotency of MSCs [21]. Our data showed that cell-deposited ECM successfully accelerated cell growth of BM-MSCs by approximately 4-fold higher than TCPS culture system while maintaining stem cell characteristics, consistent with previous studies [22]. ECM microenvironment also induced the increase and translocation of cyclin D to control cell cycle progression through G_1 phase to S phase [19]. High level of telomerase activity when cells were exposed to bone marrow-like ECM was possibly responsible for improved cell self-renewal [23].

In vivo, specific extracellular regulatory microenvironment consists of cytokines, growth factors, and a complex mixture of matrix components to control cell behavior and biological functions of stem cells. Native ECM as an essential part of stem cell microenvironment provides a structural scaffold to resist tensile and compressive stress and functions as a tight connection to cytoskeleton of cells through cell-surface receptors to enable cells to sense and respond to mechanical and chemical signals [24]. To reconstruct liver's stem cell microenvironment we attempted to decellularize native cell-derived ECM while preserving matrix compositions. The fibrillar structure of decellularized ECM was more similar to *in vivo* native ECM than monolayer system [16]. More importantly, decellularized ECM deposited by BM-MSCs were detectable for complicated matrix proteins, including type I collagen, type III collagen, fibronectin, and laminin. However, matrix component of decorin as a small proteoglycan binding to type I collagen fibrils [25] was undetectable after decellularization, suggesting that decorin was soluble and infirmly connected to ECM. The role of decorin in decellularized ECM on cell proliferation and differentiation needs to be elucidated in future.

Accumulation of intracellular ROS, such as superoxide anions and hydrogen peroxide, is thought to cause cell death and inhibit lineage-specific differentiation [26]. Although the mechanisms underlying the influence of ROS

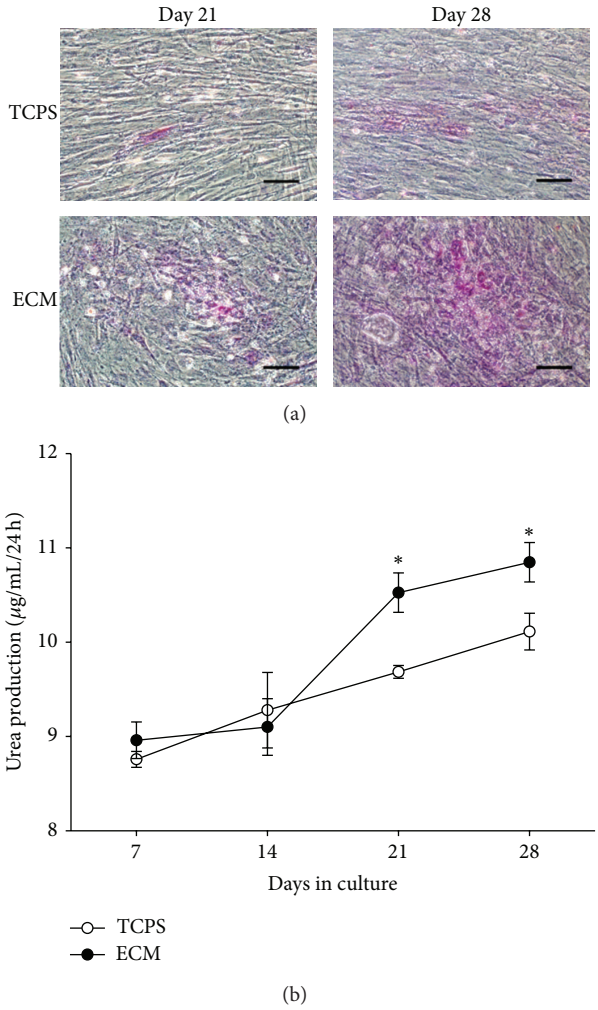


Figure 6: Cell-deposited ECM promoted hepatic differentiation of BMSCs. PAS staining of differentiated BMSCs on days 21 and 28 cultured on TCPS and decellularized ECM (a). Urea biosynthesis of differentiated BMSCs on TCPS and decellularized ECM on days 7, 14, 21, and 28, respectively, (b). Scale bar = 100 μm. All values are mean ± S.E.; $^{*}P < 0.05$.

on hepatic differentiation of MSCs are poorly understood, increased oxidative stress produced by mitochondria and nicotinamide adenine dinucleotide phosphate oxidase has been demonstrated to induce hepatocyte apoptosis and liver inflammation [27]. It has also been reported that activation of Notch signal pathway protects the survival and biological functions of hepatocytes from ischemia injury by scavenging ROS in mice [28]. Evidence obtained from our present studies showed a significantly lower level of oxidative stress in BM-MSCs cultured on ECM than TCPS. Therefore, it is possible that decellularized ECM promoted hepatic maturation of BM-MSCs by attenuating oxidative stress.

The application of whole decellularized organ is considered as a promising method to reconstruct hepatocyte specific microenvironment and improve the efficiency of MSC transdifferentiation into hepatocyte-like cells [29]. The scarce sources of autologous or allogenic organs and the risk of immunological rejection of xenogenic organs are still obstacles. In addition, various synthetic scaffolds were reported to be used in liver tissue engineering. A collagen-coated poly (lactic-coglycolic acid) (PLGA) scaffold that was fabricated to mimic 3D microenvironment of native liver was shown to support cell survival and increase expressions of liver-specific genes in MSCs [30]. However, the lack of vascular microstructure and simplicity of matrix chemistry remain issues for the design of biomodified scaffolds. To our knowledge, this is the first time to demonstrate that decellularized cell-deposited ECM promoted hepatic maturation from BM-MSCs into hepatocyte-like cells with high expression of hepatocyte-specific genes and increased levels of urea biosynthesis and glycogen storage. The hepatocyte-specific gene expression in TCPS culture system increases during differentiation period; however, long-term culture significantly alters the characteristics of MSCs, evidenced by decreased differentiation potential, high expression of aging genes [31], and shortened telomere length [32]. This jeopardizes the use of MSCs as therapeutic application. Thus, hepatocyte-specific biofunctions of MSCs, that is, improved by ECM culture system in relatively short period will benefit liver tissue engineering.

Type I collagen has been reported to promote hepatic maturation of human pluripotent stem cells [33] and to maintain differentiated hepatocyte phenotypes [34]. In addition, peptides from laminin α1 support the biological functions in hepatocytes [35]. In this study, we revealed that cell-deposited ECM was consisted of various matrix proteins that are identical to native liver [29]. Although it is known that various kinds of ECM proteins have an efficiently promotive effect on hepatogenesis of MSCs, the key bioactive component is still unidentified. Meanwhile, ECM derived from different cells supported lineage-specific differentiation, evidenced by opposite influence of osteogenic-specific and adipogenic-specific ECM on controlling differentiation of MSCs into osteoblasts and adipocytes [36] and supportive effect of decellularized ECM derived from synovium MSCs on chondrogenesis instead of osteogenesis [18]. We hypothesize that preservation of the native architecture and complex matrix chemistry provides the mix of structural and chemical signals to drive BM-MSCs differentiation into mature hepatocytes [37].

The underlying relationship between BM-MSCs and decellularized ECM was possibly related to mitogen-activated protein kinase (MAPK) signaling cascades. Decellularized ECM suppressed the phosphorylation of focal adhesion kinase (FAK) [38] but induced sustained activation of MAPK and the downstream extracellular signal regulated kinases 1 and 2 (ERK1/2) [19]. Moreover, Xu et al. demonstrated that biosysthesis of bile acid was dependent on the activation of p38 MAPK in primary hepatocytes [39]. In this regard, it is possible that decellularized ECM enhanced the activation of MAPK signaling cascades and thus improved hepatogenesis of BSMCs. However, the underlying mechanism of hepatic differentiation of BM-MSCs on decellularized ECM needs to be elucidated in future studies.

In conclusion, our results indicate that decellularization of cell-deposited ECM preserves the natural framework and

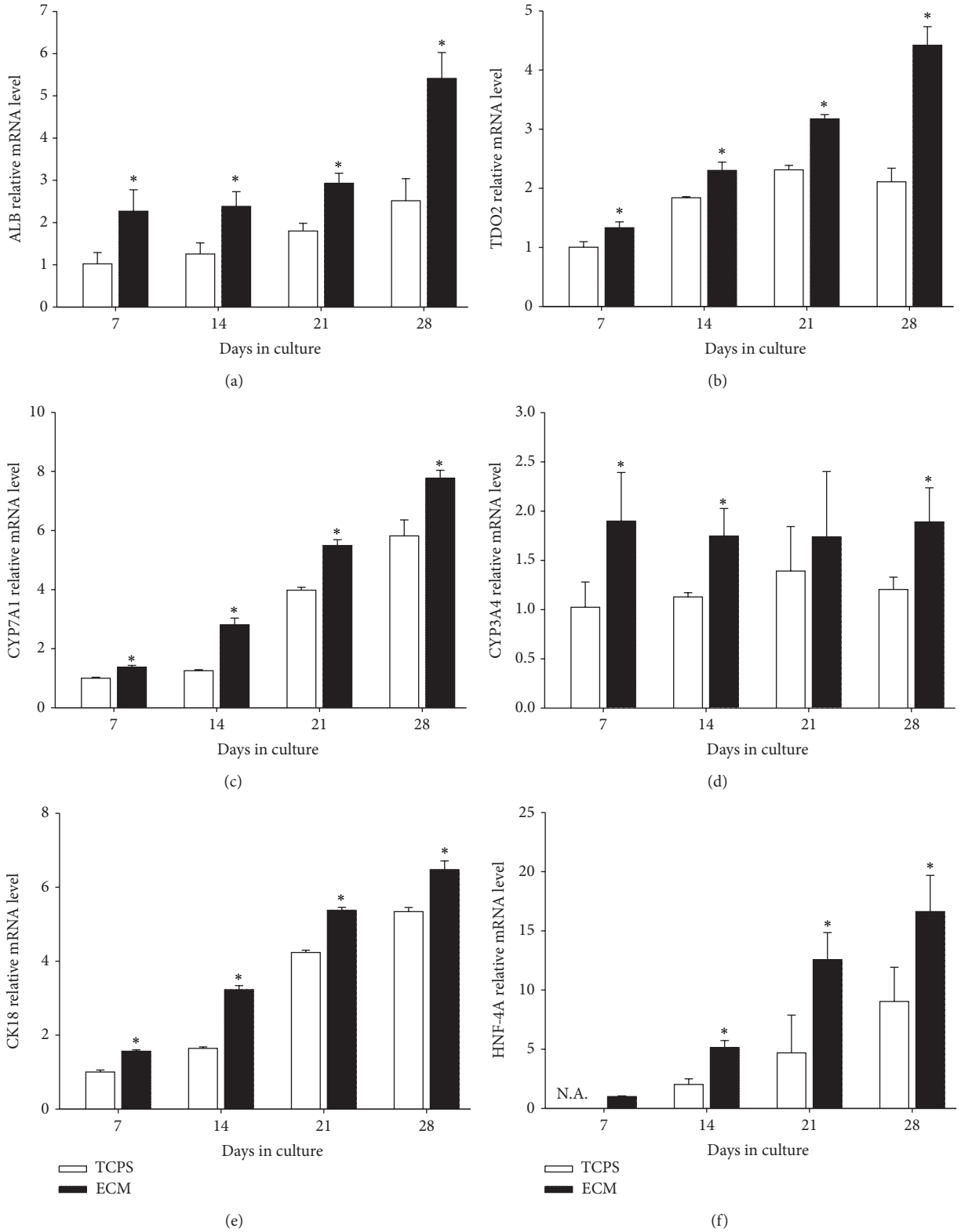


Figure 7: Expressions of hepatocyte-specific genes including ALB (a), TDO2 (b), CYP7A1 (c), CYP3A4 (d), CK18 (e), and HNF-4A (f) were examined by real-time RT-PCR on days 7, 14, 21, and 28, respectively. All values are mean ± S.E.; $^{*}P < 0.05$; N.A.: not available.

matrix proteins. ECM culture system maintains stem cell phenotypes, increases cell proliferative rate, and suppresses oxidative stress in BM-MSCs. We also demonstrate that cell-deposited ECM closely mimics *in vivo* liver's stem cell microenvironment and promotes the differentiation of BM-MSCs to adult liver fates. Our findings therefore contribute to stem-cell-based liver tissue engineering, bioartificial liver development, and clinical stem cell therapies to treat chronic liver damage.

Abbreviations

MSCs:	Mesenchymal stem cells
ECM:	Extracellular matrix
BM-MSCs:	Bone marrow mesenchymal stem cells
TCPS:	Tissue culture polystyrene
ROS:	Reactive oxygen species
SOD:	Superoxide dismutase
ALB:	Albumin
TDO2:	Tryptophan 2,3-dioxygenase
CYP7A1:	Cytochrome P450 7A1
CYP3A4:	Cytochrome P450 3A4
CK18:	Cytokeratin 18
HNF-4A:	Hepatocyte nuclear factor 4 alpha.

Conflict of Interests

Authors have no conflict of interests to disclose.

Acknowledgments

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Research Article

Isolation and Characterization of Chicken Dermis-Derived Mesenchymal Stem/Progenitor Cells

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Dermis-derived mesenchymal stem/progenitor cells (DMS/PCs) were isolated from the skin tissue of 16-day-old chick embryos and then characterized by immunofluorescence and RT-PCR. We found that primary DMS/PCs could be expanded for 15 passages. Expression of β-integrin, CD44, CD71, and CD73 was observed by immunofluorescence and RT-PCR. Passage 3 DMS/PCs were successfully induced to differentiate into osteoblasts, adipocytes, and neurocytes. The results indicate the potential for multilineage differentiation of DMS/PCs that may represent an ideal candidate for cellular transplantation therapy.

1. Introduction

Mesenchymal stem cells (MSCs) were first discovered in bone marrow (BMSCs), which have a strong proliferative capacity and can be differentiated into adipocytes [1, 2], osteoblasts [3–5], myoblasts [6–8], and neurons [9, 10].

However, the proliferation, differentiation, and number of BMSCs are significantly decreased with aging. In addition, because of possible virus infection [11], researchers began to search for MSCs in other tissues. In recent years, MSCs have been found in muscles, amniotic fluid, umbilical cord blood, fat, and other tissues [12–14].

The dermis contains mostly differentiated cells including fibroblasts that only participate in scar tissue formation during skin repair [15]. Therefore, the dermis is often regarded as a negative control for studies of stem cells [16]. Following isolation and characterization of BMSCs in the 1990s [2], significant progress has been made in studies of dermis-derived mesenchymal stem/progenitor cells (DMS/PCs), including their separation and culture. Moreover, DMS/PCs can be induced to differentiate into osteoblasts, adipocytes, and ectodermal cell types. Considering the easy accessibility of DMS/PCs, these cells have become an ideal cellular source in tissue engineering.

Current research of stem cells focuses on humans, mice, rabbits, and other mammals, but little research has been performed on poultry. As an animal model, the chicken possesses abundant dermal tissues. Furthermore, the chicken is an endemic species that is important in the global economy. In this study, we carried out a pilot study on the separation, culture, and differentiation potential of chicken DMS/PCs.

2. Materials and Methods

2.1. Isolation and Culture of DMS/PCs. Animal experiments were performed in accordance with the guidelines established by the Institutional Animal Care and Use Committee of the Chinese Academy of Agriculture of Sciences.

Dorsal skin tissues were isolated from 30 16-day-old chick embryos. The dermal layer was isolated from the epidermal layer by digestion with 0.25% dispase II (Gibco, Carlsbad, CA, USA) for 1.5–2 h at 37°C. The dermis was cut into approximately 1 cm^2 pieces and then digested with 0.25% trypsin (Gibco) for about 15 min. Then, the enzymatic activity was neutralized with fetal bovine serum (FBS) (Gibco). The digested tissue was passed through a 200 μm mesh filter and then centrifuged at 1200 r/min for 6 min at room

Table 1: Primer sequences used in RT-PCR assay.

Gene	Primer sequence	Tm (°C)	Fragment (bp)
β-integrin	F 5′ GAACGGACAGATATGCAACGG 3′ R 5′ TAGAACCAGCAGTCACCAACG 3′	60	300
CD44	F 5′ CATCGTTGCTGCCCTCCT 3′ R 5′ ACCGCTACACTCCACTCTTCAT 3′	58	290
CD71	F 5′ CCCAGGCTTCCCTTCGT 3′ R 5′ GGGCTCCAATCACAACATAC 3′	56	305
CD73	F 5′ TCCCGTTTCAAGGGTCAG 3′ R 5′ GTCCTCCAATAACAACATCCACTC 3′	52.6	310
AKP	F 5′ TTACCTCTGCGGCGTCAA 3′ R 5′ CCTGTCCAGCTCATACACCATA 3′	59.1	556
OPN	F 5′ CAGAACAGCCGGACTTTC 3′ R 5′ CTTGCTCGCCTTCACCAC 3′	51	227
PPAR-γ	F 5′ CTGTCTGCGATGGATGAT 3′ R 5′ AATAGGGAGGAGAAGGAG 3′	47.3	199
LPL	F 5′ AGTGAAGTCAGGCGAAAC 3′ R 5′ ACAAGGCACCACGATT 3′	48.7	477
NF	F 5′ CCAGTCCGACCACAACAT 3′ R 5′ TCCTGGTACTCCCTCAAAT 3′	56	231
Nestin	F 5′ CAACGAGCCTACATTGCTAA 3′ R 5′ CTCATCTGGGAACTCACATTC 3′	56	289
GAPDH	F 5′ TAAAGGCGAGATGGTGAAAG 3′ R 5′ ACGCTCCTGGAAGATAGTGAT 3′	53	244

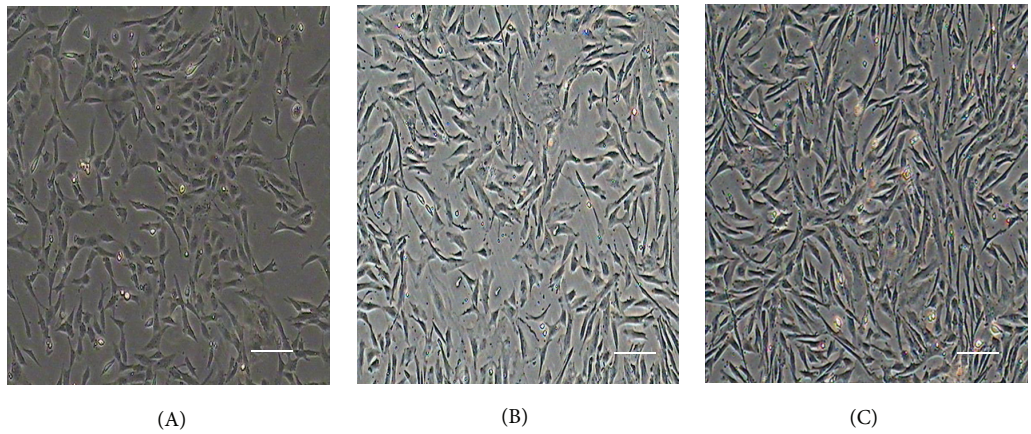
temperature. The supernatant was discarded, and the pellet was re-suspended with an optimized culture medium. The viability of DMS/PCs was determined by trypan blue exclusion. As a result, 1×10^4 cells were yielded from 1 cm^2 of chick embryo skin. The cell suspension was seeded in six-well plates and incubated at 37°C with 5% CO_2. After 48 h of culture, the cells were washed twice with PBS to remove nonadherent cells. At 70–80% confluence, the cells were passaged with 0.25% trypsin. Generally, after 3-4 passages, the cells were homogenous.

2.2. Optimization of Cell Culture Systems for DMS/PCs. DMS/PC culture at passage 3 was assessed in three culture systems: culture system I (L-Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS), culture system II (L-DMEM supplemented with 2 mM L-glutamine, 1 mM sodium pyruvate, and 10% FBS), and culture system III (L-DMEM supplemented with 20 ng/mL epidermal growth factor (EGF), 20 ng/mL basic fibroblast growth factor (bFGF), 10% FBS, 2 mM L-glutamine, and 1 mM sodium pyruvate). Cells were harvested and reseeded in six-well plates at 5×10^4 cells/well. The cells were cultured further and the generation time in each culture system was counted three times. Culture system III was subsequently used to culture DMS/PCs.

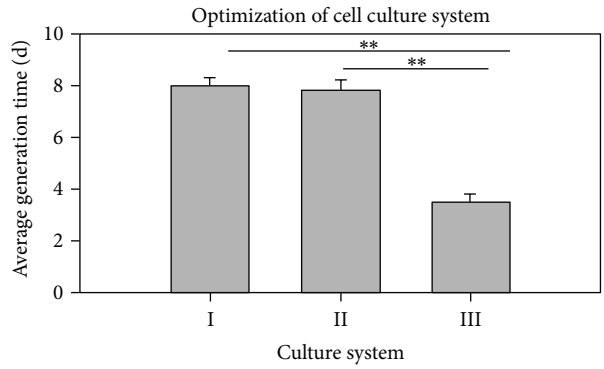
2.3. Markers of DMS/PCs. DMS/PCs were fixed in 4% paraformaldehyde for 15 min and then washed three times in PBS (5 min each). Cells were permeabilized with 0.2% Triton X-100 for 15–20 min and then washed three times (5 min each) in PBS. The cells were blocked with 10% normal goat serum (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 30 min and then incubated at room temperature for 1 h in 3% bovine serum albumin (BSA) containing the following antibodies: mouse anti-β-integrin (1 : 100; Abcam, Cambridge, MA, USA), mouse anti-CD44 (1 : 200; Abcam), mouse anti-nestin (1 : 200; Abcam), rabbit anti-synaptophysin (SYP) (1 : 100; Bioss, Beijing, China), rabbit anti-CD71 (1 : 200; Bioss), rabbit anti-CD73 (1 : 200; Bioss), rabbit anti-neurofilament (NF) (1 : 200; Bioss), or goat anti-β-III tubulin (1 : 200; Santa Cruz Biotechnology). Then, the cells were washed three times (10 min each) with PBS and then incubated in PBS containing secondary antibodies at 37°C for 1 h. Secondary antibodies were Cy5.5-conjugated goat anti-mouse and donkey anti-rabbit IgGs, and fluoroisothiocyanate-conjugated goat anti-rabbit and donkey anti-goat IgGs (Santa Cruz Biotechnology).

Cells were examined under a TE-2000-E inverted fluorescence microscope (Nikon, Yokohama, Kanagawa Japan). Cells were counterstained with DAPI (Sigma-Aldrich, St. Louis, MO, USA).

2.4. RT-PCR. RNA was isolated from cells using Trizol reagent (Invitrogen). cDNA was synthesized using a reverse transcription system (Takara, Dalian, Liaoning, China) and amplified by PCR using specific primers (Table 1). PCR products were visualized by 2% agarose gel electrophoresis.



(a)



(b)

Figure 1: (a) Morphology of primary and subcultured DMS/PCs. (A) After 24 h of primary culture, DMS/PCs exhibited a shuttle shape with clear boundaries and grew slowly. (B) After 5 days of primary culture, DMS/PCs showed polygonal and long shuttle shapes, most of which had protrusions, and gradually reached confluency. (C) Passage 3 DMS/PCs were fibroblast-like and homogeneous (scale bar: 100 μm). (b) Comparison of cell proliferation in different culture systems. Culture systems III was suitable for DMS/PC proliferation. Data are expressed as the means ± S.D. of triplicates ($^{**}P < 0.01$).

2.5. Adipogenic Differentiation of DMS/PCs. Cells were divided into two groups: induced and control groups. At 50–60% confluence, cells in the induced group were incubated in adipogenic medium containing 1 mM dexamethasone (Sigma), 0.5 mM isobutyl-methylxanthine (IBMX; Sigma), and 10 μg/mL insulin (Sigma). Cells in the control group were cultured in complete medium without any inducers. After 2 weeks of differentiation, the cells were stained with oil red O to assess intracellular lipid accumulation. RNA was also isolated for RT-PCR analysis.

2.6. Osteogenic Differentiation of DMS/PCs. Passage 3 cells were seeded in six-well plates at 1×10^4 cells/well. The cells were also divided into inducted and control groups. At 50–60% confluence, cells in the induced group were cultured in osteogenic medium containing 0.5 mM dexamethasone (Sigma), 10 mM β-glycerophosphate (Sigma), and 50 μg/mL vitamin C. Cells in the control group were cultured in complete medium without any inducers. Media were changed every 2 days. After 2 weeks of differentiation, the capacity for calcium node formation was detected by alizarin red staining and osteoblast-specific gene expression was analyzed by RT-PCR.

2.7. Neurogenic Differentiation of DMS/PCs. Cells were seeded and divided into the two groups as described above. Neural-like cell differentiation was accomplished in L-DMEM supplemented with 10% FBS, 1 μM all-trans-retinoic acid, and 100 μM 2-mercaptoethanol (Sigma) [17]. After 10 days, the cells were harvested and neural-specific marker expression was detected by immunofluorescence and RT-PCR.

3. Results

3.1. Isolation, Culture, and Morphology of DMS/PCs. Primary cells isolated from the dermis adhered to the culture plates and began to elongate after 24 h (Figure 1(a)-A). After about 5 days, the cells exhibited a fibroblast-like morphology (Figure 1(a)-B) and grew to 80–90% confluence (Figure 1(a)-C).

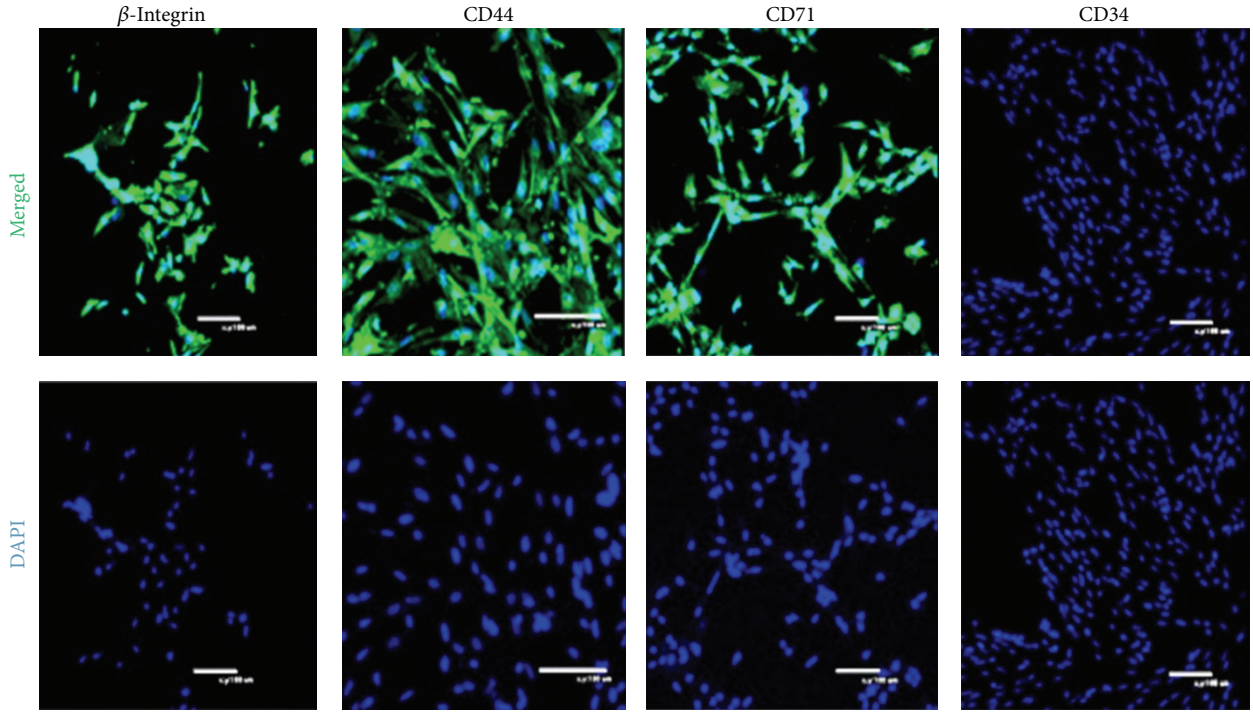


Figure 2: Surface antigen characterization of DMS/PCs at different passages. DMS/PCs expressed numerous surface markers such as β-integrin, CD44, CD71, and CD73 but not CD34 (a hematopoietic marker). Immunofluorescence showed that β-integrin and CD44 were positive, while CD34 was negative (scale bar: 100 μm).

3.2. Optimization of DMS/PC Culture. There was no significant difference between culture systems I and II ($P > 0.05$). The generation time was about 8 days for both systems. Culture system III and the other culture systems were significantly different and resulted in a generation time of about 3 days ($P < 0.01$) (Figure 1(b)). These results indicated that EGF and bFGF promote DMS/PC proliferation, and culture system III is suitable for expansion of DMS/PCs.

No obvious morphological differences were observed among passages, and the characteristics of the cells were stable after passaging. The cells were cultured to passage 16 and showed the representative appearance of senescence, such as blebbing and karyopyknosis in most cells. Moreover, cells cultured for more than 16 passages became detached from the plates.

3.3. Characterization of DMS/PCs

3.3.1. Markers of DMS/PCs. We detected markers of DMS/PCs by immunofluorescence and RT-PCR. The immunofluorescence (Figure 2) and RT-PCR (Figure 3) results showed that DMS/PCs expressed β-integrin, CD44, CD71, and CD73 but did not express CD34 (a hematopoietic cell marker). There were no apparent differences in these markers at different passages.

3.4. Adipogenic Differentiation of DMS/PCs. Adipogenic differentiation of DMS/PCs was demonstrated by oil red O staining [18]. After incubation in adipogenic medium for 7 days, DMS/PCs changed from a shuttle shape to an oblate shape and contained many intracellular lipid droplets. As differentiation progressed, the number of lipid droplets increased and aggregated to form larger droplets (Figure 4(b)). As a negative control, cells cultured in complete medium were negative for oil red O staining (Figure 4(a)).

After induction, RT-PCR results showed that the cells expressed adipocyte-specific genes peroxisome proliferator-activated receptor-γ (PPAR-γ) and lipoprotein lipase (LPL), whereas these genes were not expressed in the control group (Figure 4(c)).

3.5. Osteogenic Differentiation of DMS/PCs . After incubation in osteogenic medium for 7 days, DMS/PCs showed obvious morphological changes. After 14 days of differentiation, the cells became aggregated and formed mineralized nodules that were stained with alizarin red. In addition, the number and size of nodules were increased (Figure 5(b)), whereas control cells showed no such effects (Figure 5(a)).

Osteogenic differentiation of DMS/PCs was also analyzed by RT-PCR. Osteogenic-specific genes alkaline phosphatase (AKP) and osteopontin (OPN) were expressed in the induced group but not in the control group (Figure 5(c)).

3.6. Neurogenic Differentiation of DMS/PCs. After incubation in neural differentiation medium for 14 days, DMS/PCs exhibited elongated cell bodies with neurites (Figures 6(b) and 6(c)). There were no obvious morphological changes

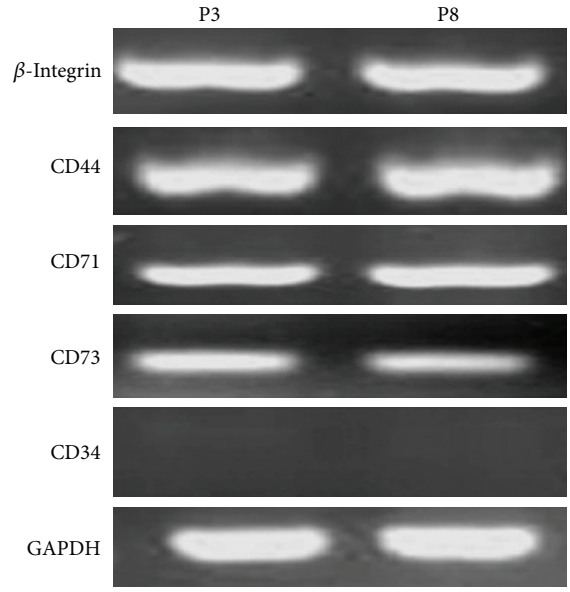


Figure 3: RT-PCR assays RT-PCR analysis showed that different passages DMS/PCs expressed β-integrin, CD44, CD71, and CD73. CD34 expression was negative. GAPDH served as the internal control.

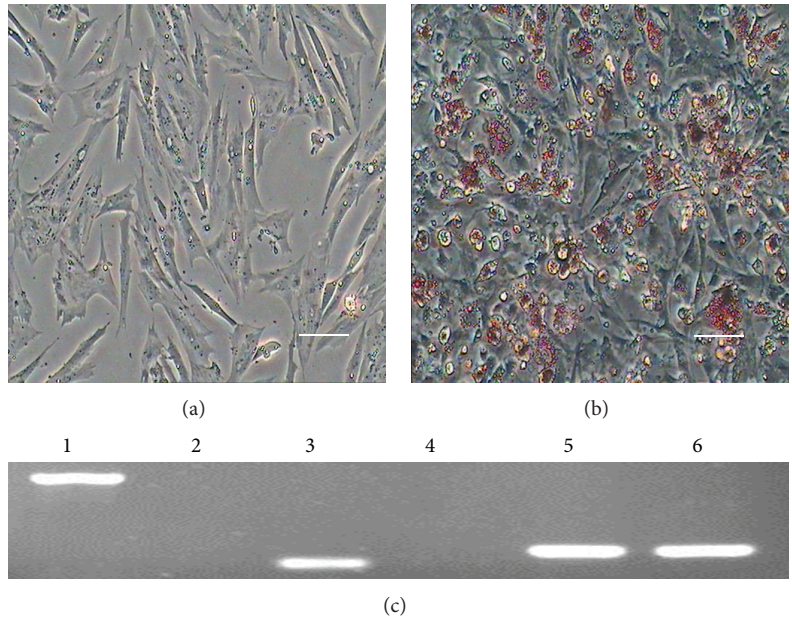


Figure 4: Adipogenic differentiation of DMS/PCs. (a) As a negative control, cells cultured in complete medium showed no changes in morphology and were negative for oil red O staining. (b) After induction for 7 days, DMS/PCs became fibroblast-like to oblate and formed many intracellular lipid droplets. Lipid droplets were stained with oil red O (scale bar: 100 μm). (c) Expression of adipocyte-specific genes LPL and PPAR-γ was detected by RT-PCR in the induced group after induction for 14 days. Adipocyte-specific genes were not expressed in the control group. Lane 1: LPL was positive in the inducted group; lane 2: LPL was negative in the control group; lane 3: PPAR-γ was positive in the inducted group; lane 4: PPAR-γ was negative in the control group; lanes 5-6 GAPDH served as the internal control.

in the control group (Figure 6(a)). Moreover, immunofluorescence demonstrated that cells in the inducted group expressed neural cell markers nestin (Figure 6(d)), β-III tubulin (Figure 6(e)), NF (Figure 6(g)), and SYP (Figure 6(h)). RT-PCR analysis demonstrated expression of the nestin gene in both inducted and control groups, but the relative expression level in the inducted group was significantly higher than that in the control group (Figure 6(j), lanes 1 and 2). In addition, the NF gene was expressed in the induction group (Figure 6(j), lanes 3 and 4). These results indicate that DMS/PCs can differentiate into neurocytes [19].

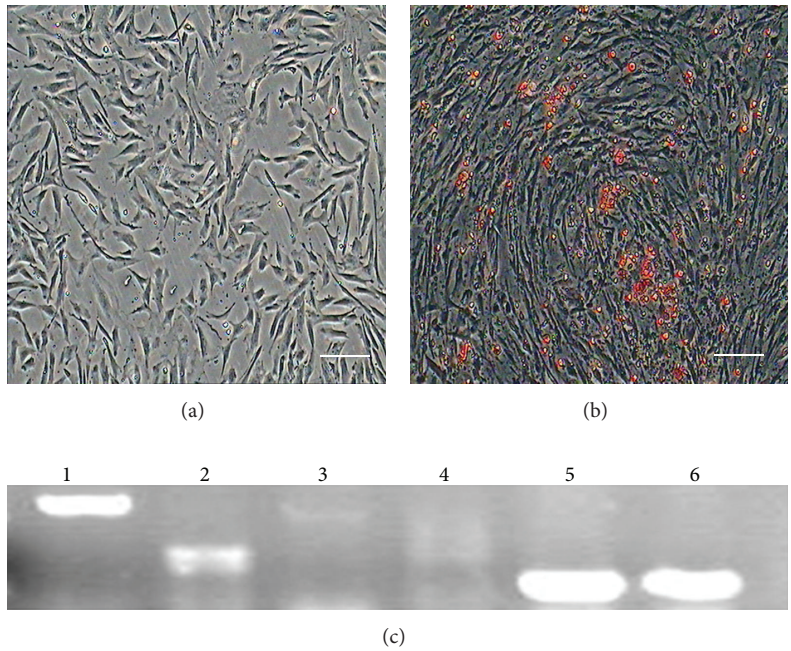


Figure 5: Osteogenic differentiation of DMS/PCs. (a) Control cells. (b) After induction in osteogenic medium for 14 days, the cells changed from fusiform to triangular in shape and were positive for alizarin red staining. Calcified nodules increased in number and became larger during induction. After about 14 days, the nodules were observed by alizarin red staining. Cells cultured in complete medium showed no morphological changes and were negative for alizarin red staining (scale bar: 100 μm). (c) After induction for 14 days, RT-PCR revealed the expression of osteoblast-specific genes ALP and OPN in the induced group, whereas these genes were not expressed in the control group. Lane 1: AKP was positive in the inducted group; lane 2: OPN was positive in the inducted group; lane 3: AKP was negative in the control group; lane 4: OPN was negative in the control group; lanes 5 and 6: GAPDH served as the internal control.

4. Discussion

In this study, DMS/PCs were successfully isolated from the dermis of 16-day-old chick embryos. Obvious differences in cell viability were observed between cells isolated from 16-day-old and 21-day-old embryos (data not show), indicating that younger animals are more suitable and the conditions to separate the dermis should be considered carefully.

The markers of DMS/PCs resemble those of BMSCs. Both cell types express some surface markers of MSCs. We examined the expression of β-integrin, CD44, CD71, and CD73 by immunofluorescence and RT-PCR. β-integrin is an integrin unit associated with very late antigen receptors. It is involved in cell adhesion and recognition in various biological processes including embryogenesis, hemostasis, tissue repair, immune responses, and metastatic diffusion of tumor cells. CD44 is a cell surface glycoprotein involved in cell-cell interactions, adhesion, and migration. This protein participates in a variety of cellular functions including lymphocyte activation, recirculation and homing, hematopoiesis, and tumor metastasis. CD71 is a member of the transferrin receptor family that is required for the import of iron into cells and is regulated in response to intracellular iron concentrations. Low iron concentrations increase the levels of transferrin receptors to increase iron intake into cells. Thus, the transferrin receptor maintains cellular iron homeostasis. CD73, also known as ecto-5′-nucleotidase, is an enzyme used as a marker of lymphocyte differentiation [20].

Multilineage differentiation of stem cells is the most notable characteristic for homotransplantation. Because of easy accessibility, DMS/PCs have become an ideal cell source in tissue engineering. In vivo, the development and function of tissue stem cells are related to transcription factors and extracellular signals [21]. However, in vitro, the mechanisms of differentiation are unclear. In our study, we differentiated chicken DMS/PCs into osteoblasts, adipocytes, and neurocytes, and then examined relevant gene expression of these cell types. The results showed that different induction factors affect the differentiation of DMS/PCs. In addition, DMS/PCs originating from mesoblastema can be induced to differentiate into mesodermal and ectodermal cells. The homotransplantation feature of DMS/PCs, together with their putative multipotency and ease of procurement, suggests that these cells are an excellent choice for many tissue engineering strategies and cell-based therapies. The chick embryo is a classic model of vertebrate developmental biology, which has been used for many decades [22]. Although the multilineage differentiation of DMS/PCs was successful in vitro, there are many drawbacks for the use of these cells in tissue regeneration in vivo, such as a higher decline rate and unstable phenotype. Therefore, more consideration may be needed for further research.

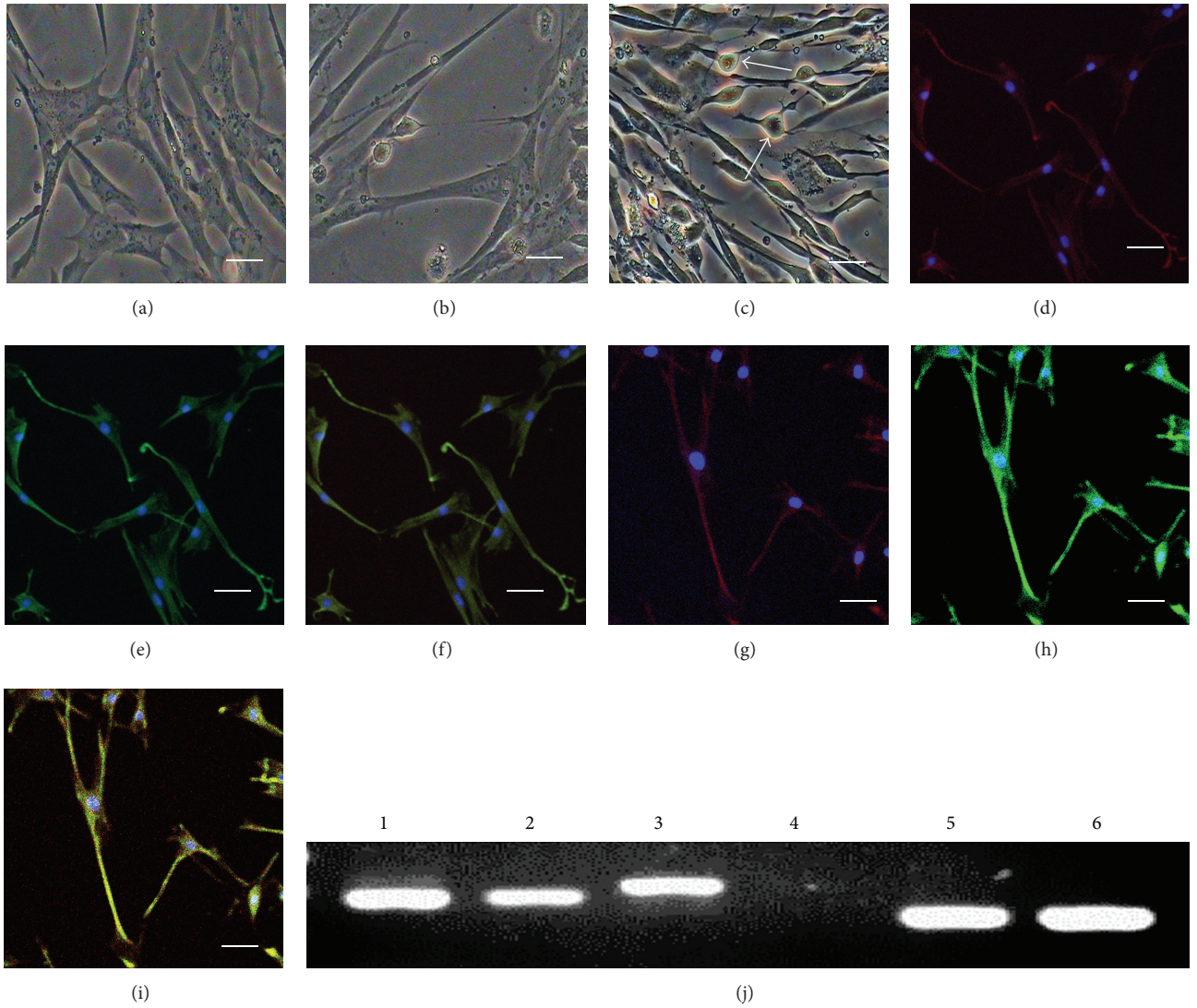


Figure 6: Neural differentiation of DMS/PCs. (a) Control cells. (b) After 1 week of induction, neural-like cells with a multipolar spindle-like shape were observed. (c) After 2 weeks of induction, neural-like cells were observed as indicated by the arrow. (d–i) Double immunofluorescence staining showed that (d) nestin (red) and (e) β-III tubulin (green) were positive in the induced cells. (f) Merged image of d and e. (g) NF (red) and SYP (h) were positive in the inducted group. (i) Merged image of g and h. Cells were counterstained with DAPI (blue) (scale bar: 100 μm). (j) After induction for 14 days, expression of neural-specific genes nestin and NF was detected by RT-PCR in the induced group, whereas these genes were not expressed in the control group. Lane 1: nestin was positive in the inducted group; lane 2: nestin was positive in the control group; line 3: NF was positive in the inducted group; lane 4 NF was negative in the control group; lanes 5 and 6: GAPDH served as the internal control.

5. Conclusions

In this study, we isolated DMS/PCs from the dermis of 16-day-old chick embryos and then examined their ability to expand and differentiate in vitro. These results have implications for the potential utility of the dermis as a source of stem cells for regenerative medical therapies.

Authors' Contribution

Yuhua Gao and Chunyu Bai contributed equally to this work.

Conflict of Interests

The authors have declared that there is no conflict of interests.

Acknowledgments

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Review Article

Nonviral Methods for Inducing Pluripotency to Cells

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The concept of inducing pluripotency to adult somatic cells by introducing reprogramming factors to them is one that has recently emerged, gained widespread acclaim and garnered much attention among the scientific community. The idea that cells can be reprogrammed, and are not unidirectionally defined opens many avenues for study. With their clear potential for use in the clinic, these reprogrammed cells stand to have a huge impact in regenerative medicine. This realization did not occur overnight but is, however, the product of many decades worth of advancements in researching this area. It was a combination of such research that led to the development of induced pluripotent stem cells as we know it today. This review delivers a brief insight in to the roots of iPS research and focuses on succinctly describing current nonviral methods of inducing pluripotency using plasmid vectors, small molecules and chemicals, and RNAs.

1. iPS: A Journey towards iPS Technology

Somatic cell nuclear transfer (SCNT) was originally hailed in the 1950s as an exciting tool that allowed scientists to probe the developmental potential of a cell. Briggs and King [1] describe this method whereby the recipient egg is activated by pricking it with a glass needle and its nucleus can then be removed using Porters technique. Following this, the egg and donor cell are prepared in a dish and with the use of specialized apparatus are drawn into a needle which damages cell membranes without harming or dispersing the contents of the cell. The cell contents are subsequently transferred to the enucleated egg. The conclusions of these experiments lead to the realization that the irreversible genetic changes that were once thought to be imposed on the genome of differentiated cells were not true, but actually they were reversible epigenetic changes. Groups from around the world continued to experiment in the field on cells derived from mammals, in some cases terminally differentiated cells, and achieved great success in demonstrating that the genomes of even fully specialized cells remained genetically totipotent. However, abnormalities in gene expression were observed in many of these "cloned" mammals which suggested that the reprogramming method was flawed [1, 2].

Work carried out on transcription factors used to switch the lineage of cells hugely contributed and influenced the discovery of inducing pluripotency to cells. These experiments involved introducing lineage-associated transcription factors to certain cells. Under normal conditions these transcription factors are involved in driving cell-type-specific genes and suppressing genes that are involved in promoting other lineages. When introduced to heterologous cells, these transcription factors allow the cell fate to be changed. This discovery was first demonstrated in fibroblasts. Myofibers were formed by transducing fibroblast cells with the skeletal muscle factor MyoD using a retroviral vector [3]. There were continued advances in this area of study where cells from different germ layers were shown to be able to cross these barriers, for instance, the work carried by Ieda et al. [4] which demonstrated fibroblasts converting to cardiomyocytes through exposure to cardiac factors Gata4, Mef2c, and Tbx5.

With past studies proving that cells remain genetically totipotent after differentiation and that it is possible to influence cells to switch between linages, the platform was set for scientists to go a step further and reprogram cells to an embryonic-like state. Although the advent of embryonic stem cell research brought with it many new and exciting

techniques that held great promise for the treatment of many diseases, iPS technology supersedes this research in two very important areas. Firstly, iPS cells do not have the same ethical issues surrounding them. This is because there is no use of human embryos as adult cells are being reprogramed. Secondly differentiated iPS cells, which are therapeutically relevant, do not face the same immune rejection following assessment in vitro and after transplantation in genetically identical recipients. This assessment found no evidence of increased amounts of T cells or antigen-specific secondary immune cells [5] (Figure 1).

2. iPS: Beginnings

It was Takahashi and Yamanaka's work [6] in 2006 that first pushed forward the subsequent wave of work that is now being carried out on iPS. This seminal work identified a series of transcription factors that when introduced to cells could reprogram them to an embryonic-like state, thus inducing pluripotency to them. An elegant experiment designed to identify factors that could reprogram somatic cells was undertaken. This experiment involved screening a set of 24 pluripotency associated genes that could activate a specific drug resistance allele. After multiple rounds of elimination, Yamanaka and Takahashi were left with 4 specific genes that they believed could reprogram somatic cells. These were Klf4, Sox2, c-Myc, and Oct 4. Upon reprogramming, the resulting iPS cells exhibited various features that are indicative of embryonic stem (ES) cells. These included expression of pluripotency markers such as Nanog: they also generated teratomas in immunocompromised mice when injected subcutaneously and contributed to different tissue development in blastocysts. These results suggested that pluripotency had been achieved, however, further analysis showed that in comparison to true ES cells, levels of pluripotency markers were markedly lower [7]. Together with failing to contribute to the germline and generate chimeras, these iPS cells had a number of issues to overcome before advancements could be made, a challenge that many groups took upon themselves to investigate. The surge in research into applying, improving and reimagining the work Yamanaka and Takahashi first started propelled iPS to the forefront of stem cell research. Rapid advancements were seen in the delivery systems, reprograming factors, and models used to reprogram cells. iPS cells have since been derived from humans [8], rats [9], and rhesus monkeys [10] as well as from different somatic cell populations ranging from keratinocytes [11], neural cells [12], and lymphocytes [13] among others.

3. iPS: A Viral Revolution

There are various means of inducing pluripotency to cells, including the use of viral vectors, nonviral vectors, using small molecules accompanied by chemical treatment and finally by RNAs. Each method of iPS has its own advantages and disadvantages. This review will focus on nonviral methods of iPS; however, a brief introduction on viral methods will be given (Figure 2).

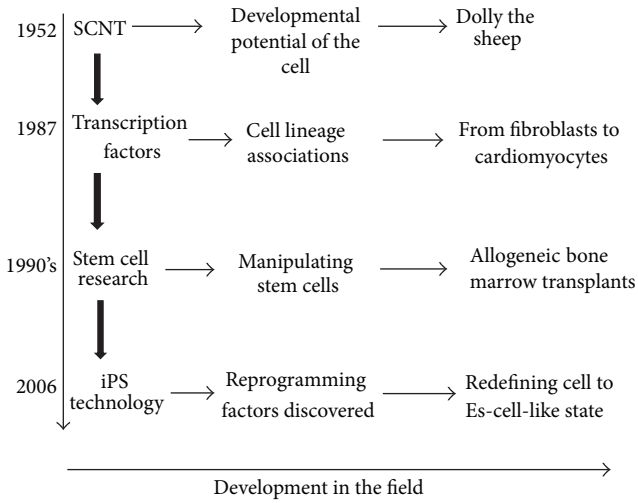

Figure 1: The journey towards iPS.

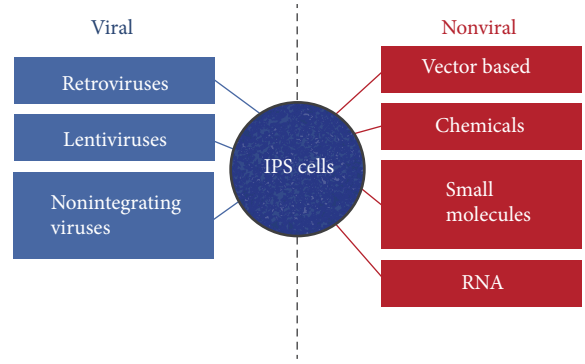

Figure 2: Viral versus nonviral methods for induction of iPS cells.

There has been a substantial amount of work carried out in the area of virally induced iPS cells which can be subcategorized into three main methods of viral iPS technology. They include the use of retroviruses, lentiviruses, and nonintegrating viruses. Retroviruses were used in producing the first iPS cells which stably integrated into the host genome and introduced the reprograming factors described by Takahashi and Yamanaka [6]. Difficulties arose with this method, however, as the reprogramming remained incomplete due to activation of methyltransferases which meant that the corresponding endogenous genes were not activated [6]. Furthermore, viral transgenes that have been integrated into iPS cells may cause tumor formation in chimeric animals [14]. Although this retroviral reprogramming method gave highly efficient iPS cells, the risk of tumor formation is too great to be applied to a clinical setting and led to other avenues being explored. Studies have taken place to examine if iPS cells can be produced in a way that does not give rise to tumors in chimeric mice which would overcome a great hurdle in viral iPS technology. To this end, Nakagawa et al. generated chimeric mice that survived 100 days using iPS cells that were produced without Myc as a factor. This achievement, however, was accompanied by a reduction in efficiency

of iPS generation [15]. Following from this experimental work, Nakagawa et al. continued experimenting with the reprogramming factor Myc in search of reducing tumor formation after reprogramming had been achieved. Results indicated that c-Myc, when used as a factor in reprogramming, was found to increase tumor formation; however, a different Myc family member, L-Myc, was found to promote reprogramming without having tumorigenic repercussions in chimeric mice [16]. An alternate method to avoid tumor formation used by researchers is inducible lentiviral vectors which would allow for the control of expression which is not possible when using retroviruses. This control is exerted by the drug doxycycline which reduces the risk of transgene expression and allows only fully reprogrammed cells to be selected [17]. These inducible vector systems have been used to generate "secondary" methods of reprogramming which do not use direct delivery of reprogramming factors to cells. This is achieved by reprogramming somatic cells using the inducible vector system and then allowing these cells to differentiate in vitro. When this has been achieved, the new somatic iPS cells are cultured in doxycycline containing media and "secondary" iPS cells are formed. These cells represent efficiency levels that are several orders of magnitude greater than the primary iPS cells that were generated [18]. The use of excision strategies has been used to avoid the problem that viral methods have yielded. These excision strategies include the use of the Cre-*lox*P recombination system and piggyBac transposition. By using these systems, undesirable sequences may be removed at a given time allowing for safe reprogramming [19, 20]. There are, however, drawbacks associated with both methods. Firstly, when the Cre-*lox*P method is used, after excision, some vector sequences may be left behind which can cause insertional mutations. Secondly, piggyBac transposition has not been reported in humans and remains a labour intensive process.

4. Nonviral/Nonintegrating iPS

4.1. Nonintegrating Vectors. As viral methods of reprogramming showed high efficiency which was desirable, they proved to be too risky to be used in a clinical setting owing to their insertional tendencies. The necessity to find an iPS method that could be used in the clinic was then sought after. Various strategies for nonviral reprogramming have been put forward and will be discussed in the following section.

Okita et al. in 2008 [21] showed that pluripotency could indeed be achieved through nonintegrating viral methods. This was achieved by repeated transfection of two expression plasmids in mouse fibroblasts. One plasmid contained complementary DNA (cDNA) of Oct3/4, Sox2, and Klf4, while the second plasmid contained c-Myc cDNA. This study, although it was carried out on embryonic fibroblasts, demonstrated the ability and potential to reprogram cells in a safe manner. The virus-free iPS cells that were obtained after four rounds of transfection expressed ES marker genes at the same level as ES cells as well as gave rise to chimeric mice, an important standard of pluripotency. Subsequent polymerase chain reaction (PCR) experiments showed no amplification of plasmid DNA in 9 of 11 positive iPS clones implying that there was no integration of the plasmid into the host genome. Southern blot analysis demonstrated that there was no integration of transgenes in the clones. Although a lower efficiency of reprogramming was exhibited, there was proof that virus-free reprogramming could be achieved.

4.2. Episomal Vectors. An alternate method of reprogramming cells to an ES-like state is described by Junying et al. in 2009 [22] following the work previously described by Okita. This method of nonviral reprogramming involved using episomal vectors and just a single transfection. In this case, reprogramming was carried out on fibroblasts by transfecting with an episomal vector oriP/EBNA1 (Epstein-Barr nuclear antigen-1) that is derived from the Epstein Barr virus. This vector was chosen as it can be used for transfection without the use of viral packaging and can be removed from cells by a drug selection method. Experimentation on reprogramming efficiency was carried out on several reprogramming factor combinations using lentiviruses. When a higher efficiency was seen, the improved combination of reprogramming factors (OCT4, SOX2, NANOG, LIN28, c-Myc, KLF4, and the SV40 large T gene (SV40LT)) was cloned into the episomal vector oriP/EBNA1, and reprogramming was carried out using IRES2 (an internal ribosome entry site for coexpression) that had been shown to work in experiments using lentivirus vectors. Following the analysis of iPS cell colonies that were found, markers indicative of ES cells were present, as well as similar morphological traits and teratoma formation after injection in immunocompromised mice. As there was no integration into the host genome as confirmed by PCR analysis and due to the loss of cellular episomal vectors in the absence of drug selection, transgene-free iPS cells may be selected through further subcloning. Despite these advantages, this method yields low reprogramming efficiency in human fibroblasts at about three to six iPS colonies per 10^6 input cells [22]. These frequencies are, however, sufficient to recover iPS cells from a reasonable number of starting cells.

4.3. Minicircle Vectors. In the following year, further advances were made in the field of iPS when Jia et al. [23] published their work on minicircle (MC) vectors that could be used to reprogram human adult cells. They reported that they had constructed a plasmid containing the four reprogramming factors Oct4, Nanog, Lin28, and Sox2 in addition to a green fluorescent protein (GFP) reporter gene. The group were able to excise the bacterial backbone from the plasmid as well as the origin of replication and drug resistance genes by taking advantage of the PhiC31-based intramolecular recombination system which cleaves away the undesired bacterial artifacts and degrades them, leaving MC DNA to be purified containing the desired reprogramming factors. The parental plasmids also contained I-SceI restriction enzyme expression cassettes under the control of an L-arabinose inducible promoter. It was claimed that MC DNA benefited from higher transfection efficiency compared to other plasmids. They also have longer ectopic

Table 1: Advantages and disadvantages of vectors for iPS.

	Advantage	Disadvantage	Efficiency
Nonintegrating vector	Nonintegrating	Low efficiency, need for multiple rounds of transfection	0.001%
Episomal	Nonintegrating, single round of transfection	Low efficiency, labour intensive	0.001%
Minicircle	Nonintegrating, higher transfection efficiency	Potentially cytotoxic	0.005%

expression which is due to the lower activation of exogenous silencing mechanisms. In this study, pluripotency was induced to human adipose stem cells. Nucleofection was carried out, and following this, two subsequent rounds were undertaken at days 4 and 6. Analysis after selection and culturing demonstrated a reprogramming efficiency of 0.05% with MC-derived iPS cells. Staining for embryonic markers was positive, and the MC-derived iPS cells exhibited all characteristics associated with pluripotency [23]. This nonviral method of iPS demonstrated a lack of integration into the host genome, an attribute that is desired if the method is to be applicable to a clinical setting, however, reprogramming efficiency still remains lower compared to that of viral methods. The work carried out on iPS MC's gained much support with other groups focusing on this as a means to induce pluripotency. Improvements in MC work was carried out by Chabot et al. [24] who demonstrated the use of electropulsation for MC with GFP delivery to cells. Results showed that there was twofold difference of GFP expression in cells after 3 days between electropulsated MC and parental plasmids. Cellular toxicity was examined, and it was found that the increase of transfection efficiency in MC electropulsated cells was not due to a lack of cellular toxicity as both samples were similarly cytotoxic. In vivo studies also showed increased GFP expression of electropulsated MC, and after day ten, it had expression 36 times higher than that of parental plasmid which could be translated to an iPS scenario [24]. An alternative improvement put forward by Yoshida et al. [25] for iPS cells was to conduct reprogramming in hypoxic conditions. It was found from their study that reprogramming in this condition improved efficiency of reprogramming using both viral and nonviral vectors such as plasmids under 5% O_2. However, further experimentation needs to be carried out to find the optimal conditions for favourable iPS generation as cytotoxicity remains problematic under such conditions.

Three general approaches are listed previously using nonviral vectors for iPS cell generation. These different methodologies have certain aspects in common; for instance, all three methods avoid integration into the host genome, a considerable achievement, and are carried out in 3 very different ways. Similarly, the three methods that have been attempted generate low reprogramming efficiency, an issue of concern, should the method be used in a clinical setting. Means of enhancing reprogramming efficiency such as culturing in hypoxic conditions and using different methods of transfection are important should iPS be used in a therapeutic approach. Other methods of reprogramming have been studied in a hope to improve upon this drawback in reprogramming efficiency, such as use of small molecules and chemical agents, which will be discussed forthwith (Table 1).

4.4. Small Molecules and Chemical Compounds. The use of small molecules and chemicals is well documented in the literature, and they are used to enhance reprogramming efficiency and iPS cell generation. The idea behind their use is to substitute Yamanaka and Takahashi's original reprogramming factors with a cocktail of chemicals or molecules which will serve to enhance the process. Shi et al. [26] describe how they screened for chemicals and molecules that could do just this. They showed that neural progenitor cells (NPCs), which endogenously express Sox2, were transduced with Oct4 and Klf4 alone (OK) and were successfully reprogrammed to iPSCs. They also showed that this process was greatly enhanced in the presence of a G9a histone methyltransferase inhibitor, BIX-01294 (BIX). Desponts and Ding [27] also carried out work in this area, screening for chemicals and molecules that could be used in conjunction and in place of currently used transcription factors. They claim that an L-channel calcium agonist, BayK8644 (BayK), does not directly cause epigenetic modifications as it works upstream in cell signaling pathways and can therefore avoid unwanted modifications. Other work carried out in this increasingly attractive field includes that of Lee et al. [28] who worked with nanoparticles and iPS generation, Lyssiotis et al. [29] who worked on generating iPS through complementation of Klf4 by chemical means, and Pasha et al. who nonvirally reprogrammed murine myoblasts with a single small molecule, DNA methyltransferase (DNMT) inhibitor, and RG108, to generate cardiac progenitor cells [30]. A prime example of the use of small molecules for replacing transcription factors for reprogramming was discovered by Ichida et al. Their RepSox2 molecule successfully replaces Sox2 by the inhibiting transforming growth factor-β (TGF-β) signaling, which in turn induces Nanog expression. After screening over 800 compounds, these researchers found that RepSox2 was the only one that could generate iPS cells in the absence of another chemical, valproic acid (VPA). Of important note, when reprogramming using this small molecule, the problematic transcription factor c-Myc was not necessary for inducing pluripotency, and the efficiency of the reprogramming was not compromised [31]. Histone deacetylases (HADC) other than VPA such as suberoylanilide hydroxamic acid (SAHA) and trichostatin A (TSA) also greatly improved reprogramming efficiency [32]. Others carrying out work on small molecules and chemical means for generating iPS cells include Lin et al. [33] who focus on chemical means of treating cells in culture to induce pluripotency [33], Wang et al. [34] who generated iPS cells by retinoic acid receptor gamma and liver receptor homolog 1, and Zhu et al. [35] who induce pluripotency in somatic human cells by OCT4 and chemical compounds. As more groups searched for compounds to replace or indeed enhance the action of known transcription

factors for reprogramming, it is interesting to note that Esteban et al., while investigating the role of vitamin C to prevent the build-up of reactive oxygen species in culture after reprogramming, discovered that its presence "alleviates the senescence roadblock during iPSC generation" and increases the performance of reprogramming. This observation was made as there was an increase of proliferation during the middle phase of reprogramming, which the group suggested to be a result of the vitamin C aiding in the bypass of senescence. The authors also postulated that the addition of vitamin c may be influencing reprogramming by interacting with various enzymes [36]. The task of searching for small molecules that can overcome low reprogramming efficiency is being undertaken by many research groups as is highlighted by the work carried out by Zhonghan and Rana on a kinase inhibitor screen for small molecules to aid in reprogramming and iPS generation. A key finding from this study showed that Aurora A kinase negatively affects reprogramming efficiency by inhibiting the inactivation of GSK3β. Therefore inhibitors of such molecules would greatly increase iPS generation. Other molecules that were identified as inhibitors of iPS in this study included p38 and inositol trisphosphate 3-kinase [37].

It can be seen from the vast number of groups working on chemicals and small molecules that there is a strong belief that these methods of reprogramming can efficiently produce genuine, stable iPS cells free of integration and mutation. The use of molecules in reprogramming is seen to be a safe method as they use discrete pathways rather than rely on modification to reprogram cells, which makes this process a safe one. It must be noted, however, that substituting a transcription factor for a chemical compound results in a decreased number of generated iPS clones, which may indicate that a single compound may not be able to entirely replace a functioning transcription factor.

4.5. RNAs. The most recent advancement and developing trend in the field of nonviral iPS work is reprogramming using RNA molecules. Very recently, highly efficient miRNA-mediated reprogramming of mouse and human somatic cells to pluripotency was reported by Anokye-Danso et al. [38] but using integrating viral vectors and not direct transfection of mature miRNAs. A study by Miyoshi et al. [39], however, successfully generated iPS cells by direct transfection of human somatic cells using mature miRNA. Researchers were able to detect GFP expression on day 14 after the transfection of mir-200c, mir-302 s, and mir-369 s family miRNAs, and by day 15, they observed approximately five GFP-positive colonies giving an apparent efficiency that is comparable to that seen with the original report of retrovirus-mediated transcription factor introduction. iPS colonies were generated and passed all standard pluripotency checks. This advancement in the field of iPS technology is exciting due to the lack of any vector meaning no risk of insertional mutagenesis. The use of synthetic RNAs has also been reported, which bypass the innate response to viruses and generate true iPS cells. This work, carried out by Warren et al. 2010. [40], generated iPS cells using this method at high efficiency. BJ fibroblasts transfected with a five-factor modified RNA cocktail (KMOSL) demonstrated iPS cell reprogramming two orders of magnitude higher than those typically reported for virus-based derivations. Moreover, this method far out performed traditional viral methods in regard to the time it took to generate iPS colonies [39]. It is therefore clear that RNA strategies for iPS have come very far in the race to achieve useable iPSCs in the clinic (Table 2).

Table 2: Key literature in the area of vector-based iPS technology, chemical-induced iPS technology, small molecules, and RNA-induced iPS technology.

Vector based	[17, 20–24]
Chemical methods	[31, 33–35]
Small molecules	[26–30]
RNAs	[39, 40]

5. Conclusions

From its beginnings in 2006, iPS and its technology have seen many advancements, particularly in the nonviral arena. Attempts to emulate the success of viral reprogramming efficiency, while avoiding the pit fall of integration of undesired DNA into the host genome, have driven this field to where it currently stands. The diverse range of strategies that have been put forward to solve this problem nonvirally demonstrates the commitment and faith the scientific community has in the idea and promise of iPS technology. The applications that may arise from these studies include disease modeling and regenerative tissue engineering which are vitally important contributors to the advancement of medical science. Although this work is yet in its infancy, the awarding of the Nobel Prize for medicine to Yamanaka for his pioneering work in the field, the future of iPS, is certainly bright.

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Research Article

Kidney-Targeted Transplantation of Mesenchymal Stem Cells by Ultrasound-Targeted Microbubble Destruction Promotes Kidney Repair in Diabetic Nephropathy Rats

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We test the hypothesis that ultrasound-targeted microbubble destruction (UTMD) technique increases the renoprotective effect of kidney-targeted transplantation of bone-marrow-derived mesenchymal stem cells (BM-MSCs) in diabetic nephropathy (DN) rats. Diabetes was induced by streptozotocin injection (60 mg/Kg, intraperitoneally) in Sprague-Dawley rats. MSCs were administered alone or in combination with UTMD to DN rats at 4 weeks after diabetes onset. Random blood glucose concentrations were measured at 1, 2, 4, and 8 weeks, and plasma insulin levels, urinary albumin excretion rate (UAER) values, the structures of pancreas and kidney, the expressions of TGF-β1, synaptopodin, and IL-10 were assessed at 8 weeks after MSCs transplantation. MSCs transplantation decreased blood glucose concentrations and attenuated pancreatic islets/β cells damage. The permeability of renal interstitial capillaries and VCAM-1 expression increased after UTMD, which enhanced homing and retention of MSCs to kidneys. MSCs transplantation together with UTMD prevented renal damage and decreased UAER values by inhibiting TGF-β1 expression and upregulating synaptopodin and IL-10 expression. We conclude that MSCs transplantation reverts hyperglycemia; UTMD technique noninvasively increases the homing of MSCs to kidneys and promotes renal repair in DN rats. This noninvasive cell delivery method may be feasible and efficient as a novel approach for personal MSCs therapy to diabetic nephropathy.

1. Introduction

Both type 1 and type 2 diabetes mellitus (DM) involve destruction and dysfunction of pancreatic islets/β cells, and their main and long-term complication is diabetic nephropathy (DN) which has evolved as a leading cause of end-stage renal disease (ESRD). At the moment, transplantation of pancreatic islet and kidney is the most preferred cell replacement therapy to DN. However, the scarcity of transplantable donors and the need for lifelong immunosuppression limit the widespread use of the curative therapy.

Bone-marrow-derived mesenchymal stem cells (BM-MSCs), which possess multipotent differentiation characteristics, capacity for self-renewal, and immunomodulatory ability, are considered as a potential therapeutic agent for treatment of DN complications [1–4]. On the other hand, their utility for targeting tissue in living animals has proved to be limited. For instance, MSCs transplantation usually resulted in an insufficient number of engrafted MSCs in injury site. In view of the drawback, we have developed a technique that applies ultrasound-targeted microbubble destruction (UTMD) to promote homing of MSCs to impaired kidney.

Ultrasound contrast agent (microbubbles) is widely used to enhance the reflectivity of perfused tissues in clinical ultrasonography. Moreover, later researches focus on its potential therapeutic effect. The application of ultrasound to small vessels containing microbubbles can change blood vessel wall permeability, resulting in the extravasation of particles into the interstitial space [5]. In addition, UTMD has the potential to change the microenvironment [6], release the transported substances into target organ to repair damage tissue [7], and promote stem cells homing [8]. Currently, the majority of researchers consider that the interaction of ultrasound pulses with these gas bodies is a form of acoustic cavitation [9] and has successfully applied for blood vessels [10], skeletal muscle [11], heart [12], lung [13], liver [14], and tumors [15]. UTMD directed expression of an adenoviral reporter and was applied to selectively deliver plasmid vectors to the heart *in vivo* [16]. The transfection efficiency of cells *in vitro* was increased under the optimal UTMD conditions [17]. Lan et al. transferred a doxycycline-regulated Smad7 gene into the kidney using an ultrasound-microbubble-mediated system, specifically blocked TGF-β signaling and inhibited renal fibrosis in a rat unilateral ureteral obstruction (UUO) model [18]. Yu et al. suggested that the combined use of microbubble and high-intensity focused ultrasound (HIFU) improved the therapeutic efficiency of HIFU in rabbit kidney study [19]. Microbubble destruction by ultrasound gene transfection treatment (1.0 W/cm^2) promoted renal recovery in acute kidney injury in rats [20]. So far, no studies have been reported whether this technique provides an equal contribution to diabetic kidney disease which acts as a complication of primary disease. Based on the above facts, we propose the hypothesis that UTMD is feasible for increasing the target transplantation of MSCs to kidney and promoting renal repair in diabetic nephropathy.

To test this hypothesis, MSCs (1×10^6 cells) were administered alone or together with UTMD to DN rats at 4 weeks after diabetes onset. Normal nondiabetic rats were as those of control group. We then evaluated blood glucose concentrations, plasma insulin levels, UAER values, and the structure of kidney and pancreas, traced MSCs homing, accessed VCAM-1 levels after UTMD, and detected the levels of TGF-β1, IL-10, and synaptopodin after MSCs transplantation.

2. Materials and Methods

2.1. Animals. All Sprague-Dawley (SD) rats were provided by the Center for Experimental Animals of the Third Military Medical University. The experiments were approved by the Animal Care and Use Committee of Third Military Medical University. Rats were housed in wire cages with free access to a standard diet and tap water. The temperature and relative humidity of the animal facility were maintained under conventionally controlled conditions (22°C, 55% humidity) with a day-night rhythm.

2.2. Isolation and Culture of BM-MSCs. Three-week-old male SD rats were sacrificed by cervical dislocation. Bone marrow cells were obtained from femurs and tibias. After isolation and centrifugation, cells were cultured (37°C, 5% CO_2) at a density of 1×10^6 nucleated cells/cm^2 with DMEM/F12 (Hyclone, Logan, UT, USA) medium containing 10% inactivated fetal bovine serum (Gibco, USA), 100 U/mL penicillin and 100 mg/mL streptomycin antibiotic solution (Gibco, USA). Medium was changed and nonadherent cells were removed after 72 hours. Adherent cells reaching 80% confluence were passaged with 0.25% trypsin-EDTA solution (Gibco, USA) and then subcultured in DMEM/F12 medium with 10% inactivated fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin antibiotic solution (complete medium).

2.3. Adipogenic, Osteogenic, and Chondrogenic Differentiation of MSCs. Adipogenic differentiation was induced by culturing MSCs with 10 nM dexamethasone (Sigma) and 5 μg/mL insulin (Sigma) for 7 days with medium change every 3 days. The intracellular lipid droplets were easily observed by phase-contrast microscopy and assessed using Oil Red O (Sigma) staining.

Osteogenic differentiation was induced by culturing MSCs with 10 nM dexamethasone (Sigma), 50 μg/mL ascorbic acid (Sigma), and 10 mM β-glycerol phosphate (Sigma) for 2 weeks with medium change every 3 days. The calcium deposits were stained with alizarin red S.

Chondrogenic differentiation was induced by culturing MSCs with 10 ng/mL rh-TGFβ1 (Sigma), 50 μg/mL ascorbic acid (Sigma), 6.25 mg/mL insulin (Sigma), 10^{-7} M dexamethasone (Sigma), and 12 μM L-glutamine (Sigma) for 7 days with medium change every 3 days. The differentiated cells were assessed using Safranin O (Sigma) staining.

2.4. Flow Cytometry Analysis of MSCs. Approximately 1×10^6 MSCs were prepared and washed 2 times by centrifugation at 900 ×g for 5 min. MSCs were then resuspended in 500 μL of PBS and incubated with phycoerythrin- or fluorescein isothiocyanate-conjugated antibodies against rat CD34, CD44, CD45, and CD90 for 30 min at 4°C. All assays were conducted according to the manufacturer's instructions. Phycoerythrin-conjugated mouse anti-rat IgG1 was used as an isotype control. Cells were collected and washed with PBS by centrifugation and fluorescence analysis was carried out with a flow cytometer (Beckman, USA).

2.5. MSCs Labeling. To track the transplanted MSCs in kidney, enhanced green fluorescent protein (eGFP, Cyagen Biosciences) was used as a cell tracker. MSCs were transfected with lentiviral vectors carrying eGFP. Briefly, MSCs after the third passage were seeded at 1×10^5 cells in 6-well plates 24 hours before transfection. The next day, 1 mL complete medium per well containing 5 μg/mL polybrene (Sigma) and viral were added to the cells at a MOI (multiplicity of infection) of 10, and then cells were incubated at 37°C in 5% CO_2 for 8 hours. After incubation, cells were washed with PBS and incubated in fresh complete medium for further experimentation. 72 hours after transfection, the infected cells were observed by fluorescent microscope.

2.6. Diabetic Nephropathy Induction. Adult male SD rats aged 7-8 weeks (160–180 g body weight) were used. All rats were lightly anesthetized by intraperitoneal injection of 2% pentobarbital sodium at a dose of 35 mg/kg and received a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ, Sigma) immediately after dissolving in 0.1 M citrate buffer at pH 4.5. Three days after STZ injection, diabetes was confirmed by random blood glucose concentrations of greater than 16.7 mmol/L for 3 consecutive days. 4 weeks after diabetes onset, diabetic rats presented mild microalbuminuria (an early sign DN) and were ready for further experiment.

2.7. Experimental Preparation. The microbubbles used in this study were self-made, which was a type of perfluoropropane-gas-filled microbubbles with lipid shell and was awarded by the National Invention Patent of China in 2005. Its concentration and mean diameter were approximately $4–9 \times 10^9$/mL and 2.13 μm, respectively. A recent study confirmed that it did not produce changes in right ventricular blood pressure, had no significant influence on rat kidney, and better enhanced the ultrasound imaging, showing a fine properties and prospects for application [21]. The administration dosage of microbubbles for each injection was 0.05 mL/kg, and microbubbles were injected into tail vein through 26-gauge needles connected with a 1 mL syringe via a 15 cm length catheter, followed by 0.5 mL saline to wash the tube. MSCs (1×10^6) were resuspended in 2 mL of PBS and administrated to anesthetized rats through tail vein. A color diagnostic ultrasound system (S2000, Siemens, Germany) was applied, a high-frequency probe of 9L4 was used, and the parameters were set as follows: center frequency of 7.00 MHz; mechanical index of 0.9; continuous irradiation; depth of 3 cm; and single focal point of 1 cm. Rats were fixed in left lateral position after satisfactory anesthesia by intraperitoneal injection of 2% pentobarbital sodium at a dose of 40 mg/kg and only the right kidney was irradiated. Right flank was shaved with a shaver, and the remaining hair was removed using a depilatory cream. Ultrasound probe was put to the right kidney and irradiated for 5 min.

2.8. Real-Time Polymerase Chain Reaction (Real-Time PCR) and Transmission Electron Microscopy (TEM). DN rats ($n = 32$) were randomly divided into four groups and received MSCs transplantation: (1) DN rats received 2 mL of PBS (phosphate-buffered saline) infusion (PBS group); (2) DN rats received ultrasonic irradiation together with microbubbles infusion (UTMD group); (3) DN rats received MSCs infusion (MSCs group); and (4) DN rats received ultrasound + microbubbles combined with MSCs infusion (UTMD + MSCs group). Three days after MSCs transplantation, rats were killed by anesthetic overdose and kidneys were rapidly dissected out. Real-time PCR analysis was performed to investigate VCAM-1 mRNA expression, and renal capillary permeability was observed by transmission electron microscopy.

2.8.1. Real-Time PCR. The whole tissue preparation procedure was carried out at 4°C. Renal cortices were weighted, minced, and homogenized in a glass homogenizer. DNA was extracted with chloroform and precipitated with ethanol, and total DNA was assayed by UV absorbance. The oligonucleotide primers were as follows: VCAM-1, 5′-CGTTGACATCCGTAAAGACCTC-3′ (sense), and 5′-TAGGAGCCAGGGCAGTAATCT-3′ (antisense). β-actin, 5′-GCGAGGTCGTTTAGAGTAGTAGC-3′ (sense), and 5′-CCTGAAAGTCAACCCAGTGA-3′ (antisense). Real-time PCR assay was performed with target DNA, VCAM-1 primers, and a fluorescent probe by using a real-time PCR instrument (Applied Biosystems).

2.8.2. TEM. Targeted homing ability of MSCs to kidneys was associated with renal capillary permeability which was assessed by TEM. Renal cortices were cut into 1 mm pieces and fixed in 2% glutaraldehyde for 1 hour. After being washed with PBS for 3 times, the samples were postfixed in 1% osmium tetroxide in cacodylate buffer (pH 7.2) for 1 hour. Subsequently, the samples were dehydrated in ethanol and embedded in epoxy resin (Agar 100). Ultrathin sections (50 nm) were cut, double stained with uranyl acetate and Reynolds lead citrate, and examined with a transmission electron microscope (Philips TECNAI 10, USA).

2.9. MSCs Tracking. DN rats ($n = 10$) were equally divided into MSCs group and UTMD + MSCs group. Three days after cells transplantation, rats were killed by anesthetic overdose. Kidneys and pancreases were separated and immediately snap-frozen in liquid nitrogen for frozen sections (5 μm in thickness). Nuclear counterstaining was performed with 4′,6-diamidino-2-phenylindole (DAPI, Beyotime, China). The survival of implanted MSCs was observed and evaluated in frozen sections by counting five randomly chosen fields under laser scanning confocal microscope (LSCM) from each rat in the two groups.

2.10. Allogenic MSCs Implantation. Figure 1 showed the experimental protocol. DN rats ($n = 40$) were randomly divided into four groups ($n = 10$/group) and a group of normal nondiabetic rats ($n = 10$) was set as a normal control group. Rats of five groups received following treatment: (1) normal nondiabetic rats without treatment (normal group); (2) DN rats received 2 mL of PBS infusion (PBS group); (3) DN rats received ultrasonic irradiation together with microbubbles infusion (UTMD group); (4) DN rats received MSCs infusion (MSCs group); (5) DN rats received ultrasound + microbubbles combined with MSCs infusion (UTMD + MSCs group). All rats of five groups were housed in wire cages for 8 weeks after treatment. Random blood glucose concentrations were measured at 1, 2, 4, and 8 weeks, plasma insulin levels, UAER values, the structures of pancreas and kidney, immunohistochemistry for TGF-β1, ELISA for IL-10, and Western blot for synaptopodin were assessed at 8 weeks.

2.11. Blood Glucose Concentrations Determination. Blood samples were collected from the tail vein from nonfasted

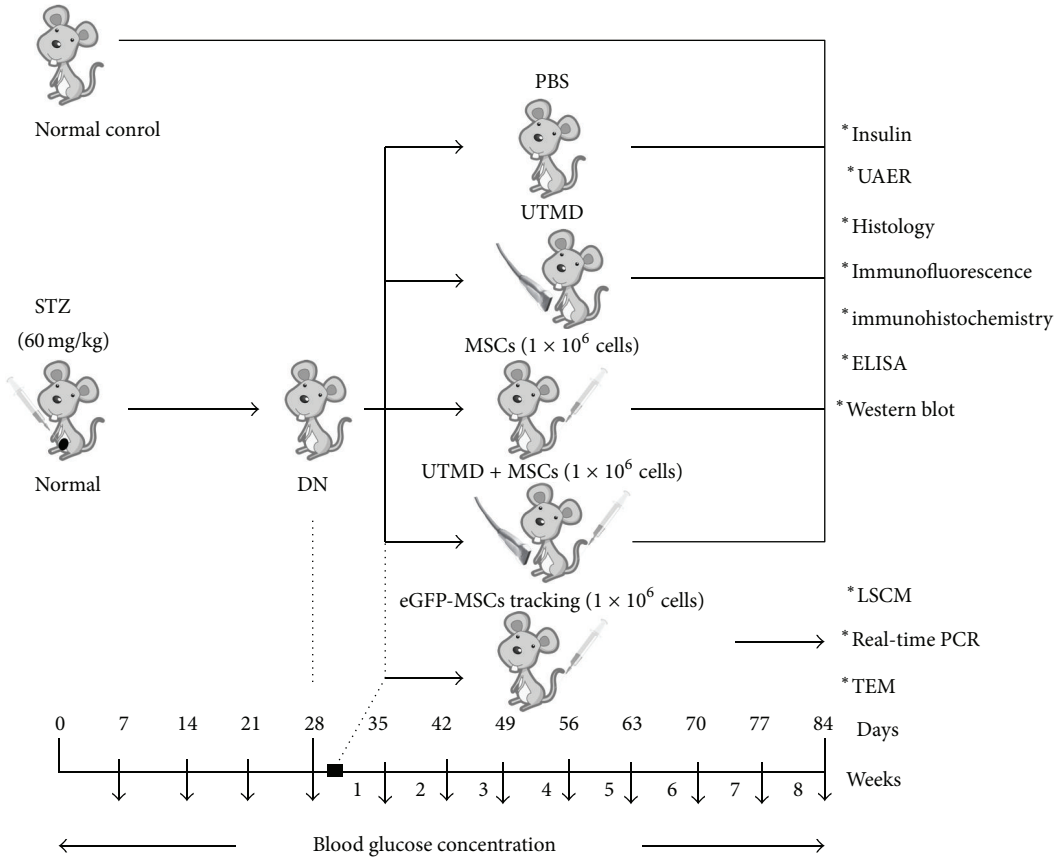

Figure 1: The experimental protocol. Four weeks after diabetes onset induced by STZ (60 mg/Kg), rats presented mild microalbuminuria (DN rats) and divided into PBS group (PBS infusion), UTMD group (ultrasound + microbubble), MSCs group (1×10^6 eGFP labeled MSCs for infusion), and UTMD + MSCs group (MSCs infusion combined with ultrasound and microbubble), and a group of normal nondiabetic rats (no treatment) was set as a normal control group. Random blood glucose concentrations were measured at 1, 2, 4, and 8 weeks, and determination of plasma insulin, UAER, histology, immunofluorescence, immunohistochemistry, ELISA, and Western blot were performed 8 weeks after MSCs treatment. To track the intrarenal localization of implanted MSCs and explore the underlying mechanism, DN rats which received ultrasonic irradiation with or without eGFP-labeled MSCs infusion were killed 3 days after treatment, and laser scanning confocal microscope (LSCM), Real-Time PCR, and transmission electron microscopy were performed.

rats and blood glucose concentrations were determined by a glucometer (Accu-Chek Aviva; Roche Applied Science).

2.12. Plasma Insulin Levels Determination. Plasma insulin levels were assayed by a radioimmunoassay (RIA) kit (Atom High-Tech Co., Ltd., Beijing, China), and the procedure was carried out in accordance with the manufacturer's instructions.

2.13. UAER Values Determination. UAER values were determined using the albumin-to-creatinine ratio (ACR) in 24-hour urine collections. The concentrations of urine albumin and urine creatinine were determined in Xinqiao Hospital clinical laboratory using an automatic biochemical analyzer (Hitachi, Japan).

2.14. Pancreas Double-Label Immunohistofluorescence. Deparaffinized pancreatic sections were incubated for 2 hours with monoclonal mouse anti-rat insulin and polyclonal rabbit anti-rat glucagon from Boster (China). After washing, the sections were incubated for 1 hour with TRITC-conjugated anti-mouse IgG and FITC-conjugated anti-rabbit IgG from Boster (China). Cross-reactivity of secondary antibodies was performed by control experiment of omitting primary antibodies. Slices were examined under laser scanning confocal microscope, and nuclear counterstaining was visualized with 4′,6-diamidino-2-phenylindole (DAPI, Beyotime, China).

2.15. Kidney and Pancreas Histology. At the end of the experiment, rats were sacrificed by anesthetic overdose. Kidneys and pancreases were rapidly removed, fixed in 10% formaldehyde, embedded in paraffin, and sectioned at 5 μm thickness. Renal sections were stained with periodic acid-Schiff (PAS) staining and pancreatic sections were stained with hematoxylin-eosin (H&E) staining. All sections were evaluated under a light microscope by a pathologist who was blinded to treatment.

2.16. Immunohistochemistry. Expression of TGF-β1 (transforming growth factor-β1) in renal tissue was assessed by immunohistochemistry. Renal sections were deparaffinized, rehydrated, and submitted to microwave antigen retrieval in citrate buffer, pH 6.0, at 95°C for 10 min. The endogenous peroxidase activity was blocked with 3% H_2O_2 for 10 min, and sections were blocked with normal goat serum for 10 min. Then, the sections were incubated with rabbit polyclonal TGF-β1 (Boster, China) antibody or a negative control reagent at 4°C, followed by biotinylated anti-rabbit IgG secondary antibody for 30 min at 37°C. Antigen-antibody reactions were visualized with diaminobenzidine (DAB) which resulted in a brown-colored precipitate at the antigen site, and hematoxylin counterstaining was performed. Five random fields of each section were photographed at a magnification of 400x and semiquantitative evaluations were assessed using Image-Pro Plus 6.0 software.

2.17. ELISA. Renal cortices were placed in cell lysis buffer and protease inhibitors on ice. Samples were homogenized and homogenates were centrifuged at 15,000 rpm for 15 min at 4°C. The amount of IL-10 in supernatants was measured using enzyme-linked immunosorbent assay (ELISA, R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

2.18. Western Blot. Renal cortices were homogenized in lysis buffer on ice for 30 minutes. Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked using 0.1% Tween-20 in Tris-buffered saline (TTBS) containing 5% bovine serum albumin (BSA) at room temperature for 2 hours. Then, membranes were washed three times with TTBS and incubated for 2 hours at room temperature with rabbit polyclonal antisynaptopodin (Abcam, 1 : 500 in TTBS) and β-Actin (stained by polyclonal anti-β-Actin 1 : 500 dilution, Abcam). The signal was detected by chemiluminescence method (PIERCE).

2.19. Statistical Analysis. Data are summarized as mean ± standard deviation for each group. Student's t-test was used to determine the significant difference between two groups. One-way ANOVA and post hoc comparisons (Bonferroni's test) were used to determine the significant difference among multiple groups. A P value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Characteristics, Identification, and Labeling of MSCs. Most of cultured MSCs were spindle shaped, attached to the culture dish tightly, proliferated in culture medium, and became more uniform after several passages (Figure 2(a)). Adipogenic, osteogenic, and chondrogenic differentiation were successfully induced in MSCs after 1 week and 2 weeks of treatment, respectively. The intracellular lipid droplets, calcium depositions and chondrogenic cells were observed by phase-contrast microscope (Figures 2(b)–2(d)). Flow cytometry analysis showed that these expanded MSCs were positive for CD44 (99.44%, Figure 2(g)), CD90 (99.37%, Figure 2(h)), and negative for the leukocyte common antigens CD34 (1.17%, Figure 2(i)), CD45 (7.12%, Figure 2(j)).

MSCs transfected by lentiviral vectors carrying eGFP showed bright green fluorescence 72 hours after transfection (Figure 2(e)) under fluorescence microscopy. It was worth noting that eGFP-MSCs mostly localized in outer region of interstitial capillary, the peritubular area (Figures 2(k) and 2(m)). A small number of eGFP-MSCs located in glomeruli and approximately 10% glomeruli presented about 1 to 3 eGFP-MSCs. Quantification analysis of eGFP-MSCs in renal tissue showed that there was a significant difference between MSCs group and UTMD + MSCs group. The number of eGFP-MSCs in UTMD + MSCs group (18.3 ± 2.9, Figure 2(m)) was much more than that of MSCs group (5.7 ± 0.8, Figure 2(k)). Figure 2(l) represents the histogram of the quantification analysis. No MSCs were found in pancreas.

3.2. VCAM-1 mRNA Expression. VCAM-1 mRNA expressions in each group are shown in Figure 2(n). The result revealed that VCAM-1 mRNA expression was increased both in UTMD group and MSCs group and it was markedly increased in UTMD + MSCs group compared with PBS group ($P < 0.01$), UTMD group ($P < 0.01$), and MSCs group ($P < 0.01$).

3.3. Increase of Interstitial Capillary Permeability. Transmission electron microscope was used to observe the ultrastructural change of renal capillary, and we found that a part of interstitial capillary walls became thinner, discontinuous, and roughened in UTMD group and UTMD + MSCs group (Figures 3(a) and 3(b)) and they kept intact in PBS group and MSCs group (Figure 3(c)), suggesting that a mild injury of endothelial cells was responsible for the changes and interstitial capillary permeability was increased by UTMD.

3.4. The Changes of Blood Glucose Concentrations, Plasma Insulin Levels, and UAER Values. In order to observe the improvement of pancreatic damage, blood glucose concentrations and plasma insulin levels were assessed. Four weeks after STZ injection, blood glucose concentrations of DN rats increased from normal level (5.2 ± 0.7 mmol/L, normal group) to severe hyperglycemia (27.3 ± 6.5 mmol/L, PBS group). Within 1 week after cell therapy, blood glucose concentrations significantly decreased in MSCs group (22.5 ± 5.3 mmol/L) and UTMD + MSCs group (22.1 ± 5.0 mmol/L), reached the lowest levels at 2 weeks, and lasted at least during the observation period (Figure 3(d)). In contrast, untreated PBS group (28.4 ± 6.2 mmol/L) and UTMD group (27.9 ± 6.5 mmol/L) remained a high level of blood glucose until the end of the experiment. No obvious difference was found in blood glucose concentration between MSCs group (23.8 ± 5.5 mmol/L) and UTMD + MSCs group (24.0 ± 4.6 mmol/L), $P = 0.395$, which was the same as that between PBS group (28.4 ± 6.2 mmol/L) and UTMD group (27.9 ± 6.5 mmol/L), $P = 0.816$.

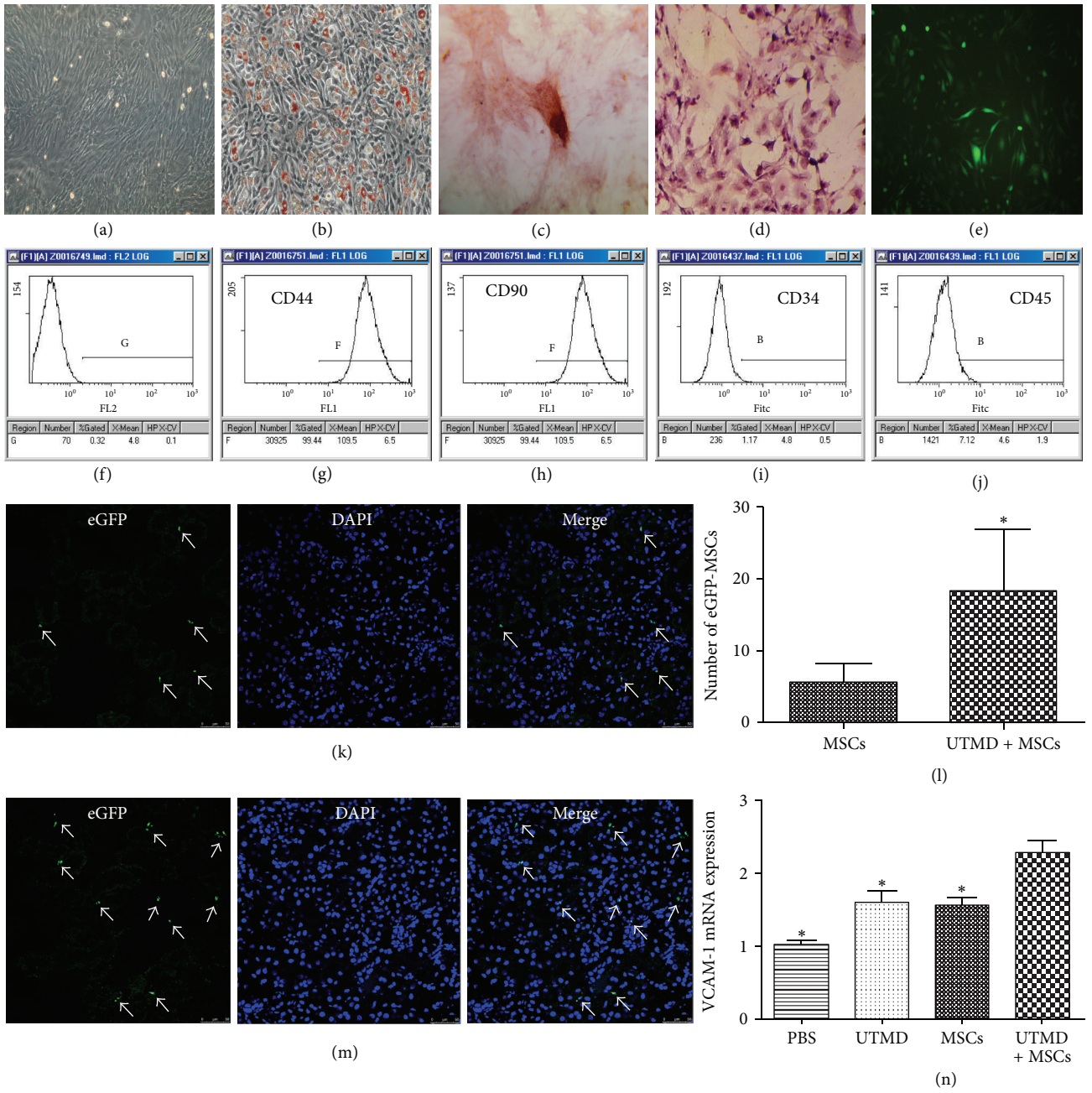

Figure 2: BM-MSCs culture, characteristics, and tracking. (a) After 10–14 days of primary culture, MSCs were nearly 80%–90% confluent (×100). The intracellular lipid droplets stained with oil red O staining (b), the calcium depositions stained with alizarin red S staining (c), the chondrogenic cells stained with Safranin O staining (d) were observed after 1 week and 2 week of treatment, respectively. (e) MSCs transfected by lentiviral vectors carrying eGFP showed bright green fluorescence. The phenotype of rat MSCs was shown to be positive for CD44 (99.44%, (g)), CD90 (99.37%, (h)), and negative for CD34 (1.17%, (i)), CD45 (7.12%, (j)). (f) represents the isotype control. There were much more eGFP-labeled MSCs localized in the kidney in UTMD + MSCs group (m) than those in MSCs group (k). (l) Comparison of the eGFP-labeled MSCs between MSCs group and UTMD + MSCs group, $^{*}P < 0.01$ versus groups, independent t-test. (n) VCAM-1 mRNA expression was increased by UTMD and/or MSCs infusion and it had a significant increase after MSCs infusion together with UTMD, $^{*}P < 0.01$ versus groups.

The increased blood glucose concentrations were associated with the deceased plasma insulin levels. Compared with normal group (25.27 ± 2.34 mIU/L), plasma insulin levels decreased in PBS group (16.42 ± 2.17 mIU/L) and UTMD group (16.67 ± 2.33 mIU/L) and improved in MSCs group (19.31 ± 1.68 mIU/L) and UTMD + MSCs group (19.53 ± 1.59 mIU/L) (Figure 3(e)). There was no significant difference in plasma insulin levels between MSCs group and UTMD + MSCs group ($P = 0.574$), which was the same as that between PBS group and UTMD group ($P = 0.564$).

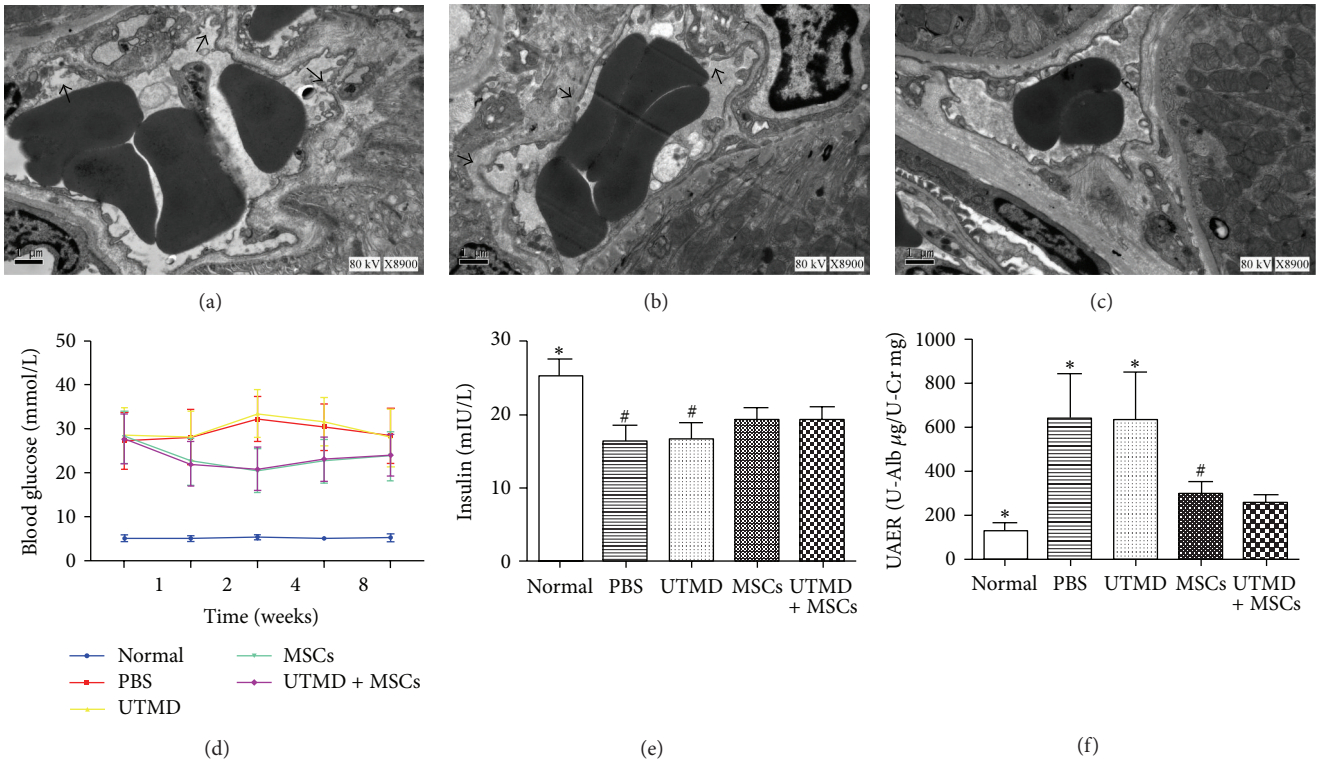

Figure 3: The endothelial cells of renal interstitial capillary were mildly injured under TEM and a part of interstitial capillary walls became thinner, discontinuous, and roughened in UTMD group ((a), arrow) and UTMD + MSCs group ((b), arrow), while the endothelial cells and interstitial capillary walls were kept intact in PBS group and MSCs group (c). (d) A trend graph for the change of blood glucose concentrations at 1, 2, 4, and 8 weeks after MSCs transplantation alone or together with UTMD. (e) 8 weeks after cell therapy, plasma insulin levels increased in MSCs group and UTMD + MSCs group, compared with PBS group and UTMD group, respectively. $^{*}P < 0.01$ versus groups and $^{\#}P < 0.05$ versus groups. (f) MSCs transplantation with or without UTMD significantly decreased the increased UAER values of PBS group and UTMD group, and there was a statistical difference between MSCs group and UTMD + MSCs group. $^{*}P < 0.01$ versus groups and $^{\#}P < 0.05$ versus groups.

Rats displayed mild microalbuminuria 4 weeks after STZ injection and UAER values of DN rats increased from 57.79 ± 13.42 μg/mg (normal group) to 85.47 ± 19.43 μg/mg (PBS group). At the end of the experiment, UAER values maintained a high level in PBS group (643.25 ± 204.58 μg/mg) and UTMD group (637.29 ± 212.24 μg/mg), and both of them were more than fourfold higher than that of age-matched normal control rats (128.57 ± 36.93 μg/mg) (Figure 3(f)). As a comparison, UAER values reduced in MSCs group (302.41 ± 49.21 μg/mg) and UTMD + MSCs group (252.83 ± 39.58 μg/mg). Furthermore, UAER values were significantly milder in UTMD + MSCs group (252.83 ± 39.58 μg/mg) than that in MSCs groups (302.41 ± 49.21 μg/mg). Although UAER values in MSCs group and UTMD + MSCs group never reached the normal level, UTMD + MSCs group showed an obvious fall in the level than MSCs group.

3.5. Pathological Changes of Pancreas and Kidney. At the end of the experiment, PBS group and UTMD group showed a massive destruction of pancreatic islets/β cells observed by pancreatic pathology and double-label immunohistofluorescence. Compared to normal pancreatic islets/β cells (Figures 4(a) and 4(f)), morphological irregularity, volume reduction, and less insulin- and more glucagon-positive cells were observed in PBS group and UTMD group (Figures 4(b), 4(c), 4(g), and 4(h)). As expected, MSCs group and UTMD + MSCs group exhibited a good recovery in amount, architecture and volume of pancreatic islets/β cells (Figures 4(d), 4(e), 4(i), and 4(j)).

Renal histological analysis showed that there have been similar changes both in PBS group and UTMD group in glomeruli and tubules which displayed glomerular sclerosis, mesangial expansion, and tubular dilatation observed by light microscope (Figures 4(l), 4(m), 4(q), and 4(r)). However, the extent of such changes of glomeruli and tubules was extenuated in MSCs group and UTMD + MSCs group (Figures 4(n), 4(o), 4(s), and 4(t)). Only a small part of glomerular hyalinosis and tubular dilatation were observed in MSCs group. Further improvements in structure or even nearly normal structure of glomeruli and tubules were observed in UTMD + MSCs group.

3.6. Immunohistochemistry. TGF-β1 was slightly expressed in glomeruli and tubules in normal group (Figure 5(a)) and strongly expressed in tubules in PBS group (Figure 5(b)) and UTMD group (Figure 5(c)). TGF-β1 expression decreased

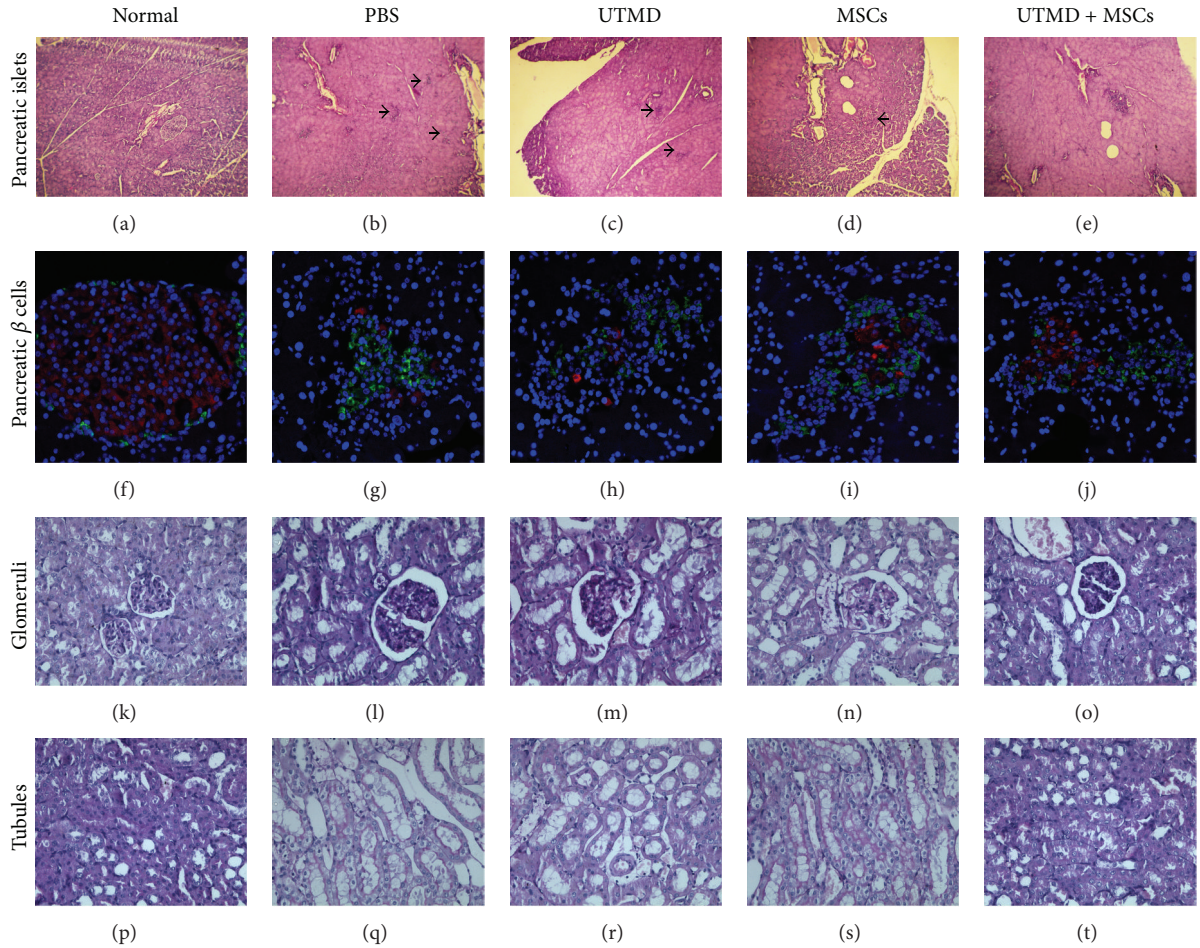

Figure 4: (a–e) Pancreatic histological sections were stained with H&E staining and observed under light microscope. Pancreatic islets structures were indicated by black arrows (b–d). (f–j) The amounts of insulin- and glucagon-producing cells (red and green, resp.) were observed by pancreatic double-label immunohistofluorescence. (k–t) Renal histological sections were stained with PAS staining and observed under light microscope. Normal rats glomeruli (k) and tubules (p). Glomerular sclerosis and glomerular mesangial expansion (l and m) and tubular dilatation (q and r) were observed in PBS group and UTMD group. MSCs-treated rats glomeruli (n) and tubules (s). Even nearly normal structure of glomeruli (o) and tubules (t) were observed in UTMD + MSCs group.

in MSCs group (Figure 5(d)) and UTMD + MSCs group (Figure 5(e)) after MSCs administration, and a further decrease was observed in UTMD + MSCs group. Figure 5(f) represents the quantification analysis of integrated optical density (IOD) of pictures of five groups.

3.7. ELISA. As shown in Figure 5(g), renal cortical IL-10 levels significantly decreased in PBS group (18.24 ± 2.17 pg/mg protein) and UTMD group (19.32 ± 2.25 pg/mg protein), compared with nNormal group (36.92 ± 1.27 pg/mg protein). After MSCs treatment, IL-10 levels notable increased in MSCs group (25.43 ± 3.46 pg/mg protein) and UTMD + MSCs group (31.15±3.19 pg/mg protein), compared with PBS group (18.24 ± 2.17 pg/mg protein). IL-10 levels in UTMD + MSCs group (31.15 ± 3.19 pg/mg protein) were higher than those in MSCs group (25.43 ± 3.46 pg/mg protein).

3.8. Western Blot. Consistent with the result of ELISA, synaptopodin protein expression in renal cortices decreased in PBS group and UTMD group, compared with normal group. After MSCs implantation, synaptopodin protein expression increased both in MSCs group and UTMD + MSCs group, and a further improvement in UTMD + MSCs group was detected.

4. Discussion

Ultrasound contrast agent (microbubbles) has developed to produce intense echoes and enhance blood-to-tissue contrast in clinical ultrasonography. Moreover, microbubbles destruction under ultrasonic irradiation has been proposed as a new, appealing technique for site-specific drug and gene delivery *in vitro* and *in vivo* and has been introduced in a variety of application areas.

Mesenchymal stem cells (MSCs) are the stromal component of bone marrow (BM) and easily obtained from BM, have the potential to differentiate into several cell types, and show immunomodulatory properties. MSCs are

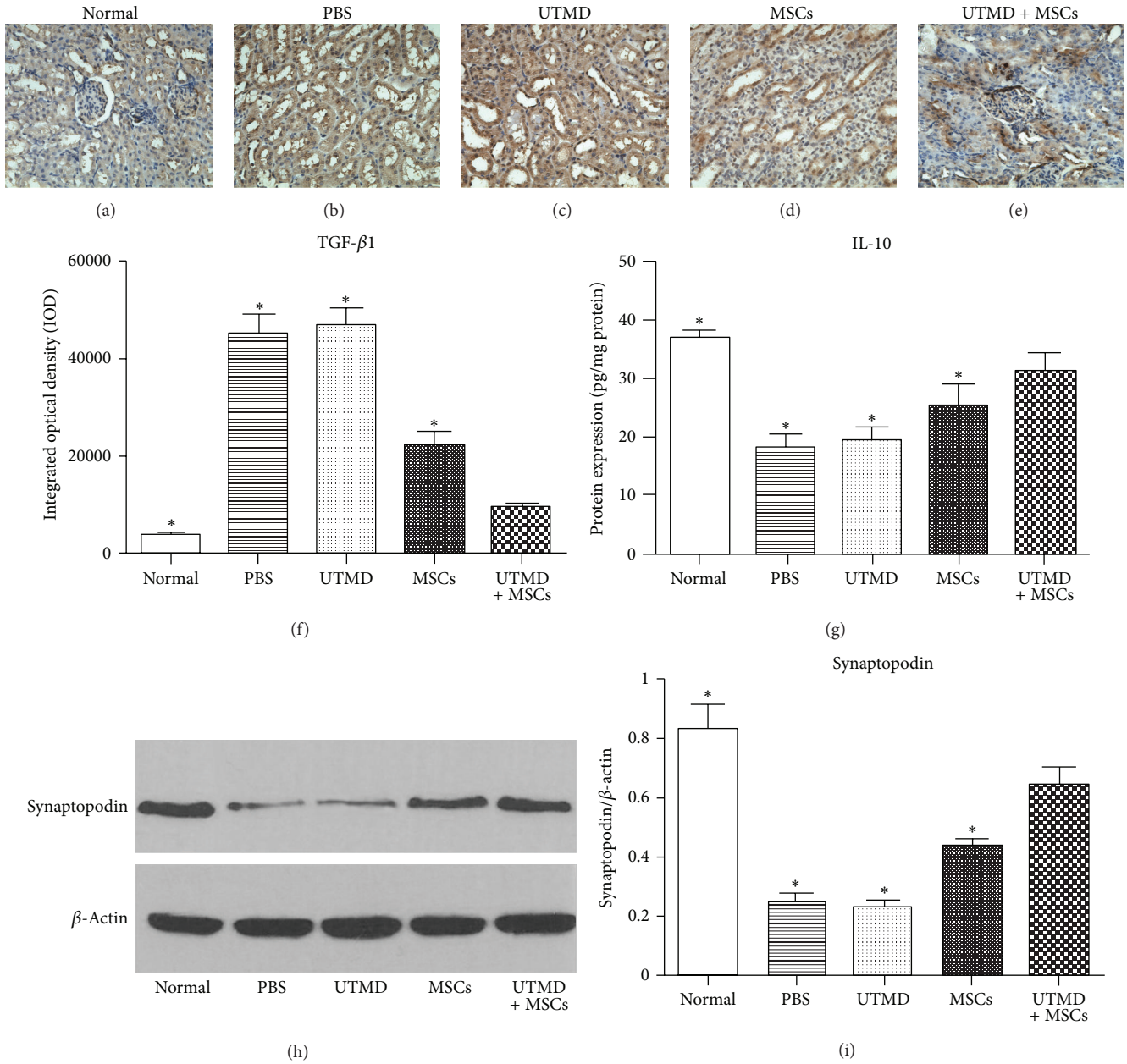

Figure 5: (a–e) Immunohistochemical staining of TGF-β1 in kidney of normal group (a), PBS group (b), UTMD group (c), MSCs group (d), and UTMD + MSCs group (e). The brown area was the positive expression of TGF-β1 and TGF-β1 expression significantly decreased after MSCs transplantation combined with UTMD technique. $^{*}P < 0.01$ versus groups. (f) represents the histogram of integrated optical density (IOD) of TGF-β1 expression analyzed using Image-Pro plus 6.0 software. (g) IL-10 levels in renal cortices were determined by ELISA and they were obviously higher in UTMD + MSCs group than those in PBS group, UTMD group, and MSCs group. $^{*}P < 0.01$ versus groups. (h and i) represents Western blot (h) and summary of densitometric analyses (i) for synaptopodin expression, which showed that synaptopodin expression increased after MSCs infusion and there was a greater improvement in UTMD + MSCs group. $^{*}P < 0.01$ versus groups.

capable of differentiating into functional insulin-producing cells *in vitro*, which can reverse hyperglycemia in diabetes rats [22]. MSCs also have the potential to differentiate into renal cells *in vivo*, which can repair the destroyed kidney [23]. Therefore, MSCs are regarded as an attractive strategy to ameliorate hyperglycemia and improve renal function [24, 25] through intracardiac injection, intra-arterial or intravenous injection, and intrarenal injection.

The use of MSCs for cell therapies relies on the capacity of these cells to home and engraft in the long term into the appropriate target tissue [26]. In this experiment, we showed here that the number of eGFP-labeled MSCs in the kidney of UTMD + MSCs group was significantly larger than those of MSCs group, which demonstrated that UTMD has the capability of increasing tropism of MSCs to damage kidney tissue. To prove this point of view, transmission

electronic microscope was performed to observe the ultrastructure of renal capillary after ultrasonic irradiation and we found that parts of interstitial capillary walls became thinner, discontinuous, and roughened after UTMD. It directly proved that interstitial capillary permeability was increased by UTMD, which facilitated the homing and gathering of MSCs to target tissue. VCAM-1 mRNA expression was performed after UTMD to clarify its underlying mechanism. The result suggests that VCAM-1 expression increases after MSCs implantation or UTMD, while a higher expression of VCAM-1 was shown in UTMD + MSCs group. It means that paracrine effect of the transplanted MSCs or ultrasound-targeted microbubble destruction leads to the upregulation of adhesion molecule, respectively. The combined effect of UTMD and MSCs induces a higher expression of VCAM-1 on the targeted vascular endothelium in UTMD + MSCs group, which causes the enhanced attachment of transfused MSCs onto the targeted endothelial layer [8]. Therefore, the targeted delivery and the enhanced homing of implanted MSCs to kidney might be induced by the increased interstitial capillary permeability under UTMD and the higher expression of VCAM-1 caused by acoustic cavitation of ultrasound and paracrine effect of MSCs.

No significant difference was found between MSCs group and UTMD + MSCs group in blood glucose concentration, the regeneration of insulin cells, and the structural recovery of pancreatic islets. A possible explanation was that only kidney, not pancreas, received ultrasonic irradiation before MSCs transplantation. There was no difference in the state of pancreatic vessels before and after MSCs transplantation. Therefore, there might be an equivalent amount of MSCs engrafted into pancreatic tissue and might have an equal therapeutic effect in MSCs group and UTMD + MSCs group. This phenomenon confirmed that UTMD played the role of specific target transplantation at the same time.

There is now strong evidence that the onset and progression of diabetic complications is significantly delayed by improving glycaemic control [27]. MSCs administration results in the reduction of blood glucose levels and prevents renal damage in diabetic mice [28]. Although no MSCs were found in pancreas, our experiment shows that MSCs transplantation reduces blood glucose concentrations, enhances insulin levels, and improves the morphology, structure, and quantity of pancreatic islets/β cells. MSCs acting through paracrine action might be a reasonable interpretation. In addition, as the kidneys receive approximately 25% of the cardiac output, therefore they have a richer blood supply than pancreas. This means that implanted MSCs through tail vein have more chance to engraft into renal interstitium, and thus it might be relatively easier to track eGFP-labeled MSCs in kidney than in pancreas. There was a significant difference in UAER value, the pathological change of kidneys, and renal cortical cytokine and protein expression after MSCs treatment between MSCs group and UTMD + MSCs group, and there was almost no apparent difference in these observed items between PBS group and UTMD group. This was possible for three reasons: (1) recent studies confirmed that MSCs crossed the endothelial cell layer and recruited to damage tissues analogous to those of inflammatory cells. In our study, interstitial capillary permeability in renal tubulointerstitial area and renal cortical VCAM-1 levels were increased by UTMD, which made MSCs more easily to attach and cross the thinner and discontinuous capillary walls and gather to damage kidney; (2) correspondingly, UTMD used alone may have negligible impact on the tissue repair for no MSCs supply in time during the change of permeability and the levels of adhesion molecule; (3) the reduction of blood glucose concentration in MSCs-treated rats depends on MSCs therapy. Renoprotection in MSCs-treated rats is correlated not only with MSCs therapy, but also with an improvement of endocrine pancreatic function. That is to say, the lower blood glucose concentrations play a facilitative effect for protecting the kidney. To sum up, there are more MSCs homing, retention, and participation for renal therapy after UTMD than MSCs transplantation alone. MSCs transplantation in combination with UTMD was not only a noninvasive method for cell-targeted delivery, but also an efficient approach for cell therapy.

TGF-β is a prototypical hypertrophic and fibrogenic cytokine [29]. It stimulates renal cell hypertrophy and extracellular matrix accumulation which are two hallmarks of diabetic nephropathy. It causes glomerular basement membrane (GBM) thickening and may promote podocyte apoptosis or detachment [30]. Cellular hypertrophy and matrix production are stimulated by high glucose concentrations in tissue culture studies. High glucose, in turn, appears to act through the TGF-β system because high glucose increases TGF-β expression, and the hypertrophic and matrix-stimulatory effects of high glucose are prevented by anti-TGF-β therapy [31]. The role of MSCs in antifibrotic therapy is still a matter of controversy. Ezquer et al. showed that MSCs administration prevented the onset of nonalcoholic steatohepatitis in obese mice [32]. However, Carvalho et al. indicated that MSCs were unable to reduce fibrosis or improve liver function in a rat model of severe chronic liver injury [33]. TGF-β1 is considered the most fibrogenic isoform of TGF-β. In our finding, MSCs transplantation prevents the development of renal hypertrophy, mesangial matrix expansion, and tubular dilatation, and decreases TGF-β1 expression, which means that glomerulosclerosis and renal interstitial fibrosis are prevented effectively. It might be correlated both with the decrease of blood glucose and with the inhibition of TGF-β1 expression by MSCs.

IL-10, the main anti-inflammatory cytokine and immunosuppressive cytokine, is produced by several types of immune cells such as T regulatory and Th2 lymphocytes, activated macrophages, B regulatory lymphocytes as well as other cell types [34]. It plays a key role in the regulation of immune responses, has a potent deactivator of monocyte/macrophage proinflammatory cytokine synthesis, and inhibits leukocyte infiltration, inflammation, and tissue damage associated with immunological response [35]. Diabetic nephropathy is an inflammatory disease and accumulating evidence now indicates that immunologic and inflammatory mechanisms play a significant role in its development and progression [36, 37]. Inflammatory factors promote macrophage accumulation and activation and accelerate the progression of diabetic renal injury [38]. Therefore, IL-10,

as an immunoregulatory cytokine, may be correlated with diabetic nephropathy and its anti-inflammatory properties have been similarly demonstrated in animal models [39]. In our model, DN rats were manifest by albuminuria, glomerular hypertrophy, tubular dilatation, and an apparent reduction of IL-10 amount, while most manifestations were ameliorated after MSCs transplantation. These data suggest that MSCs transplantation attenuates inflammatory response of kidney in diabetic nephropathy, leads to modulation of the inflammation through upregulation of IL-10 cytokine, and delays the progression of diabetic nephropathy. It also shows that IL-10 is involved in the pathogenesis of diabetic nephropathy and the attenuation of inflammation could mitigate renal interstitial injury. This beneficial effect is thought to be due to the anti-inflammatory factors, such as IL-10, produced by MSCs that are secreted in a paracrine fashion [39].

Although the quantity of implanted MSCs in glomeruli were relatively less than that in renal interstitium, glomerular repair was obvious as well. Podocyte lines the outer aspect of glomerular basement membrane (GBM), therefore forming the final barrier to protein loss [40]. Podocyte injury has been suggested to be associated with the pathogenesis of albuminuria in diabetic nephropathy and predicts the progressive course of diabetic nephropathy. Synaptopodin is a proline-rich, actin-associated protein which may play an important role in modulating podocyte foot processes (FP), and highly expressed in telencephalic dendrites and renal podocytes [41, 42]. From this experiment, podocytes repair was assessed by detecting synaptopodin protein expression and UAER values. The results show that UAER values significantly increase and synaptopodin protein expression significantly decreases 8 weeks after diabetes onset, indicating a serious injury in podocyte. MSCs transplantation ameliorates podocyte injury for decreasing UAER values and increasing synaptopodin expression. Furthermore, kidney-targeted transplantation of MSCs mediated by UTMD showed a better improvement in UAER values and upregulated the more synaptopodin expression, suggesting an apparent podocyte repair.

In this paper, we provide an intuitive proof (transmission electron microscope) to prove that ultrasonic cavitation increases interstitial capillary permeability, which facilitates the homing of MSCs to injured kidney. UTMD technique combined with MSCs transplantation induces a higher expression of the adherent molecule (VCAM-1), which promotes the attachment and gathering of MSCs to interstitial capillary endothelium of damage kidney. Target transplanted MSCs secrete anti-inflammatory cytokine (IL-10) by a paracrine action, which leads to the increase of IL-10 levels and the reduction of inflammatory response. Infused MSCs repair the podocyte and renal interstitial injury by upregulating synaptopodin expression and downregulating TGF-β1 expression, resulting in that the manifestations such as glomerular sclerosis, mesangial expansion, and tubular dilatation were obviously improved.

In conclusion, MSCs transplantation reverts hyperglycimia and kidney-targeted transplantation of MSCs mediated by UTMD increases MSCs homing to damage renal tissue in diabetic nephropathy rats and prevents nephropathy, therefore providing a potential means for targeting therapeutic agents to kidney.

5. Conclusions

MSCs transplantation reverts hyperglycemia, UTMD technique noninvasively increases the homing of MSCs to kidneys and promotes renal repair in DN rats. The key mechanism may be due to that UTMD promotes the more MSCs homing to diabetic kidney by increasing the permeability of renal interstitial capillary and VCAM-1 expression, which suppresses the inflammatory reaction by enhancing IL-10 levels, repairs the damaged glomeruli and tubules by inhibiting TGF-β1 expression and upregulating synaptopodin expression.

Conflict of Interests

The authors declare that there is no conflict of interests with the trademarks mentioned in their paper.

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Research Article

Nonviral Gene Targeting at rDNA Locus of Human Mesenchymal Stem Cells

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Background. Genetic modification, such as the addition of exogenous genes to the MSC genome, is crucial to their use as cellular vehicles. Due to the risks associated with viral vectors such as insertional mutagenesis, the safer nonviral vectors have drawn a great deal of attention. *Methods*. VEGF, bFGF, vitamin C, and insulin-transferrin-selenium-X were supplemented in the MSC culture medium. The cells' proliferation and survival capacity was measured by MTT, determination of the cumulative number of cells, and a colony-forming efficiency assay. The plasmid pHr2-NL was constructed and nucleofected into MSCs. The recombinants were selected using G418 and characterized using PCR and Southern blotting. *Results*. BFGF is critical to MSC growth and it acted synergistically with vitamin C, VEGF, and ITS-X, causing the cells to expand significantly. The neomycin gene was targeted to the rDNA locus of human MSCs using a nonviral human ribosomal targeting vector. The recombinant MSCs retained multipotential differentiation capacity, typical levels of hMSC surface marker expression, and a normal karyotype, and none were tumorigenic in nude mice. *Conclusions*. Exogenous genes can be targeted to the rDNA locus of human MSCs while maintaining the characteristics of MSCs. This is the first nonviral gene targeting of hMSCs.

1. Introduction

Human mesenchymal stem cells (hMSCs) are an attractive source of adult stem cells for autologous cell and gene therapy. They have immunosuppressive property and ability to differentiate into multiple cell types present in several tissues [1–3], making them to be a promising cellular vehicle for gene therapy [4, 5]. Current methods most commonly used for genetically modifying hMSCs are based on random transgene integration; however, the uncertainty of the integration site brings problems. Random integration may take place in heterochromatin, leading to silencing [6], or in coding regions, causing disruption of an endogenous gene or interference in the transcription of neighboring sequences [7]. These issues can be addressed by gene targeting, a primary alternative method. Unfortunately, efficient gene targeting in hMSCs has been poorly developed. To our knowledge, only four cases of gene targeting in hMSCs have been reported to date and all of them were based on viral transfer methods. Due to safety concerns related to random integration, nonviral gene targeting at appropriate transgene harbor deserves great attention, while no actual case based on nonviral delivery has been described in hMSCs.

Human cells have approximately 400 copies of a 45S ribosomal DNA (rDNA) repeat that encodes ribosomal RNA (rRNA) and is distributed over the short arm of the five acrocentric chromosomes 13, 14, 15, 21, and 22. The rRNA gene is transcriptionally active and produces approximately 80% of the total RNA in rapidly dividing cells [8]. It is well known that the human rDNA copy number variations are common among healthy individuals, and a balanced chromosomal translocation involving the rRNA cluster occurs without apparent phenotypic effect. The rDNA cluster exhibited strikingly variable lengths between and within human individuals and showed high intrinsic recombinational instability during both meiosis and mitosis [9]. In addition, the human rRNA gene cluster consists of hundreds copies of tandemly repeated rDNA units. Targeting an exogenous gene into one or a few of the rDNA repeats may not cause loss of function effects of the rRNA genes owing to high copy number of this gene. These

properties indicate that the rDNA locus may hold a high intrinsic homologous recombination (HR) activity and this locus is considered to be an ideal safe locus for transgene integration [10, 11]. Several studies have reported that efficient targeted gene addition at the rDNA 28s locus could be achieved based on viral transfer methods. However, because the majority of the integration was randomly located in the genome, the risks of the random integration were unavoidable for these methods [10–12]. Alternatively, based on a nonviral vector, in our previous studies, gene targeting at the rDNA 18S locus had been achieved, and the transgene could be stably expressed ectopically in targeted cells [13, 14]. In this study, a nonviral gene-targeting vector was constructed and targeted gene addition in MSCs was performed at the rDNA locus.

In fact, MSCs are a rare population that comprises only approximately 0.001% ~ 0.01% of the total bone marrow mononuclear cells. In addition, gene-targeting frequency in MSCs is intrinsically low. The limited number of the targeted MSCs may be a hurdle for their therapeutic use. Therefore, MSCs genetically modified for clinical application typically require extensive expansion *in vitro*. Unlike embryonic stem cells that have an unlimited proliferative lifespan, adult MSCs *in vitro* display a restricted proliferative longevity, a diminishing replication capacity, and an increased loss of differentiation potential [15–17]. The role of growth factors in enhancing the proliferation and survival of MSCs has been widely studied over the past few years. Several suitable factors have been found to improve *ex vivo* expansion in MSCs without altering their stem cell phenotype and multipotent differentiation potentials, including fibroblast-growth-factor- (FGF-) 2, epidermal growth factor (EGF) [18], and platelet-derived-growth-factor- (PDGF-) BB [19–21]. The combined effects of the factors have appeared quite robust [17, 19–21]. The aim of this study was to target an exogenous gene at the rDNA locus of human MSCs using the nonviral rDNA-targeting vector. The proliferation capacity of the MSCs was improved significantly by adding growth factors. The exogenous neomycin (Neo) gene was targeted at the rDNA locus of MSCs. The recombinant MSCs were compared to control MSCs with respect to phenotype, plasticity, multipotency, karyotype, and tumorigenicity. This is the first report of any nonviral gene targeting of human MSCs and this method may be an optimal approach to MSC-based disease modeling and gene therapy.

2. Materials and Methods

2.1. Construction of pHr2-NL. The pHr2-NL plasmid contains a long homologous arm (LHA) corresponding to rDNA +937 to ++6523 and a short homologous arm (SHA) corresponding to rDNA +6523 to +7643. It was generated in five steps. Firstly, a fragment homologous to the rDNA +6523 to +7684 region was amplified from human genomic DNA with the primers (5′-AATCGATTTTGATATCTGAGGCAACCCCCTCTCCTCTTGGGC-3′/5′-GTCGCCGCCGGGGACACGCGAA-3′). A fragment homologous to the rDNA +5513 to +6523 was amplified from human genomic DNA with the primers (5′-GCGGAAGGATCATTAACGGAGCCCGGA-3′/5′-ATAATCGATAGAGGAGAGGGGTTGCCTCAGGCC-3′). The PCR products were cloned into pGEMT, resulting in pGEM-T-SHA and pGEM-T-LS, respectively. Secondly, the expression cassette ECMV-IRES-Neo-SV40PolyA was amplified from the pHrneo [14] plasmid with the primers (5′-ATAATCGATATAACTTCGTATAATGTATGCTATACGAAGTTATTCTTAAGGAATTCCCCCTCTCCCT-3′/5′-ATAGATATCATAACTTCGTATAATGTATGCTATACGAAGTTATTAGACGGTCGACCCGTGCGGA-3′). The PCR product (1.8 kb) was cloned into pGEMT, resulting in pGEM-T-INL. Thirdly, the 1.8 kb *ClaI*/*SacI* fragment from pGEM-T-LS was inserted into the *ClaI* and *SacI* sites of pGEM-T-SHA, generating the plasmid pGEM-T-LS-SHA. Next, the *ClaI/EcoRV* fragment from pGEM-T-INL was inserted into the *ClaI* and *EcoRV* sites of pGEM-T-LS-SHA, generating the plasmid pHr1-NL. Finally, the 5.6 kb *MfeI*/*SacI* fragment from the T-pHr (constructed previously [14], containing the long homologous arm corresponding to rDNA +937 to +6523) and the 5.5 kb *AAT II*/*ClaI* fragment from the pHr1-NL, treated with T4 DNA polymerase (FERMENTS) and fastAP thermo sensitive alkaline phosphatase (FERMENTS), respectively, were ligated to generate pHr2-NL.

2.2. Isolation of MSCs from Bone Marrow and Culture Conditions. Informed consent was obtained from all participants according to a protocol approved by the Ethics Committee of State Key Laboratory of Medical Genetics of China (no. 2010-HUMAN-004). Bone marrow was obtained from the iliac bones of two volunteers. MSCs were isolated by using a Histopaque-1077 density gradient (Sigma) as previously described [22, 23]. The cells were cultured in MSC basal medium (L-glucose Dulbecco minimum essential medium (DMEM-L, Sigma-Aldrich, China) supplemented with 10% foetal bovine serum (GIBCO), 100 U of sodium penicillin/mL, and 100 U of streptomycin sulphate/mL). The mononuclear cells were plated at a density of approximately 5×10^5 cells/cm^2. Symmetrical colonies became visible on days 5 to 7, and the cells were subcultured at a seeding density of 1×10^4 cells/cm^2. Growth factors including VEGF (5 ng/μL, PEPRO TECH 100–200), bFGF (10 ng/μL, Invitrogen PHG0263), vitamin C (Vc) (50 μg/mL, Sigma A4544), and ITS-X (insulin-transferrin-selenium-X) (100x Invitrogen 51500056) were added [19, 24, 25].

2.3. Fluorescence-Activated Cell Sorting (FACS) Analysis. Surface markers CD31, CD44, CD45, CD73, CD90, and CD105 (BD Pharmingen, Hunan, China) were analyzed according to the manufacturer's instructions.

2.4. Colony-Forming Unit-Fibroblast (CFU-F) Assay. MSC cells were reseeded at a concentration of 150 cells per 100 mm dish (2.7 cells/cm^2). After 14 days, cultures were stained with 0.5% crystal violet (Sigma). Colonies less than 2 mm in

diameter and faintly stained colonies were ignored. Colony-forming efficiency was expressed as the relative number of colonies generated from the number of cells seeded.

2.5. Cell Proliferation Assay. Numbers of cells and cell viability were measured by counting cells on a hemocytometer using the Trypan Blue dye exclusion method. Cells were cultured in 96-well plates (1×10^4 cells/cm^2). Seventy-two hours later, the number of viable cells was determined using an MTT assay. The plates were analyzed using a microplate reader at 570 nm.

2.6. Stable Transfection in MSCs. MSCs were stably transfected with the plasmid pHr2-NL and linearized with *Ahd*I, by nucleofection using the c-17 pulsing program. The DNA/cells ration was 2 μg DNA/5×10^5 cells. The transfected MSCs were plated in 100 mm dishes at a density of 1×10^3 cells/cm^2. Twenty-four hours after transfection, the medium was replaced with fresh medium. After culture for another 48 hours, 50 μg/mL G418 was added to the culture medium. The medium was refreshed every third day, and the concentrations of G418 on days 3, 6, 9, and 12 were 200 μg/mL, 100 μg/mL, 100 μg/mL, and 15 μg/mL, respectively. Medium without G418 was added on day 14, and the drug-resistant cells were cultured without G418 for another 3 weeks. Individual colonies were picked and expanded, and the genomic DNA was extracted using PCR and Southern blotting to detect recombinants.

2.7. PCR Identification of the Site-Integration Colonies. The primer t-up (5′-GTTATCCGCTCACAATTCCACACA-ACATACGA-3′) and the primer t-re (5′-GGAGGTCGG-GGGGACGGGTCCGAGGA-3′) were used.

2.8. Sequencing the PCR Fragment. PCR products were isolated after migration on 0.8% LMP agarose gels, cloned into the pGEM-T vector, and sequenced with primer-T7 (5′-TAATACGACTCACTATAGGG-3′) and primer-SP6 (5′-CATACGATTTAGGTGACACTATAG-3′).

2.9. Southern Blotting. After overnight digestion with restriction enzymes *Pvu II, Nco I, EcoR I,* and *Hind III* (New England Biolabs, Ipswich, MA, USA), 3 μg of genomic DNA per sample was electrophoresed on a 0.8% agarose gel overnight and then transferred to positively charged nylon membranes (Hybond-N+, Amersham, Piscataway, NJ, USA). DNA molecular weight marker III, digoxigenin-labeled DNA (Roche Diagnostics, Indianapolis, IN, USA), and lambda DNA *Hind III* (TaKaRa, Dalian, China) were used as molecular weight markers. The blots were hybridized with DIG-dUTP-labeled probes overnight at 42°C. After incubation with AP-conjugated DIG antibody (Roche Diagnostics, Indianapolis, IN, USA) and appropriate washing, the signals were detected using CDP-Star (Roche Diagnostics, Indianapolis, IN, USA) as a substrate for chemiluminescence. Probes were generated using DIG-High Prime (Roche Diagnostics, Indianapolis, IN, USA), and the templates were generated using PCR amplification from pHr2-NL. The primers used for probe 1 (P1) were 5′-CCCGGAAACCTGGCCCTGTCTT-3′ and 5′-CTTCGCCCAATAGCAGCCAGTC C-3′, and primers for probe 2 (P2) were 5′-AATGGCCGCTTTTCTGGA-3′ and 5′-TGTGATGCTATTGCTTTATTTGTA-3′.

2.10. Karyotyping. About 5×10^5 cells from each of the four targeted MSC colonies were treated with 0.08 μg/mL colcemid (Sigma, St. Louis, MO, USA) for 2.5 hours. Then cells were trypsinized, centrifuged, and incubated in 0.075 M KCl for 30 minutes at 37°C. After fixing with Carnoy fixative, metaphase chromosome spreads were prepared using the air drying method. Thirty metaphase spreads were evaluated per colony.

2.11. In Vivo Implantation Assay. All animal protocols were approved by the Animal Ethics Committee of the State Key Laboratory of Medical Genetics of China. Twenty-four SCID mice were divided into four groups of six mice each. PBS and a total of 2×10^6 cells of each of the three cell types (heterogenous MSCs derived from the four targeted MSC colonies, wild-type MSCs, and HT1080) were injected subcutaneously over the right ribcage. The skin and underlying soft tissue of the relevant area were dissected, fixed in 4% paraformaldehyde, stained with hematoxylin and eosin, and investigated for possible tumor growth.

2.12. Differentiation Assays. The four MSC colonies subjected to site-specific integration (1-1, 1-2, 2-1, 2-2) were assessed for adipogenic, osteogenic, and chondrogenic potential. Assays of *in vitro* differentiation to osteocytes, chondrocytes, and adipocytes were performed using StemPro Osteogenesis Differentiation Kit, StemPro Chondrogenesis Differentiation Kit, and a StemPro Adipogenesis Differentiation Kit according to the manufacturer's protocol.

2.13. Statistical Analysis. Data sets were expressed as the mean value and standard deviation. The significance of colony-forming efficiency was determined using the Student's *t*-test. The viable cell numbers derived from media with different additives and the number of oil-red-O-positive cells derived from wild-type and targeted MSCs were analyzed using one-way ANOVA. Differences were considered significant at $P < 0.05$.

3. Results

3.1. Proliferation and Survival of MSCs Treated with Growth Factors. First, the effects of several growth factors, including bFGF, VEGF, Vc, and ITS-X were individually evaluated on the proliferation of MSCs. During a 5-day culture period bFGF significantly increased the number of viable cells relative to cells exposed to plain basal medium, but Vc, ITS-X, and VEGF did not (Figure 1(a)). The cumulative numbers of MSCs cultured in the mediums supplemented with bFGF, Vc, VEGF, and ITS-X were 1.47-, 1.05-, 0.78-, and 1.13-fold higher than those in the basal medium. Next, the effects of

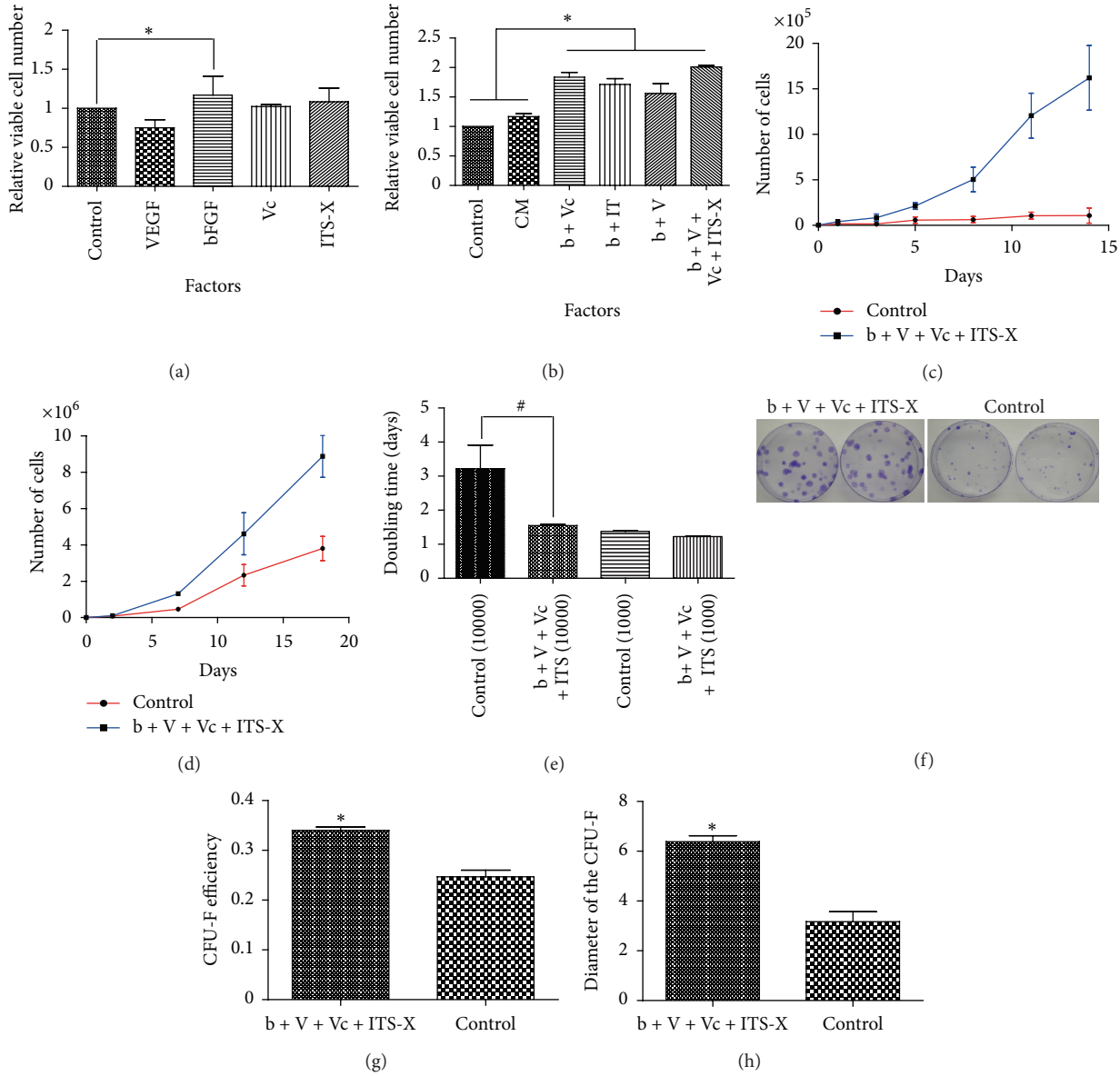

Figure 1: Effects of VEGF, bFGF, VC, and ITS-X on MSC proliferation. The effects of culture conditions with growth factors alone (a) and (b) in combination were examined on viable cell yield as assayed by MTT. MSC growth curves were generated at plating densities of (c) 1×10^4 cells/cm^2 and (d) 1×10^3 cells/cm^2. (e) Doubling time was calculated ($n = 3$) at these two plating densities. $^{\#}P < 0.05$. (f) CFU-Fs were stained with crystal violet and captured using a camera (Sony). (g) CFU-Fs efficiency ($n = 3$). (h) The average diameter of CFU-Fs from each set of culture conditions. b, bFGF; V, VEGF; Vc, vitamin C; ITS-X, insulin-transferrin-selenium-X; CM, commercial medium from Stem Cell Technologies; control, DMEM with 10% FBS. $^{*}P < 0.05$.

the basal medium supplemented with various combinations of growth factors on the proliferation of MSCs were evaluated. The results showed that all combinations of growth factors increased proliferation. The cumulative numbers of MSCs cultured in the medium supplemented with Vc+bFGF, VEGF+bFGF, ITS-X+bFGF, and VEGF+bFGF+Vc+ITS-X were 2.06-, 1.77-, 1.63-, and 2.39-fold higher than those in the basal medium (Figure 1(b)). At the plating density of 1×10^4 cells/cm^2, the cumulative cell numbers from 3×10^3 cells at passage 6 were on average 15-fold (Figure 1(c)) higher than those in the basal medium after 14 days of incubation. They showed a doubling time of about 1.6 days. At a plating density of 1×10^3 cells/cm^2, the cumulative cell numbers were on average 2.7-fold higher than the basal medium after incubation for 18 days (Figure 1(d)), with a doubling time of about 1.25 days (Figure 1(e)). When the total cell populations were evaluated using a CFU-F assay, the colony-forming efficiency was 34% with the combination of VEGF+bFGF+Vc+ITS-X, which was significantly higher than that in the basal medium (24.7%). The colonies in the VEGF+bFGF+Vc+ITS-X group were clearly larger than the colonies in the basal medium (Figures 1(f)–1(h)).

Table 1: Gene targeting in HT1080 cells.

Exp.	N	C	T	S	ATF	RTE
1	1.0	210	6	12	105	50.0%
2	3.0	529	16	30	94	53.3%

Exp.: experiment performed. N: number of cells nucleofected ($\times 10^6$). C: total number of resistant colonies obtained from each experiment. S: number of colonies screened. T: number of colonies screened as targeted recombinants. ATF: absolute targeting frequency ($\times 10^{-6}$) = TC/NS. RTE: relative targeting efficiency = T/S.

Table 2: Gene targeting in MSCs.

Don	N	C	S	T	ATF	RTE
1#	0.5	17	9	2	7.6	22.2%
2#	1.0	36	23	3	4.7	13.0%
2#	3.0	98	50	11	7.2	22.0%

Don: donor of bone marrow. N: number of cells nucleofected ($\times 10^6$). C: total number of resistant colonies obtained from each experiment. S: number of colonies screened. T: number of colonies screened as targeted recombinants. ATF: absolute targeting frequency ($\times 10^{-6}$) = TC/NS. RTE: relative targeting efficiency = T/S. 1#, 2#: bone marrow donors.

3.2. Gene Targeting of Human MSCs. We constructed an rDNA-targeting plasmid, pHr2-NL, which introduced a promoterless neomycin (Neo) cassette flanked by two loxP sites into the 45S pre-RNA gene. The cassette was flanked by a long homologous arm (5.6 kb) and a short homologous arm (1.1 kb). The cassette contained an encephalomyocarditis virus internal ribosomal entry site (EMCV-IRES), which enabled resistant gene expression under the control of endogenous RNA polymerase I (Pol I) promoter upstream after HR (Figure 2(a)).

A targeting experiment was first carried out in HT1080 cells. The enrichment efficiency was 50% and the targeting efficiency was 0.01% (Table 1). Then the targeting experiment was performed in triplicate on two groups of MSCs. In the groups exposed to basal medium, a few of drug-resistant cells can be observed but there were no colonies (Figure 2(b)). When exposed to VEGF+bFGF+Vc+ITS-X, many tight colonies were observed (Figure 2(c)). PCR was initially used to detect the positive recombinants; 2 out of 9, 3 out of 23, and 11 out of 50 drug-resistant colonies were found to contain positive recombinants (Table 2) (Figure 2(d)). PCR-positive recombinants were detected by Southern blotting after 5 passages; the results showed only one 8.3 kb band, which indicates that the site-specific integration of the exogenous cassette at the rDNA locus without random integration was present in 4 out of 5 representative PCR-positive colonies. However, an unexpected extra band appeared in one of the PCR-positive colonies, indicating that random integration also took place (Figure 2(e)). Consistent results were produced when the genomic DNA was cut with *Nco I, EcoR I,* and *Hind III* (Figure 2(f)).

The MSC colonies that underwent gene targeting were expanded and the cell numbers were counted. On average, 1×10^7 cells were obtained from one targeted MSC colony (Figure 3(a)). The expanded MSCs retained the MSC surface antigene expression (Figures 3(b) and 3(c)) and the ability to differentiate into chondrocytes, adipocytes, and osteocytes *in vitro* (Figure 3(d)). Quantitative analysis indicated that the adipogenic differentiation partially decreased compared with the normal MSCs at passage 6 (Figure 3(e)). They retained a normal karyotype (Figures 4(a) and 4(b)) and failed to develop tumors *in vivo* (Figures 4(c)–4(h)).

4. Discussion

Recent advances have shown that the use of MSCs as therapeutic vehicles may be feasible. The development of gene-targeting methods based on nonviral transfer for hMSCs deserves attention with respect to the advantages of nonviral vectors. The advantages of nonviral gene transfer include low acute toxicity, simplicity, few restrictions on the size of the gene of interest, and feasibility to be produced on a large scale [26–29]. Here, we established a nonviral method and demonstrated that the exogenous Neo gene could be targeted to the rDNA locus of MSCs. The gene-targeted MSCs maintained uniform surface antigen expression and a normal karyotype and did not develop tumors *in vivo*. Nonviral methods based on transposons such as Sleeping Beauty and piggyBac have been reported to be efficient in gene therapy and to be comparable in time-consuming compared with this method. However, safety issues about the transposons are reported. The first safety issue is about the presence of the SB transposase gene and the potential for remobilization of transposons already sited in the recipient genome. The second one is the insertional mutagenesis. The Sleeping Beauty transposon has the most random integration preference of the vectors currently in use for gene therapy [30, 31]. In this study, following antibiotic selection using G418 based on a promoter-strap strategy, the site-specific integration recombinants were selected by PCR and Southern blot assays and expanded *in vitro*. The random recombinants were eliminated.

The low integration efficiency of nonviral gene-targeting addition in mammalian cells has been a major limitation to its application [32]. It is thought that nonhomologous end joining (NHEJ) and HR DNA-repair pathways mediate random integration and site-specific integration, respectively. NHEJ is believed to occur at rates that are three to four orders of magnitude higher than those of HR [33], which makes it relatively easier to obtain colonies that carry a randomly integrated transgene. In this study, the relative gene-targeting frequencies achieved in the rDNA locus were observed to be 13%–22% in hMSCs and 50% in HT1080 cells. By using the nonviral delivery method, the absolute targeting frequency is more than 20-fold higher than that in HT1080 cells at *HPRT*, a most commonly targeted locus [34]. In previous reports, by including 28S rDNA homology arms into the vector design, the integration frequency of a recombinant adeno-associated viral vector in rat hepatocytes was enhanced by 30-fold [10]. The underlying mechanism appears to be the relatively high intrinsic activity of HR at the rDNA locus. Studies on Arabidopsis thaliana and yeast cells have suggested that the rDNA region may have functional components that stimulate HR [35]. Based on the similarity of rDNA structures between different eukaryotic cells, the rDNA region may be

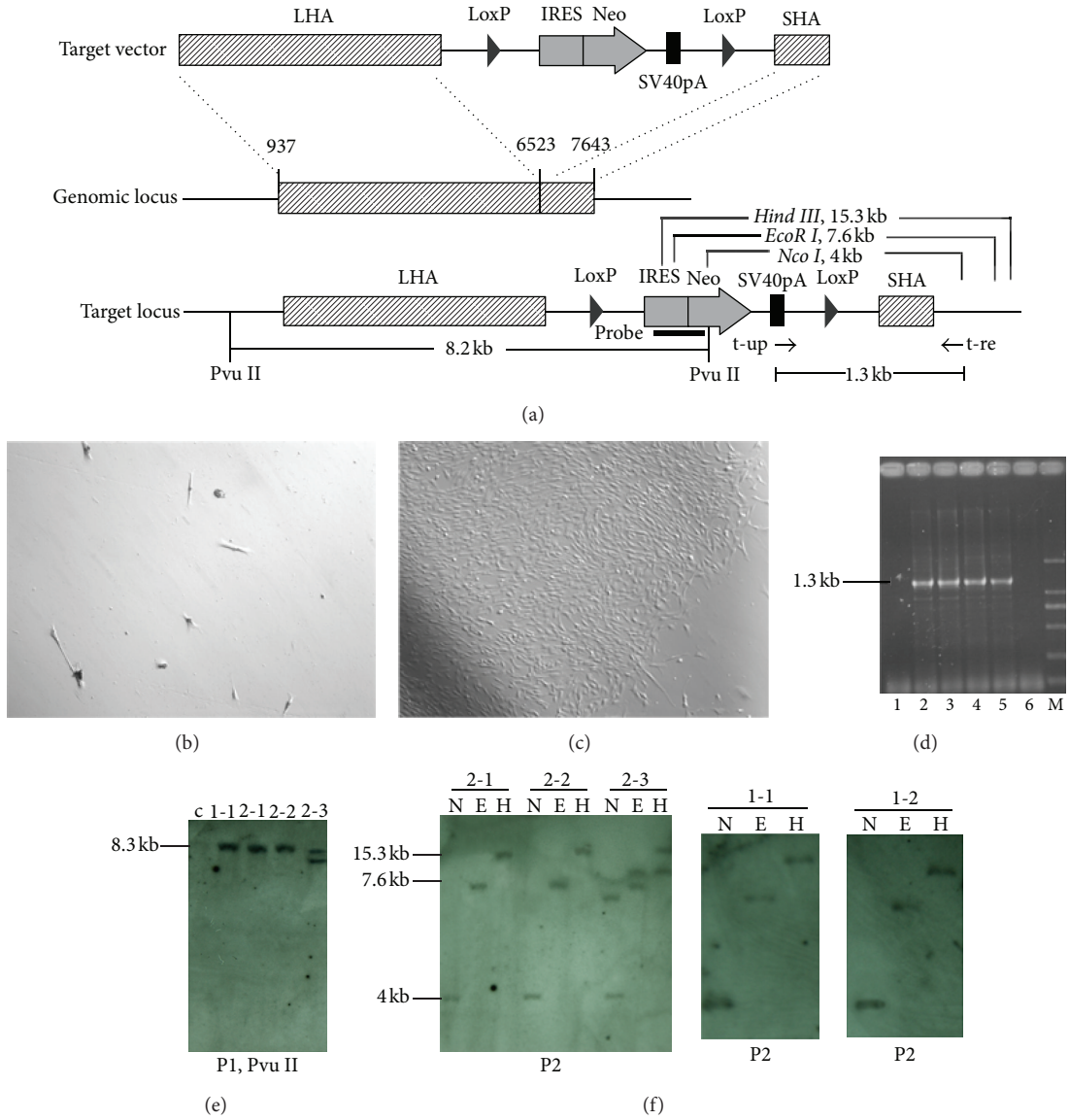

Figure 2: Site-specific integration at the rDNA locus of MSCs. (a) Schematic of the construction of pHr2-NL. pHr2-NL contained two inverted expression cassettes, one consisting of an IRES element from the encephalomyocarditis virus, the coding region of the Neo gene, the SV40 polyA signal (SV40pA), and two loxP sites with the same orientation. LoxP sites were recognized by CRE enzyme to remove the Neo cassette after gene targeting. LHA, long homologous arm (U13369:937-6523); SHA, short homologous arm (U13369:6523–7643). The genomic locus indicates the 6.7 kb fragment (U13369:937-7643) required for homologous recombination at the internal transcribed spacer 1 (ITS1) of the rRNA gene. Single cutting sites for restricted enzymes of *Nco I, EcoR I, Hind III*, and *Pvu II* are located at the IRES-Neo frame and outside of the long homologous fragment. The fragment between the two *Pvu II* sites was 8285 bp in size, and it was detected using probe 1 (P1). The expected sizes of the restriction fragments produced by *Nco I, EcoR I, and Hind III* were 4001 bp, 7628 bp, and 15,316 bp, respectively. These were detected using probe 2 (P2). Primer t-up was located at the SV40 polyA. Primer t-re was located outside of the SHA at the hrDNA locus. (b) Drug-resistant cell in basal medium. (c) Drug-resistant colonies in the medium supplemented with VEGF+bFGF+Vc+ITS-X. (d) Identification of colonies with site-specific integration by PCR. The expected fragment, 1.3 kb in size, was amplified from the genomic DNA of colonies using site-specific integration. M, DL200 DNA marker; 1, negative colony; 2–5, positive colonies; 6, wild-type MSCs. (e–f) Southern blotting analysis of the representative recombinants. Genomic DNA digested with *Pvu II*, *Nco I, EcoR I,* and *Hind III* was analyzed. A specific band was consistently detected in colonies 1-1, 1-2, 2-1, and 2-2. An additional band beside the specific band was detected in colony 2-3. c, control (untransfected MSCs); *N, Nco I; E, EcoR I; H, Hind III*.

a common HR hotspot in the majority of eukaryotic cells [36]. Despite recent success of gene targeting mediated by zinc-finger nuclease (ZFN) [4], we chose not to pursue this strategy because of the laborious design process and the toxicity resulted from the "off-target" effects [37].

To obtain enough cell for clinical use, the MSCs modified at a low efficiency need extensive expansion *in vitro*. Because the gene-targeting efficiency of nonviral vectors was relatively low, there exists a need to expand the targeted cells to get the requisite cell number. Previous reports have shown that by

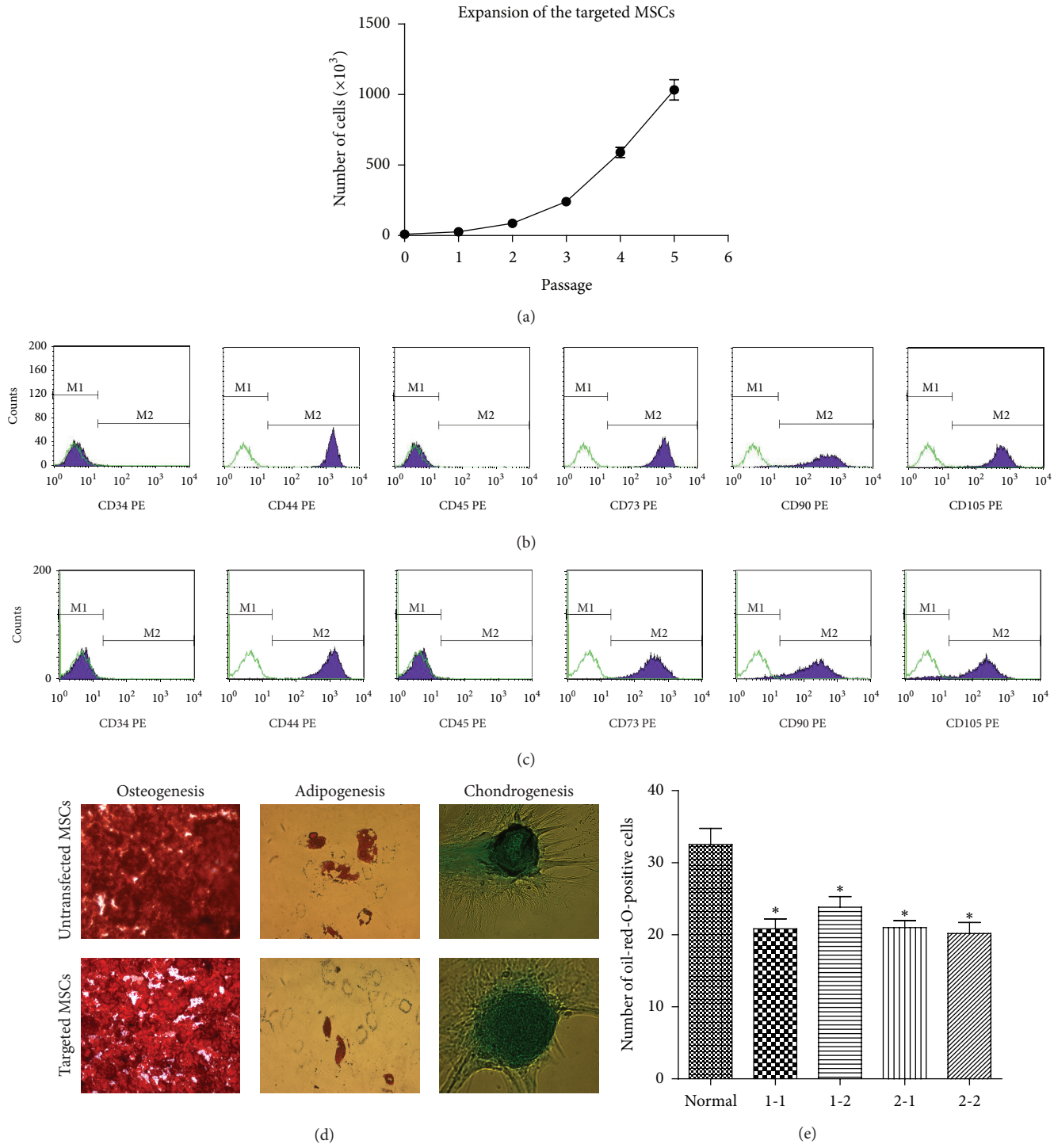

FIGURE 3: MSC surface antigen and differentiation potential detection. (a) Expansion of the targeted MSCs ($n = 3$). About 1×10^4 cells from every targeted colony were expanded. Cell count was performed at every passage. The expanded MSCs were subjected to MSC surface antigen and differentiation potential detection. (b) Flow cytometry analysis of the surface antigen expression of hMSCs untransfected. Green curves represent isotype controls and blue curves represent the specific antibodies. (c) Flow cytometry analysis of the surface antigen of expanded MSCs with gene targeting. (d) Adipogenic, osteogenic, and chondrogenic potential of hMSCs untransfected (upper) or with gene targeting (down). The adipogenic cultures were stained with oil red O to measure the accumulation of intracellular lipids. The osteogenic cultures were stained with alizarin red S to detect calcium deposition. For chondrogenic induction, the pellet sections were stained with alcian blue dye to detect proteoglycans. (e) Quantitative analysis of the adipogenic differentiation. Normal MSCs at passage 6 (normal) and targeted MSCs (1-1, 1-2, 2-1, 2-2) were differentiated into adipocytes. For each colony, the number of the oil-Red-O-positive cells was counted under 6 microscope fields. $^*P < 0.05$. The bar indicates 50 μm.

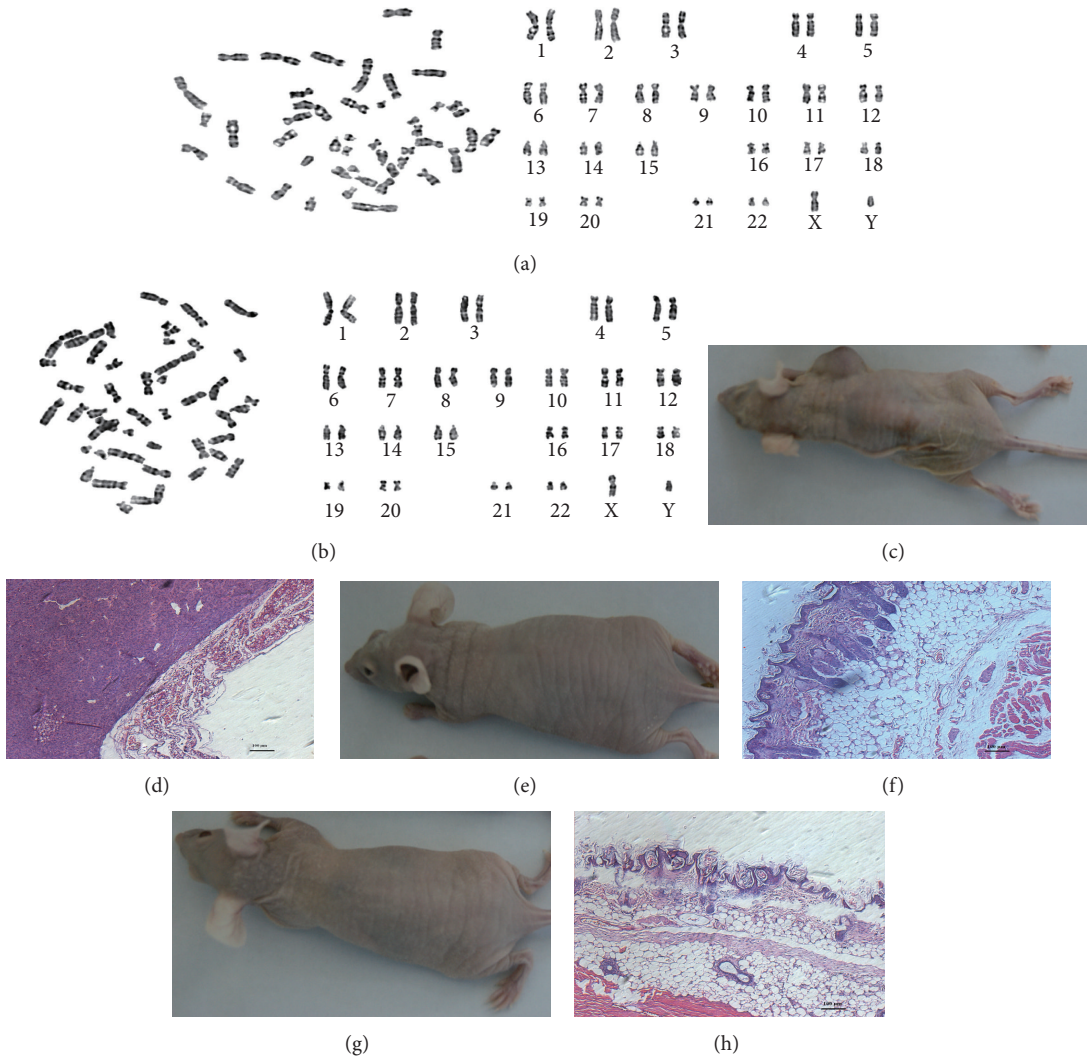

Figure 4: Karyotyping and *in vivo* tumor formation. Karyotyping revealed that the (a) untransfected MSCs and (b) MSCs subjected to gene targeting maintained a normal karyotype: 46, xy. (c–h) *In vivo* tumor formation. Untransfected MSCs and those targeted with an exogenous gene did not show (e, g) macroscopic or (f, h) microscopic staining after 8 weeks. (c) In contrast, HT1080 formed macroscopically visible tumors within 4 weeks. (d) Hematoxylin and eosin staining revealed a characteristic tumor growth. The bar indicates 50 μm.

supplementing growth factors the culture conditions could be optimized for inducing proliferation of MSC while maintaining their multipotency. Although combination of two or more supplements have been used [20, 21], the synergistical effects of the factors on the proliferation of MSCs were rarely reported [19]. Our results show that bFGF is critical to MSC growth and that it acted synergistically with vitamin C, VEGF, and ITS-X, causing the cells to expand significantly. Masahiro found continuous FGF stimulation to be necessary for the maintenance of VEGFR2 levels in mice modulating sensitivity to VEGF stimulation [38]. This may explain the synergistic effects of VEGF and bFGF on the proliferation of MSCs. Although the details of the mechanism by which VEGF, bFGF, Vc, and ITS-X synergistically increase cell proliferation are unclear, the most robust growth stimulation was observed with VEGF+bFGF+Vc+ITS-X (Figures 1(c) and 1(d)). In the medium without supplements, after antibiotic selection, for example, using G418, a few of the resistant MSCs could be observed but they did not form large cell clones and even did not expand to sufficient numbers for characterization (Figure 2(b)). By adding the supplements to the culture medium, the proliferation capacity of the MSCs was obviously improved and at least 1×10^7 cells could be obtained from one targeted recombinant colony. As 11 targeted colonies can be obtained from 3×10^6 MSCs transfected, the total cell number could be calculated as 1.1×10^8. The amount of this level could meet requirement of clinical use (10^7~10^8) [39]. The expanded MSCs retained multipotency, although the adipogenic differentiation partially decreased.

In addition, a second cell behavior critical for the successful use of MSCs is the survival of the cultured cells. The increased survival of MSCs stimulated by growth factors, such as VEGF [25] and Vc [40], may help single targeted MSCs to form colonies. Transferrin and selenite can reduce

toxic levels of oxygen radicals and be used as antioxidant in the medium [41]. During the selection process by G418, untargeted MSCs killed may release cytokines to the culture medium. This may increase the survival stress of the targeted MSCs. It was reported that high survival stress such as the oxidative stress could promote cell senescence [42]. This may be why the untargeted MSCs could form colonies but the targeted MSCs cannot form colonies in the basal medium without growth factors. Further improvements proliferation and survival of MSCs by formulation optimization of the different additives and improvement targeting efficiency by optimization of the targeting conditions may help to get more MSCs with targeted modification.

In summary, this study is the first to describe gene targeting of hMSCs using a nonviral delivery system. Exogenous therapeutic genes could be targeted to the rDNA locus of MSCs using the hrDNA vector described herein, and desirable number of the targeted cell could be obtained by improving the proliferation capacity of the MSCs using growth factors. This shows that MSCs have potential as a cellular vehicle for clinical use, and we believe that this method may be useful for autologous therapy of monogenic inheritance disease. Based on the fact that hFVIII integrated at the rDNA locus of several human cell lines expressed efficiently [14] and MSCs can home to sites of ongoing injury/inflammation to release FVIII [43], hFVIII-expressing MSCs generated using the method described herein may bring great hope for the autologous therapy of the hemophilia A, which is the most common inheritable deficiency of coagulation.

Conflict of Interests

No competing financial interests exist.

Acknowledgments

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Research Article

No Immunogenicity of IPS Cells in Syngeneic Host Studied by *In Vivo* Injection and 3D Scaffold Experiments

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Induced Pluripotent Stem Cells (IPSCs) open the great possibility to employ patient's own tissue to the previously incurable diseases. However these cells can be used in cell therapy only if they are not rejected when transplanted back into the syngeneic host. We found that the injection of iPSCs derived from different ages of mice into syngeneic C57BL/6 mice produced teratoma and was not rejected. Then we cultured iPSCs and myeloid differentiated iPSCs in three-dimensional porous scaffold and transplanted to C57BL/6 mice and BALB/C mice. After transplantation, we could observe the cell density inside the scaffold increased rapidly in syngeneic mice compared to the allogeneic mice indicating the favorable conditions supporting the growth of iPSCs *in vivo*. Unlike the allogeneic counterpart, we could not observe few infiltrating T cells inside the scaffold of syngeneic mice. These results contribute to the optimistic view of iPSCs for regenerative medicine in near future.

1. Introduction

Induced Pluripotent Stem Cells (iPSCs), one of the greatest inventions of the 21st century are nothing but embryonic stem cell like cells generated from somatic cells by the introduction of defined transcription factors Oct3/4, Sox2, Klf4, and c-Myc [1, 2]. Since iPSCs possess the ability to differentiate into various kinds of cells, successful differentiation of iPSCs to various somatic cells pertaining to different germ layers like neurons, cardiovascular progenitor cells, hepatocytes, and so forth is being reported repeatedly in recent years [3–6]. Unlike ES cells the use of IPSCs does not evoke moral or ethical objections [2, 7]. Also, ES cell transplantation causes HLA barrier, and their transplantation needs immunosuppressive drugs, which cause several side effects. Hence iPSCs have a great potential in regenerative medicine to treat innumerable diseases and clinical conditions. However the survival of transplanted iPSCs *in vivo* is questioned in a recent paper by Zhao et al. stating that transplantation of iPSCs derived from Mouse Embryonic Fibroblasts by retroviral reprogramming evokes an acute immune response in syngeneic recipients [8]. To treat damaged tissues or dysfunctional organs in elderly patients, it is necessary to establish iPSCs from the patient's own tissue. We succeeded in establishing iPS clones (aged iPSCs) using bone marrow (BM) of 21-month-old EGFP-C57BL/6 (EGFP-C57BL/6) mice that had been cultured for four days in granulocyte macrophage-colony stimulating factor (GM-CSF) [9].

2. Materials and Methods

2.1. Mice. C57BL/6 mice and BALB/C were purchased from SLC Japan. All mice were maintained in the Animal Research Facility at the Nagoya University Graduate school of Medicine under specific pathogen-free conditions and used according to institutional guidelines.

2.2. Cell Culture. MEF-iPSCs and aged iPSCs were previously established [9] from MEFs of C57BL/6 mice and bone marrow dendritic cells derived from 21-month-old EGFP-C57BL/6 mice (C14-Y01-FM1310sb) carrying pCAG-EGFP (CAGpromoter-EGFP). 15-month-old iPSCs were also established from Bone Marrow dendritic cells of 15 month old C57BL/6 mice. SNL76/7 feeder cells were clonally derived from the STO cell line transfected with $G418^R$ and an LIF expression construct [10].

All iPS cells were maintained by culturing in KO-DMEM (Gibco) supplemented with 1% L-glutamine, 1% nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 5.5 mM 2-mercaptoethanol, and 15% FBS (iPS medium) at 37°C with 5% CO_2.

Myeloid-differentiated MEF-iPS cells were previously generated from C57BL/6 mice [9] and cultured in RPMI 1640 medium (10%FBS), 300 g/L L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 50 mM 2-mercaptoethanol (Sigma Aldrich) containing 0.3% GM-CSF supernatant. (from murine GM-CSF producing Chinese hamster ovary cells, a gift from Dr T.Sudo, Toray Silicon, Tokyo, Japan). This medium is referred to as complete RPMI medium hereafter.

2.3. 3D Scaffolds. 3D scaffolds (code: LG/HP-BL005) were purchased from GC Biolabs Funakoshi, Japan. GC scaffold is a biodegradable material for the paradigm of stem cell and tissue engineering. It is a PLGA (poly D,L-Lactic acid glycolic acid) based product with validated biocompatibility. MEF-iPSCs and Myeloid-differentiated MEF-iPSCs were seeded (1×10^5) into the scaffolds and cultured in 96-well plates with similar culture conditions as mentioned above. The scaffolds were transplanted into the dorsal flanks of syngeneic or allogeneic mice for different time periods and surgically operated.

2.4. Teratoma Formation Assay. iPSCs were collected and washed twice with PBS, and a total of 100 μL of the cell suspension (1×10^7) were injected subcutaneously into the dorsal flank of C57BL/6 mice. Three weeks after injection the teratoma was surgically dissected from the mice.

2.5. Histological Analysis and Immunostaining. The samples were fixed with 4% formaldehyde. The sections were stained with hematoxylin for 2 min, washed in tap water for 5 min, then stained with eosin for 4 min, and washed in tap water for 5 min. After dehydration, the sections were mounted with mounting medium (Malinol), observed, and then photographed using a Keyence BZ-8000 microscope (Osaka) or Olympus FSX 100 microscope. Frozen tissue samples and scaffolds were sectioned at 5–10 μm thickness with the cryostat. The cryosections were then fixed in acetone, and nonspecific binding sites were blocked with 0.2% bovine serum albumin and 1% goat serum in PBS. The sections were then incubated with rat anti-mouse CD4 or anti-CD8 monoclonal antibody (eBioscience) conjugated to FITC and PE, respectively. After incubation the specimen was counterstained with Dapi. We observed and took pictures by using Nikon A1Rsi confocal Microscope.

2.6. Electron Microscope. Teratoma samples were immediately immersed in 2.5% glutaraldehyde and prepared for electron microscope as described previously [11].

2.7. Statistical Analysis. Statistical analysis was expressed as mean ± standard deviation (SD). Student's *t*-test was used to determine statistical significance. Results were considered significant if the *P* value was <0.05.

Table 1

Type of iPSC	Type of mouse	No. of mice	Teratoma after injection
21-month-aged iPSC clone I	BALB/c	4	0/4
MEF iPSC clone I	BALB/c	4	0/4
21-month-aged iPSC clone I	C57BL/6	22	22/22
21-month-aged iPSC clone II	C57BL/6	5	5/5
MEF iPSC clone I	C57BL/6	14	13/14
MEF iPSC clone II	C57BL/6	6	6/6
15-month-old iPSCs clone I	C57BL/6	12	12/12
15-month-old iPSCs clone II	C57BL/6	10	10/10

3. Results

In this study we analyzed the immunogenicity of iPSCs clones derived from different ages of mice. MEF iPSCs, aged-iPSCs derived from 21-month-old male were already described [9]. We added newly established iPSCs clones (clone 1 and clone 2) derived from 15 months old C57BL/6 mice, by selecting pluripotent markers (data not shown). All these iPSCs were established by retroviral reprogramming. We investigated the teratoma formation potential of different iPSCs clones by subcutaneous injection of the cells in C57BL/6 and BALB/c mice. We found that the injection of two clones of 21-month-old iPSCs and two clones of 15-month-old iPSCs resulted in teratoma after 3 weeks, whereas the teratoma forming ability in MEF iPSCs is 92.86%. All the teratoma continued to grow and did not regress. There was no teratoma formation by any of the iPSCs in allogeneic BALB/c mice (Table 1). We confirmed the presence of tissue types of all three germ layers by hematoxylin and Eosin staining in newly established 15-month-old iPSCs, clone 2 of 21-month-old iPSCs and clone 2 of MEF-iPSCs (data not shown). By performing Transmission Electron Microscopy (TEM), we confirmed the presence of different cell types pertaining to ectoderm, endoderm, and mesoderm in teratoma of MEF-derived iPSCs. The number of immune cells that could be observed by TEM is highly negligible (Figure 1).

Then, we try to evaluate immune cell infiltration into teratoma made by the transplantation of iPSCs either into C57BL/6 or BALB/c mice. The potential of teratoma formation can be evaluated only after 3 weeks in syngeneic host and failure of teratoma formation in allogeneic mice becomes difficult to comparative analysis of the teratoma tissue in syngeneic and allogeneic host backgrounds. We asked whether by any other means we could analyze the rate of acceptance of these cells inside the host even before a teratoma could be observed visually. Hence we made use of three-dimensional (3D) porous scaffold made by PLGA (poly D, L- Lactic acid glycolic acid) for the *in vitro* culture and transplantation of iPSCs subcutaneously into the host (Figure 2). We investigated the growth rate of iPSCs in syngeneic and allogeneic hosts. A total of 1×10^5 cells of MEF iPSCs were seeded in the scaffold and after 2 weeks of continuous culture in 96-well plate; the scaffolds were washed in PBS and transplanted

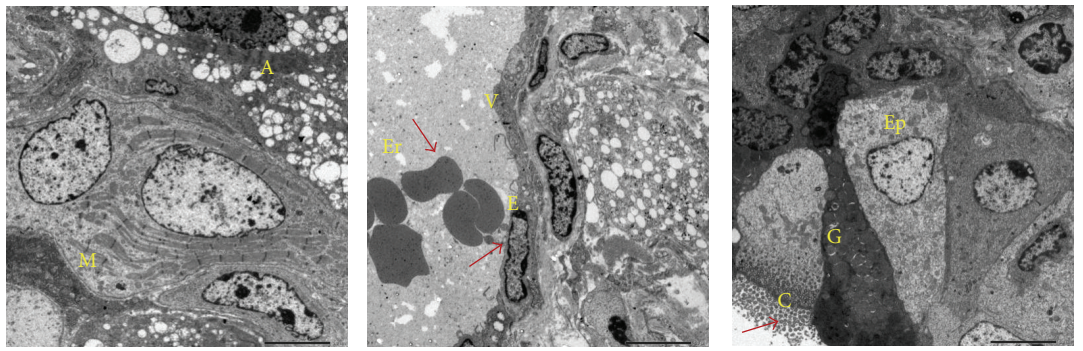

Figure 1: Three germ lines in clone 1 of MEF iPSCs. Electron microscopic observation of the teratoma showed different structures including muscle cell (M) and adipose cell (A), Venue (V) showing erythrocyte (Er) and endothelial cells (E) and ciliated columnar epithelial cell (Ep) and goblet cell (G) with cilia (C) (×1000).

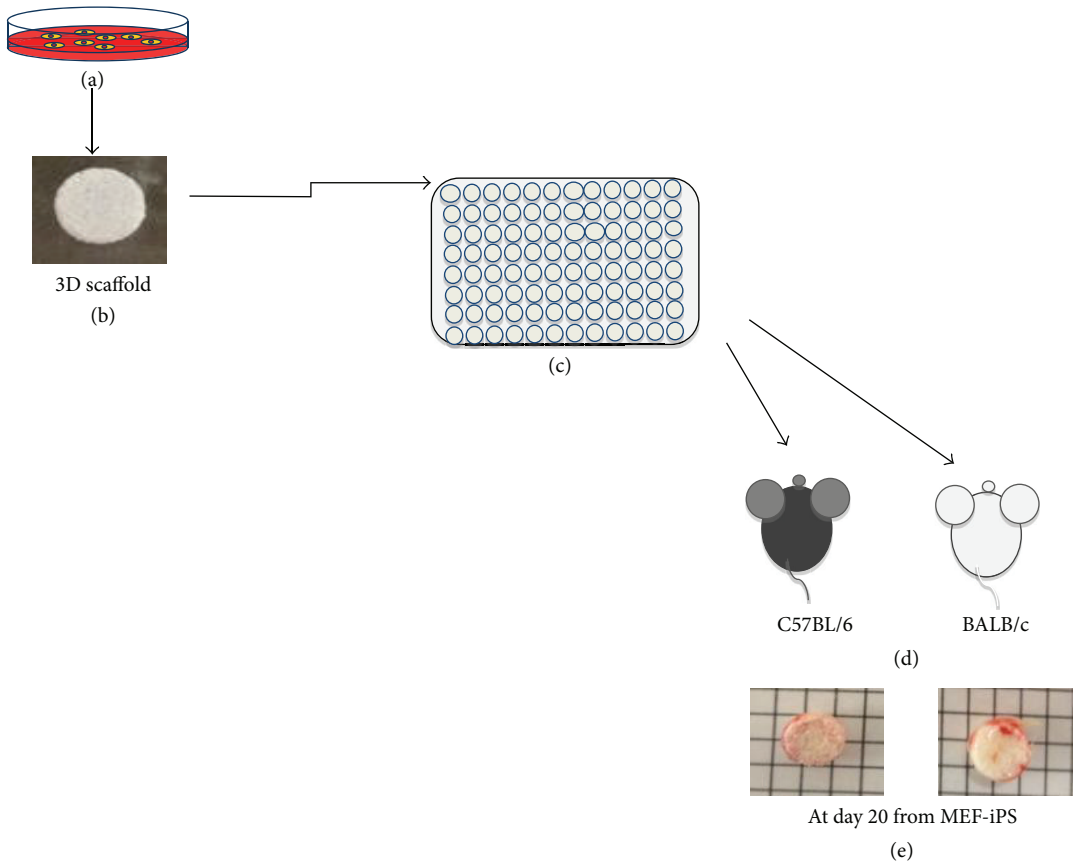

Figure 2: Overview of 3D culture in scaffold of iPSCs and transplantation to syngeneic and allogeneic mice for immune rejection analysis. Clone 1 of MEF iPSCs cultured on feeder (a) was trypsinyzed and washed and total of 1×10^5 cells of MEF iPSCs were seeded in the 3D scaffold (b). After 2 weeks of culture in 96-well plate (c), they were subcutaneously transplanted into C57BL/6 mice and BALB/c mice (d). After different time periods the scaffolds were removed from the mice and frozen in OCT (e).

into syngeneic C57BL/6 and allogeneic BALB/c mice. At different days after transplantation, the scaffolds were removed (Figure 2(e)). Hematoxylin and Eosin staining of the scaffolds shows that the cell density inside the scaffolds transplanted to syngeneic C57BL/6 mice increases rapidly, whereas the scaffolds transplanted to allogeneic BALB/c mice had low cell density (Figure 3). We next investigated the T cell infiltration in transplanted scaffolds. Neither in BALB/c nor in C57BL/6 mice could we observe CD4 or CD8 T cell infiltration at day 6 after transplantation. However at day 20 there is increase in the number of CD8 T cells in allogeneic host compared to the syngeneic host (Figure 4(a)), but we could not observe a CD4 positive cell in the syngeneic and allogeneic host (Figure 4(b)). These results indicate that there is no induction

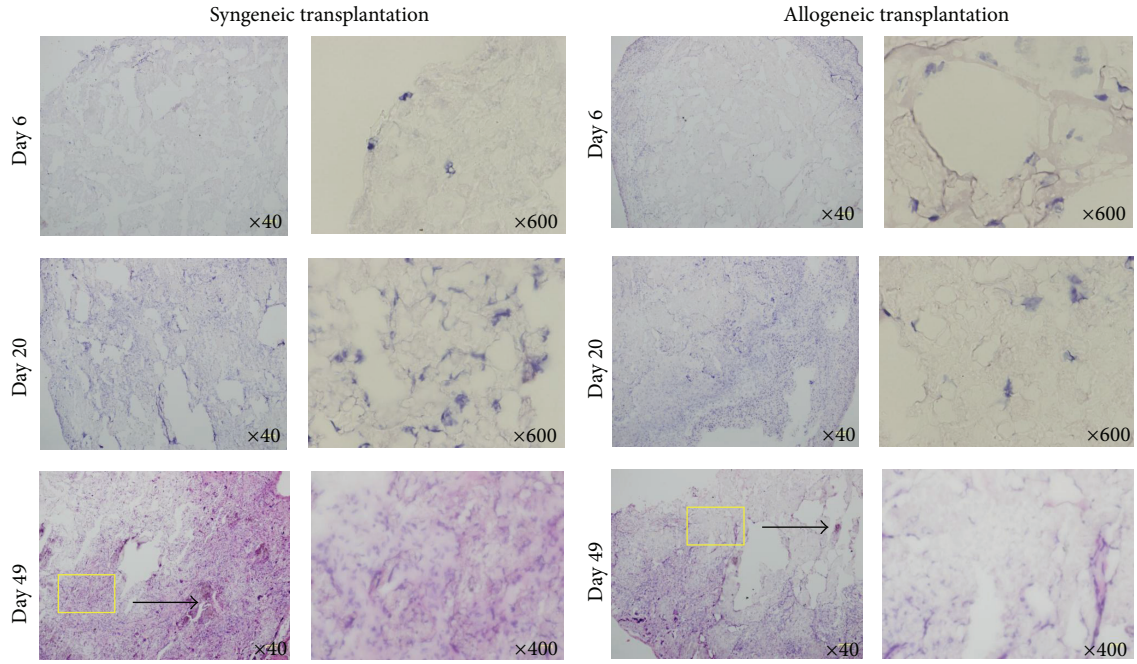

Figure 3: Growth of MEF iPSCs in scaffold transplanted in syngeneic mice and rejection of iPSCs in scaffold transplanted in allogeneic mice. MEF iPSCs (1×10^5) were cultured in scaffolds for 2 weeks and transplanted into C57BL/6 mice or BALB/c mice. After different time periods the scaffolds were removed from the mice and frozen in OCT. Hematoxylin and Eosin staining of the scaffolds operated at day 6, 20, and 49 from the syngeneic and allogeneic host is shown.

of T cell infiltration by MEF iPSCs upon syngeneic transplantation. We also examined secondary challenge of MEF iPSCs in scaffold. We first injected a total of 2×10^6 cells of splenocytes from C57BL/6 mouse into BALB/c or C57BL/6 mice as a primary challenge followed by transplantation of iPSCs cultured inside scaffold after 2 weeks. At day 4 and day 8, we could observe immediate increase in CD8 as well as CD4 T cell number in the allogeneic explants. In contrast, we could not detect any CD8 or CD4 positive T cells at day 4 and only a few CD4 cells could be detected at day 8 in syngeneic explants. These results signify that there is little or no evidence of immune response induced by iPSCs transplantation in syngeneic host even after secondary challenge (Figures 4(c) and 4(d)).

In order to apply iPSCs from patient's own tissue, we must differentiate these iPSCs to diseased tissue cells and transplant these cells back to patient. Thus we next explored the immunogenicity of completely differentiated iPSCs. Hence, we made use of myeloid differentiated C57BL/6-derived MEF iPSCs, which were previously established [9]. Similar to the MEF-iPSCs scaffolds experiments, differentiated macrophages from MEF iPSCs were also cultured in 3D scaffolds and transplanted into syngeneic and allogeneic hosts for evaluating the immune response. We found that the cell proliferation rate was relatively slow and not significantly different in syngeneic and allogeneic hosts up to 6 days after transplantation. However after 20 days, the cell density in syngeneic explants was found to be 70% greater than the allogeneic one (Figures 5(a) and 5(b)). In the case of evaluating T cell infiltration, neither CD4 nor CD8 positive cells could be observed until 6 days after transplantation of the scaffolds in any of the recipients. But, after 20 days numerous number of infiltrating CD8 positive cells were detected in the scaffolds transplanted to BALB/c mouse, which accounts for 87% more than the syngeneic counterpart. However the number of CD4 positive cells that could be detected is highly negligible in both types (Figures 5(c) and 5(d)). These findings conclude that there is no significant immune response induced by myeloid differentiated MEF iPSCs when transplanted into syngeneic hosts.

4. Discussion

Zhao et al. have shown that iPSCs made by four retrovirus are immunogenic. They showed that teratomas developed by retrovirus-induced iPSCs were rejected by syngeneic mice by T cells [8]. They have shown that abnormal gene expression in some cells differentiated from iPSCs can induce T-cell-dependent immune response in syngeneic recipients. They implicated immunogenicity-causing Zg16 and Hormad1 genes [8]. Their results are contradictive to our results. Okita et al. commented on their results. Zhao et al. used only one line of iPSCs to compare immunogenicity [12]. One possibility of differences between our results and the results of Zhao et al. is that during the course of iPSCs production some iPSCs get copy number variation [13, 14], retro element stability and infrequent DNA rearrangement [15], and chromosomal aberrations [16] somatic mosaicism [17, 18]. These changes may induce neoantigens in iPSCs or after differentiation, which will be recognized by syngeneic

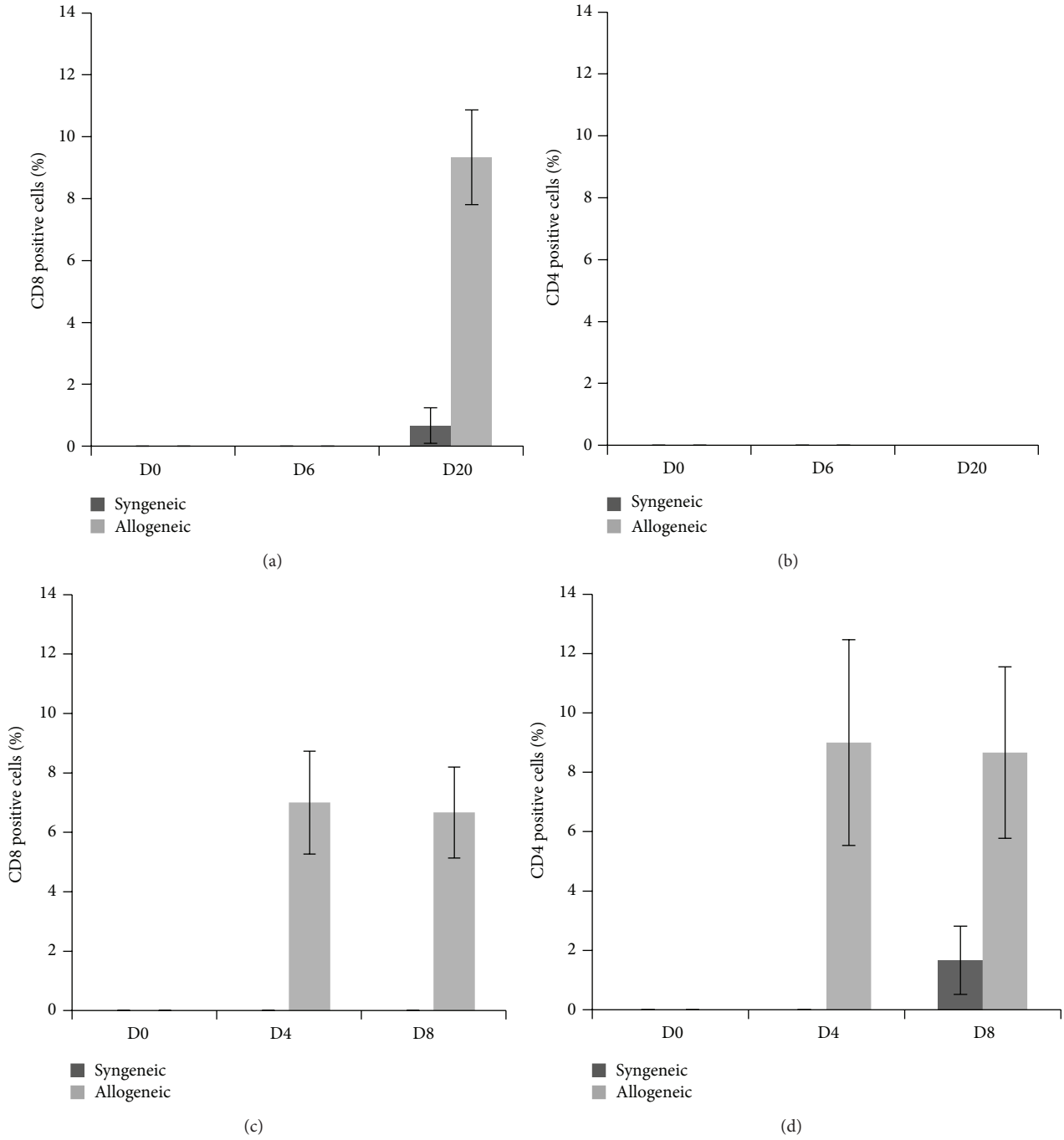

Figure 4: Lack of immunogenicity of MEF iPSCs in syngenic recipient. (a, b) MEF iPSCs (1×10^5) derived from C57BL/6 were cultured in scaffolds for 2 weeks. They were transplanted into C57BL/6 or BALB/c mice. Tissue sections of the explanted scaffold were stained with FITC-labeled anti-CD4 and PE-labeled anti-CD8. The graph shows the average number of CD8 (a) or CD4 (b) positive cells at day 0, day 6, and day 20 ($n = 3$). (c, d) C57BL/6 derived splenocytes (2×10^6) were injected into BALB/c or C57BL/6 mice as a primary challenge. After 2 weeks MEF-iPSCs, which were cultured inside scaffold for 2 weeks, were transplanted to immunized BALB/c or C57BL/6 mice. The graph shows the average number of CD8 or CD4 positive cells at day 0, day 6, and day 20 ($n = 3$). Error bars represent standard deviation.

host. These changes may be included in some cells in one clone, which are derived from fibroblasts in skin. In our experiments, only one in 14 trials of teratoma transplantation derived from MEF iPSCs was rejected, which might be recognized in syngeneic host by inducing T cells. However, all clones derived from bone marrow were not rejected by syngeneic host. We think that if there exist some iPSCs, which carry some abnormalities in genes and produced neoantigens, these cells are rejected by T cells in some stages of development. However, other iPSCs, which have normal genes, can grow in syngeneic host. Here we analyzed teratoma formation of several iPSCs clones derived from MEF or

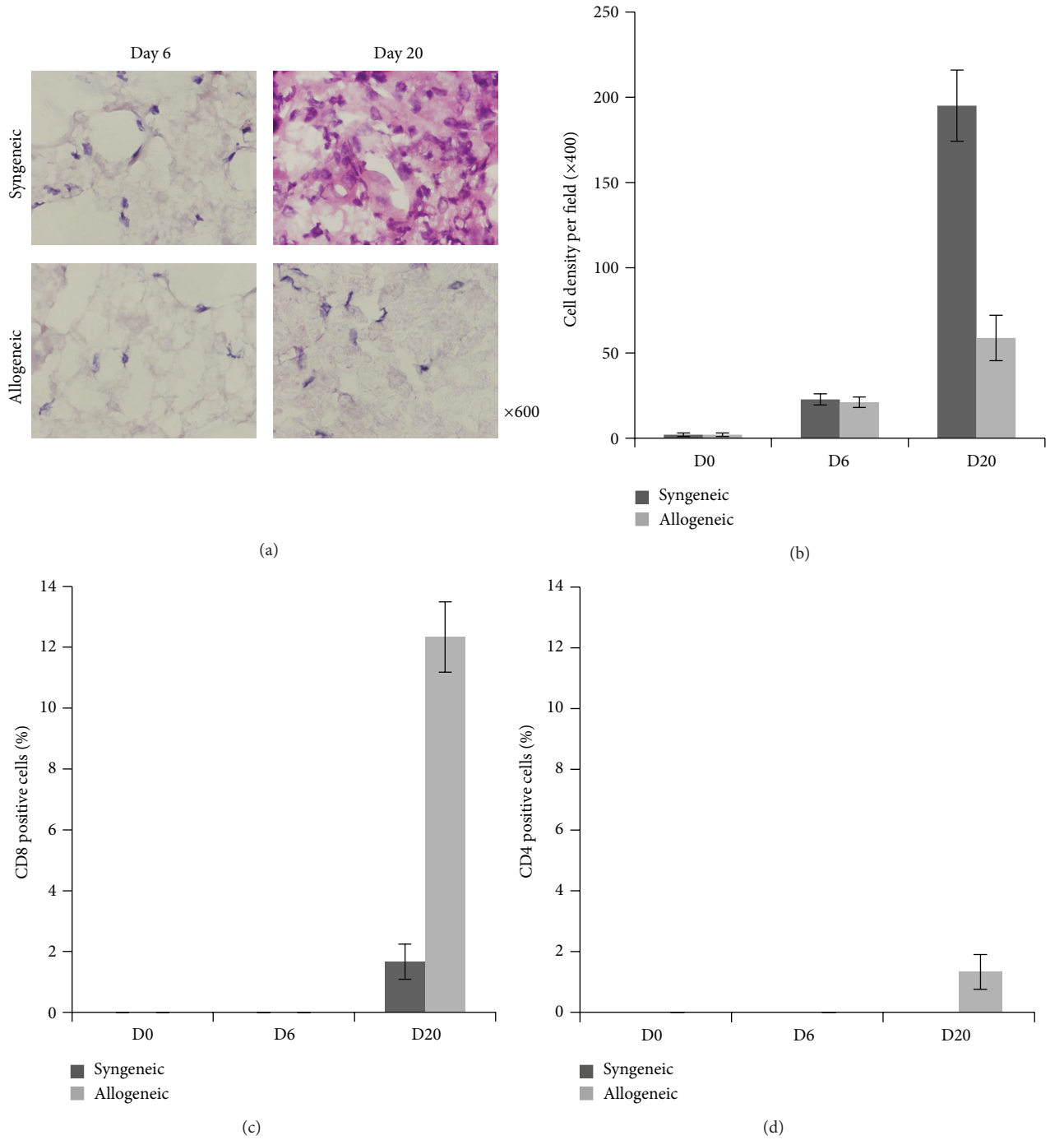

Figure 5: Lack of immunogenicity of myeloid differentiated MEF iPSCs in syngenic recipient. Myeloid differentiated MEF iPSCs (1×10^5) cultured in scaffolds for 2 weeks. They were transplanted into C57BL/6 or BALB/c mice. (a) Hematoxylin and eosin staining of the scaffolds operated at day 6 and 20. (b) The number of cells in the scaffold was counted at a magnification of 400x. ($n = 3$). Tissue sections of the explanted scaffold are with FITC-labeled anti-CD4 and PE-labeled anti-CD8. The graph shows the average number of CD8 (c) or CD4 (d) positive cells at day 0, day 6 and day 20, respectively ($n = 3$). Error bars represent standard deviation.

macrophages from different ages of mice. Almost all clones were accepted in syngeneic mice but not allogeneic mice.

For clinical application, immunogenicity of differentiated cells must be examined. We [4, 19, 20] have reported model transplantation works of differentiated iPSCs into syngeneic mice and have shown that transplanted cells have been detected *in vivo*. Although we could detect differentiated iPSCs *in vivo*, the number of detected iPSCs was few. First possibility is that transplanted iPSCs might be low ability to grow *in vivo*, which might be dead *in vivo* and phagocytosed by macrophages. Second possibility is that transplanted iPSCs are diffused *in vivo*, thus it is difficult to detect by

tissue staining. Third possibility is that differentiated iPSCs expressed altered-self, which might be rejected by immune T cells. Here we clearly showed that differentiated macrophages were not rejected by T cells of syngeneic mice by using scaffold transplantation. Thus our previous difficulties might be caused by migration of transplanted cells to other site, which induce the difficulty of detection of transplanted iPSCs. We could avoid diffusion of injected iPSCs *in vivo* by using scaffold. During the course of preparing manuscript, Araki et al. reported that terminally differentiated cells derived from iPSCs are not immunogenic. They used skin and bone marrow cells from chimera mice developed from iPSCs. Differentiated iPSCs-derived skin or bone marrow cells transplanted to syngeneic host were not rejected by T cells [21]. Considering these results and our results presented here, iPSCs have no immunogenicity in syngeneic recipient with or without differentiated form.

5. Conclusions

Here we showed that iPSCs are not rejected in syngeneic host. Nonimmunogenicity of iPSCs is not restricted to MEF-derived iPSCs. Aged-iPSCs are also not rejected in syngeneic mice. These findings favor the possibility to use iPSCs from patient own tissue to treat incurable diseases such as liver, lung, and kidney failure.

Authors' Contributions

Ken-ichi Isobe designed the experiments. Sachiko Ito and Naomi Nishio developed the methodology. Suganya Thanasegaran and Zhao Cheng performed the experiments. Ken-ichi Isobe and Suganya Thanasegaran wrote the paper.

Conflict of Interests

The authors declare that they have no conflict of interests.

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