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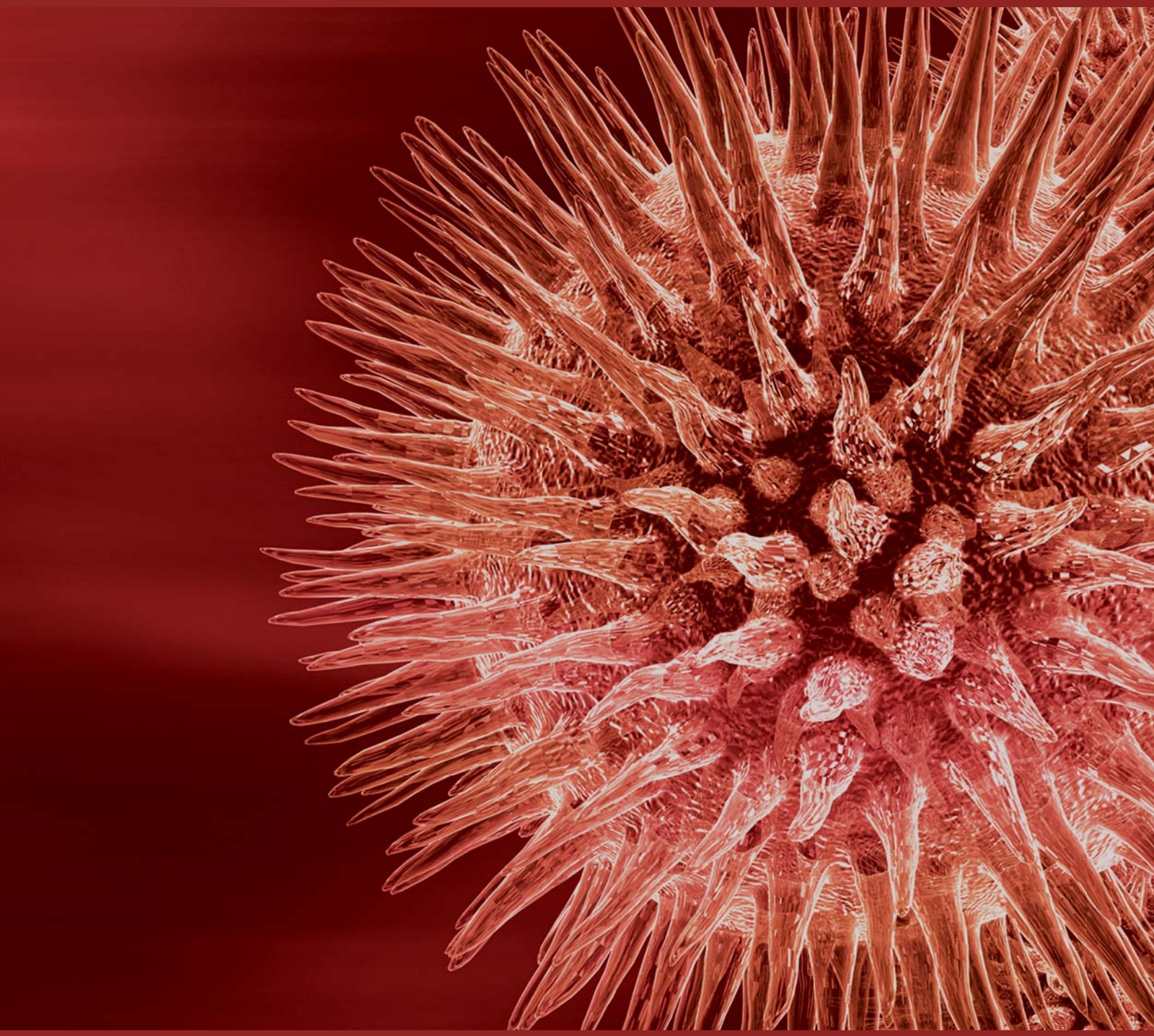
Toward Personalized Cell Therapies by Using Stem Cells

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



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Editorial

Toward Personalized Cell Therapies by Using Stem Cells

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Recent discovery of induced pluripotent stem cells (iPSCs) open the great possibility to use patient own tissue to the previously incurable diseases. Multipotent adult stem/progenitor cells have been identified in nearly all human organs. These cells are expanded *in vitro* and are possible to be used for personalized cell therapy. This special issue collected the articles which discussed recent progresses in this field.

Our body has self-defense systems to recover from damages. However, once tissues are damaged extensively, damaged tissues cannot be recovered. These include hepatic (C. H. Chiang, M. A. Puglisi), kidney, lung, and heart failures. Also in neurodegenerative diseases including Parkinson's disease, Alzheimer diseases, and ALS, patient cells are difficult to recover (H. Y. Ha). External stimuli such as high dose of irradiation caused by cancer treatment (B. Phulpin) will induce irreversible damages. There exist genetic disorders for which there is no cure including progressive muscular dystrophies (E. Zucconi), cystic fibrosis (V. Paracchini). Further age-related dysfunctions are difficult to be recovered. One example is articular cartilage (S. Seo). Currently transplantation has been shown to be an effective treatment for these tissue failures. However, transplantation has serious problems: shortage of organs to be transplanted and immune rejection caused by great heterogeneity of human HLA. Recent progresses of biotechnology open a new hope to treat incurable diseases by using patient own tissues. This special issue aimed to gather scientific papers about recent progresses in this field.

iPSCs are novel stem cell populations induced from mouse and human adult somatic cells through reprogramming by transduction of defined transcription factors. These cells open the great possibility to personalized cell therapy to treat incurable diseases (K. Seiler, T. Kunkanjanawan, H. Y. Ha). Compared to ES cells, iPSCs are not rejected by host immune cells theoretically. At least iPS cells are not rejected once these cells are differentiated (C. H. Chiang).

Mesenchymal stromal cells (MSCs) from various sources including bone marrow (S. W. Gendebien), adipose tissue (W. Lattanzi), dental pulp, placenta, umbilical cord (E. Zucconi, S. Park), menstrual blood (M. C. Rodrigues) and amniotic mesenchymal stromal cells (V. Paracchini) have been studied extensively by animal models.

Tissue stem cells are also used for personalized stem cell therapy. Muscle-derived stem cells for bone formation (X. Li), blood vessel-derived stem cells regenerate myofibers in injured and dystrophic skeletal muscles as well as improve cardiac function after myocardial infarction (C. W. Chen). Olfactory ensheathing cells (OECs) into nerve or spinal cord injuries can promote axonal regeneration and remyelination and restore functional recovery (C. Radtke). Some stem-like cells reside in the transition area between the peripheral corneal endothelium (CE) and the anterior nonfiltering portion of the trabecular meshwork (TM) replacing the lost CE (W. Y. Yu).

Cell culture on scaffold enhances cell growth and differentiation. Hydroxyapatite (HA) coating of (PCL/PVA) nanofibers enhances bone repair (E. Prosecka).

There are a number of reports for limitations of current technologies that hinder iPSCs into practical use. Immunogenicity to syngeneic mice and tumor formation are reported. MSCs also might promote tumor growth. MSCs

immunomodulatory properties affect greatly the host. More works are sure to be needed to overcome these problems.

Acknowledgments

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Ken-ichi Isobe
Herman S. Cheung
Ji Wu

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Review Article

Autism Spectrum Disorders: Is Mesenchymal Stem Cell Personalized Therapy the Future?

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Autism and autism spectrum disorders (ASDs) are heterogeneous neurodevelopmental disorders. They are enigmatic conditions that have their origins in the interaction of genes and environmental factors. ASDs are characterized by dysfunctions in social interaction and communication skills, in addition to repetitive and stereotypic verbal and nonverbal behaviours. Immune dysfunction has been confirmed with autistic children. There are no defined mechanisms of pathogenesis or curative therapy presently available. Indeed, ASDs are still untreatable. Available treatments for autism can be divided into behavioural, nutritional, and medical approaches, although no defined standard approach exists. Nowadays, stem cell therapy represents the great promise for the future of molecular medicine. Among the stem cell population, mesenchymal stem cells (MSCs) show probably best potential good results in medical research. Due to the particular immune and neural dysregulation observed in ASDs, mesenchymal stem cell transplantation could offer a unique tool to provide better resolution for this disease.

1. Autism Spectrum Disorders

Autism and autism spectrum disorders (ASDs) are heterogeneous neurodevelopmental disorders [1]. They are enigmatic conditions that have their origins in the interaction of genes and environmental factors. ASDs are characterized by dysfunctions in social interaction and communication skills, in addition to repetitive and stereotypic verbal and nonverbal behaviours [2, 3]. Several biochemical events are associated with ASDs: oxidative stress; endoplasmic reticulum stress; decreased methylation capacity; limited production of glutathione; mitochondrial dysfunction; intestinal dysbiosis; increased toxic metal burden; immune dysregulation; immune activation of neuroglial cells [4]. The exact aetiology of ASDs is unknown, likely it results from a complex combination of genetic, environmental, and immunological factors [5, 6]. This heritable disorder derives from genetic variations in multiple genes [7], making its treatment particularly difficult. Environment (i.e., air pollution, organophosphates, and heavy metals) also contributes to the incidence of ASDs [8].

Frequency of these disorders is increasing: 56% reported increase in paediatric prevalence between 1991 and 1997 [9] until present rates of about 60 cases per 10,000 children, according to Center for Disease Control [10, 11]. ASDs are increasingly being recognized as a public health problem [12]. Pathophysiology and defined mechanisms of pathogenesis of autism remain still unclear. There are no

drugs effective for treatment of core symptoms of ASDs [10]. Indeed, ASDs are still untreatable. Current available treatments for autism can be divided into behavioural, nutritional, and pharmacological options, in addition to individual and family psychotherapy and other nonpharmacologic interventions [13]. However, no defined standard approach exists [14]. Pharmacological approaches are direct towards neuropsychiatric disorders coassociated with ASDs. Psycho-stimulants, alpha-2 agonists, beta blockers, lithium, anticonvulsant mood stabilizers, atypical antipsychotics, traditional antipsychotics, selective serotonin reuptake inhibitors, antidepressants, and antipsychotics, are drugs commonly prescribed [14–16]. Catatonia is treated with lorazepam and bilateral electroconvulsive therapy [17]. Selective serotonin reuptake inhibitors are prescribed for the treatment of depression, anxiety, and obsessive-compulsive ASD-associated behaviours [2].

Other nonpsychotropic drugs which are supported by at least 1 or 2 prospective randomized controlled trials or 1 systematic review include melatonin, acetylcholinesterase inhibitors, naltrexone, carnitine, tetrahydrobiopterin, vitamin C, hyperbaric oxygen treatment, immunomodulation and anti-inflammatory treatments, oxytocin, and even music therapy and vision therapy [18].

Alternative and complementary treatments, not sufficiently supported by medical literature, include herbal remedies, vitamin and mineral therapies, piracetam, elimination diets, chelation, cyproheptadine, famotidine, glutamate antagonists, special dietary supplements, acupuncture, neurofeedback, and sensory integration training [14, 19, 20]. On the other hand, behavioural treatment could represent the effective intervention strategy for autism [21–23]. A plethora of behavioural strategies and social skill trainings have been used [24–26]. However, it has been demonstrated that no definitive behavioural intervention completely improves all symptoms for all ASD patients [27, 28].

Summarizing, all these therapies indicate that further research is needed to better address treatment of several medical conditions experienced by ASD patients [29].

2. Mesenchymal Stem Cells

Nowadays, stem cell therapy represents the great promise for the future of molecular medicine. The progression of several diseases can be slowed or even blocked by stem cell transplantation [30].

Among the stem cell population, mesenchymal stem cells (MSCs) show probably best potential good results in medical research [31–33]. These cells are nonhematopoietic stem cells having a multilineage potential, as they have the capacity of differentiating into both mesenchymal and nonmesenchymal lineages. MSCs are a population of progenitor cells of mesodermal origin found principally in the bone marrow of adults, giving rise to skeletal muscle cells, blood, fat, vascular, and urogenital systems, and to connective tissues throughout the body [34–36]. According to the International Society of Cellular Therapy, MSCs are defined by the following minimal set of criteria: (1) grown in adherence to plastic surface of dishes when maintained in standard culture conditions; (2) express cytospecific cell surface markers, that is, CD105, CD90, and CD73, to be negative for other cell surface markers, that is, CD45, CD34, CD14, and CD11b; (3) possess the capacity to differentiate into mesenchymal lineages, under appropriate *in vitro* conditions [37]. MSCs can be isolated from different tissues other than bone marrow: adipose tissue, liver, tendons, synovial membrane, amniotic fluid, placenta, umbilical cord, and teeth. MSCs show a high expansion potential, genetic stability, stable phenotype, high proliferation rate as adherent cells, and self-renew capacity and can be easily collected and shipped from the laboratory to the bedside and are compatible with different delivery methods and formulations [38, 39]. In addition, MSCs have two other extraordinary properties: they are able to migrate to sites of tissue injury, where they are able to inhibit the release of proinflammatory cytokines and have strong immunosuppressive activity that renders them a useful tool for successful autologous, as well as heterologous, transplantations without requiring pharmacological immunosuppression [40–43]. Besides, MSCs are easily isolated from a small aspirate of bone marrow and expanded with high efficiency [44]. Given that MSCs are multipotent cells with a number of potential therapeutic applications, and they represent a future powerful tool in regenerative medicine, including ASDs. Mesenchymal stem cells could be transplanted directly without genetic modification or pretreatments. They simply eventually differentiate according to cues from the surrounding tissues and do not give uncontrollable growth or tumours. In clinical application, there is no problem with immune rejection because of their *in vivo* immunosuppressive properties [45, 46]. In addition, MSCs can readily be isolated from the patients requiring transplant or from their parents. There is also no tumour formation on transplantation [47]. No moral objection or ethical controversies are involved [48].

In principle, mesenchymal stem cells can act through several possible mechanisms, that is, stimulating the plastic response in the host damaged tissue, secreting survival-promoting growth factors, restoring synaptic transmitter release by providing local reinnervations, integrating into existing neural and synaptic network, and reestablishing functional afferent and efferent connections [49]. Since MSCs have the capability to produce a large array of trophic and growth factors both *in vivo* and *in vitro.* (MSCs constitutively secrete interleukins (IL)-6, IL-7, IL-8, IL-11, IL-12, IL-14, IL-15, macrophage colony-stimulating factor, Flt-3 ligand, and stem-cell factor [50]). A more reasonable explanation for the functional benefit derived from MSC transplantation is their paracrine activity, by which these cells are able to produce factors that activate endogenous restorative mechanisms within injured tissues contributing to recovery of function lost as a result of lesions [49, 51].

3. Autism, Personalized Therapy through Mesenchymal Stem Cells

MSCs have a strong long-lasting immunosuppressive capacity [52]. This extraordinary property is mediated via soluble

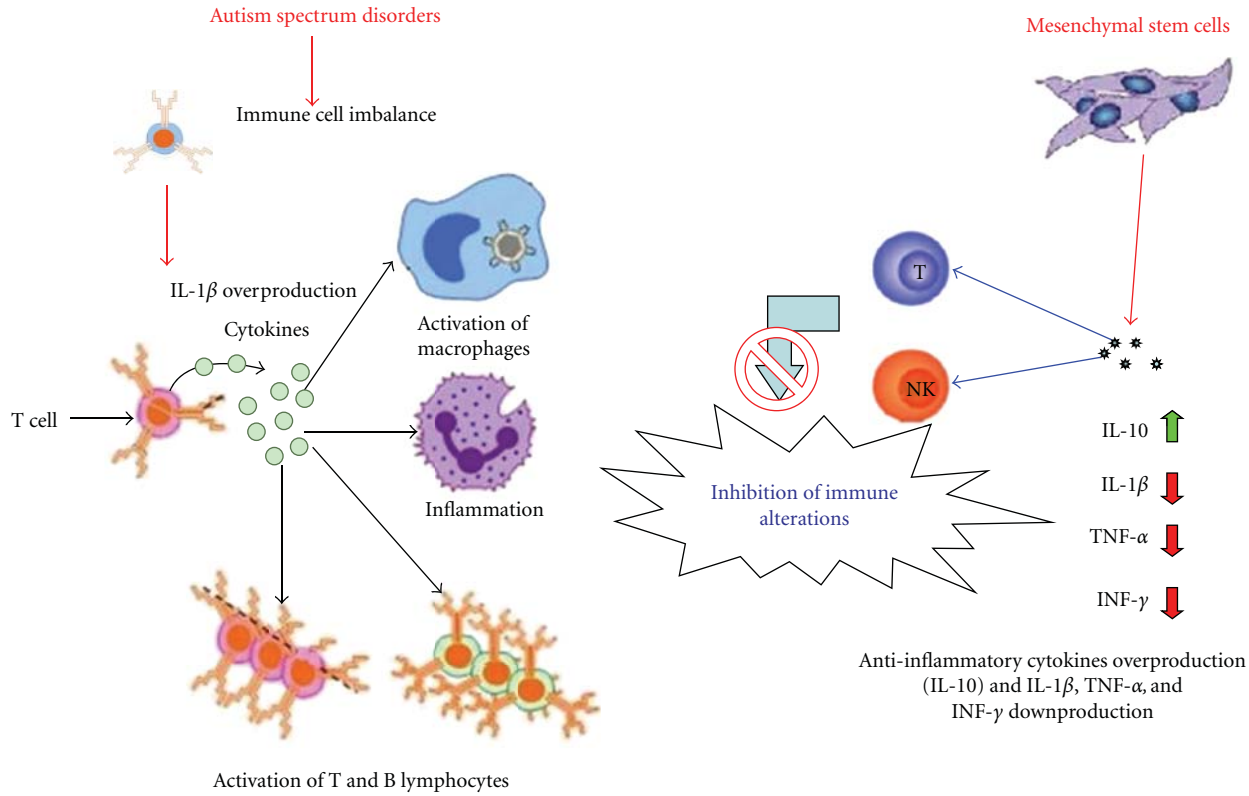

FIGURE 1: Paracrine and immunomodulatory effects as possible mechanisms of action of mesenchymal stem cells (MSCs) in autism spectrum disorder (ASD) treatment. In humans, ASDs are associated with immune alterations and pro-inflammatory cytokines (i.e., IL-1β) overproduction. These cytokines are able to trigger pro-inflammatory cellular events. Data from *in vitro* models show that MSCs are able to affect not only T cells, but also other cells of the immune system (i.e., NK cells). Immunoregulatory properties of MSCs are through secretion of large amounts of several bioactive molecules (paracrine activity), that is, PGE-2, IL-10. These molecules cause the inhibition or the unresponsiveness of T-cell mediated responses.

factors. MSCs are able to inhibit the proliferation of $CD8^+$ and $CD4^+$ T lymphocytes and natural killer (NK) cells, to suppress the immunoglobulin production by plasma cells, to inhibit the maturation of dendritic cells (DCs) and the proliferation of regulatory T cells [53]. It has been demonstrated that MSCs are also able to inhibit T lymphocyte pro-inflammatory cytokine production *in vitro* [54, 55], as well as *in vivo* [56]. Their ability to modulate the immune system opens a wide range of cell-mediated applications, not only for autoimmune diseases and graft-versus-host disease. Due to the particular immune system dysregulation observed in ASDs [57, 58], mesenchymal stem cell transplantation could offer a unique tool to provide better resolution for this disease. Indeed, in ASDs pathogenesis, innate and adaptive immunity changes have been reported [59]. ASD patients show an imbalance in $CD3^+$, $CD4^+$, and $CD8^+$ T cells, as well as in NK cells. In addition, peripheral blood mononuclear cells (PBMCs) extracted from ASD patients are able to overproduce IL-1β resulting in long-term immune alterations [60]. MSC-mediated immune suppressive activity could restore this immune imbalance (Figure 1). Indeed, MSC immunoregulatory effects strongly inhibit T-cell recognition and expansion by inhibiting TNF-α and INF-γ production and increasing IL-10 levels [51].

It has been demonstrated that in postmortem brains from ASD patients there is evidence of abnormal functioning and cerebellum alterations [61–63]. Indeed, ASD subjects show a decreased number of Purkinje cells in the cerebellum [64]. These changes could reflect defective cortical organization in ASDs development. In addition, autism is associated with dysregulation in the maturation and plasticity of dendritic spine morphology [65]. Restoring injured brain functioning could be achieved by stem-cell-based cell replacement [66]. Indeed, transplanted MSCs are able to promote synaptic plasticity and functional recovery and rescue cerebellar Purkinje cells [67, 68]. Challenging newest study from Deng et al. suggests that granulocyte colony-stimulating factor (G-CSF) is able to mobilize MSCs into peripheral blood. These mobilized MSCs are incorporated and integrate into damaged brain in craniocerebral injured mice, ameliorating the effect of trauma [69]. It is noteworthy that MSC ability to migrate to the sites of injury and participate in the repair process is a key issue in tissue repair [70]. Also by this way, MSC therapy could restore the altered brain organization seen in autistic subjects (Table 1).

A key dilemma in stem-cell-based therapy for autism treatment is whether endogenous or exogenous MSC administration is the best way of stem cell delivery. Endogenous

Table 1: Potential ameliorative effects mediated by MSCs in ASD treatment.

ASD-induced changes in human brain	Potential MSC ameliorative roles seen in preclinical models
Abnormal functioning	Improving functional recovery
Cerebellum alterations	Integrating in altered brain and restoring damaged functions
Decreased number of Purkinje cells (PCs)	Restoring cerebellar PCs
Defective cortical organization	Reinforcing cortical plasticity
Altered plasticity of dendritic spine morphology	Promoting synaptic plasticity

strategy could be limited by the availability of MSCs. Exogenous MSCs could show low rate of engraftment to provide cellular replacement. It is unclear if differentiated cells are able to develop functional interconnections with the intrinsic cells of the recipient host [49]. Controversy, few exogenous MSCs are able to exert paracrine activity. Indeed, exogenously applied MSCs have been shown to home to injured tissues and repair them by producing chemokines, or by cell or nuclear fusion with host cells [71]. On the other hand, exogenous culture-expanded MSCs could address endogenous MSCs in order to activate them and guide intrinsic repair [72]. In addition, exogenous delivery bypasses surgical intervention on the autistic child.

Cellular therapy could represent a new frontier in the treatment of several diseases. Despite the fact that MSCs have been enrolled in several clinical trials, long-term safety of MSC-based therapies is not yet well established; this fact could be one major limitation to clinical translation [73]. At the present, there are no preclinical studies on the use of MSCs in ASD models. There is just one clinical trial (NCT01343511 http://www.clinicaltrial.gov/) concerning the safety and efficacy of human umbilical cord mesenchymal stem cells (hUC-MSCs) and human cord blood mononuclear cells (hCB-MNCs) transplantation in patients with autism by Shenzhen Beike Bio-Technology Co., China. Results are not yet posted.

However, personalized stem cell therapy will be the most effective treatment for a specific autistic child, opening a new era in autism management in the next future.

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Research Article

Human Umbilical Cord Blood-Derived Mesenchymal Stem Cell Therapy Promotes Functional Recovery of Contused Rat Spinal Cord through Enhancement of Endogenous Cell Proliferation and Oligogenesis

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Numerous studies have shown the benefits of mesenchymal stem cells (MSCs) on the repair of spinal cord injury (SCI) model and on behavioral improvement, but the underlying mechanisms remain unclear. In this study, to investigate possible mechanisms by which MSCs contribute to the alleviation of neurologic deficits, we examined the potential effect of human umbilical cord blood-derived MSCs (hUCB-MSCs) on the endogenous cell proliferation and oligogenesis after SCI. SCI was injured by contusion using a weight-drop impactor and hUCB-MSCs were transplanted into the boundary zone of the injured site. Animals received a daily injection of bromodeoxyuridine (BrdU) for 7 days after treatment to identity newly synthesized cells of ependymal and periependymal cells that immunohistochemically resembled stem/progenitor cells was evident. Behavior analysis revealed that locomotor functions of hUCB-MSCs group were restored significantly and the cavity volume was smaller in the MSCs-transplanted rats compared to the control group. In MSCs-transplanted group, TUNEL-positive cells were decreased and BrdU-positive cells were significantly increased rats compared with control group. In addition, more of BrdU-positive cells expressed neural stem/progenitor cell nestin and oligo-lineage cell such as NG2, CNPase, MBP and glial fibrillary acidic protein typical of astrocytes in the MSC-transplanted rats. Thus, endogenous cell proliferation and oligogenesis contribute to MSC-promoted functional recovery following SCI.

1. Introduction

Recovery following spinal cord injury (SCI) is limited because of axonal damage [1], demyelination, and scar formation [2]. In addition to the formation of a central hemorrhagic lesion devoid of normal neurons and glia, oligodendrocytes and astrocytes in the white matter near the impact site are reduced by about 50% by 24 h after injury [3].

Recently, the use of stem cell for neurodegenerative disease has been widely investigated as a therapeutic strategy [4–6]. Neural stem cells have been used for the treatment of neurological diseases such as SCI [7] or stroke [8]. Numerous studies have reported that the survival and differentiation of grafted cells into neural cells correlate with behavior improvement. However, these cells are limited for clinical application because of insufficient cell supply, risk of immune rejection, and ethical problems. Since mesenchymal stem cells (MSCs) can be readily isolated and their numbers increased *in vitro* and differentiated into several types of mature cells including neurons, adipocytes, cartilage, and skeletal hepatocytes under appropriate conditions [9], a new therapeutic strategy has been a valuable source for central nervous stem (CNS) disease [10, 11]. Human umbilical cord blood-derived MSCs (hUCB-MSCs) have therapeutic

potential and are attractive because these cells are readily available and are less immunogenic as compared to other sources of stem cells, such as bone marrow or adipose [12].

An alternative strategy of stem cell therapy is protection of injured cells and promotion of endogenous cell regeneration. Several studies have reported that stem cells might provide a better environment for damaged tissue and save remaining neurons by neurotrophic factors or cytokines [13, 14]. However, the specific mechanism of the MSCs for these assertions remains controversial and ill-explored. Nevertheless, MSC treatment of SCI has been reported as a candidate that supplies angiogenic, antiapoptotic, and mitogenic factors as well as migration toward damaged tissue [15]. Recently, MSCs have been used in clinical treatment and were shown to be effective in the treatment of various pathologies although evidence for distinct therapeutic mechanism was lacking [16].

The normal spinal cord contains endogenous neural progenitor cells (NPC) and oligodendrocyte precursor cells (OPCs) [17]. Nevertheless, production of new neurons and oligodendrocytes by endogenous cells into the spinal cord may be very restricted after injury [18]. Furthermore, cell transplantation studies have demonstrated that exogenous stem cells differentiate only very poorly when grafted into the spinal cord. Thus, the environment of the spinal cord appears to be highly restrictive for the differentiation of OPCs. If this environmental restriction can be changed by hUCB-MSC in SCI, OPCs may be able to supply new neurons and oligodendrocytes. However, it is not known whether survival and differentiation generated from endogenous cells are influenced by transplanted hUCB-MSCs.

In the present study, we show that the transplantation of hUCB-MSCs confers therapeutic effects in a rat experimental SCI model. We investigated whether transplantation of hUCB-MSCs improved the functional recovery and improved the proliferation and genesis of resident endogenous cells within the spinal cord by hUCB-MSCs.

2. Materials and Methods

2.1. Human UCB-Derived MSCs. Human UCBs were obtained from normal full-term pregnant woman. The protocol for human subjects adhered to the guidelines outlined by the institutional review IRB board of the Catholic University of Korea (Seoul, Republic of Korea). hUCB-MSCs were isolated and expanded using a previously described protocol [12].

2.2. Animal Model. All animal protocols were approved by the Institutional Animal Care and Use Committee of Catholic University Medical School. Forty-five adult male Sprague-Dawley rats weighting between 270 and 300 g were employed in our experiments. Surgical techniques were similar to those described previously [19]. Briefly, rats were deeply anesthetized with ketamine-xylazine cocktail (80 mg/kg of ketamine, 10 mg/kg of xylazine). Under a dissecting microscope, the skin and muscles overlying the thoracic cord were separated and retracted, the T9 vertebral level was removed by laminectomy, and the underlying spinal cord segment was exposed by slitting the dural sheath. The impact rod of the NYU impactor was centered above T9 and dropped from a height of 25 mm to induce an incomplete partial SCI. Following lesion, the dorsal back musculature was sutured and the skin closed with surgical clips. After surgery, the animals were kept on a thermostatically regulated heating pad until completely awake. The urinary bladder of all rats was emptied manually two times per day until recovery of urinary function.

2.3. Cell Transplantation. Rats were assigned randomly to one of the following two major groups: one group of rats were treated with 5 μL phosphate-buffered saline (PBS) as the control group. The second group of rats received transplanted with hUCB-MSCs (3×10^5 cells/5 μL). In experiment 1, the hUCB-MSCs was designed to test the therapeutic effectiveness ($n = 26$), and, in experiment 2, these cells were designed to evaluate the proliferation of endogenous cells after transplantation ($n = 12$).

Initial locomotor scores were equalized between groups. The weight-drop injury level was based on our experience with the model to produce spontaneous recovery at a Basso-Beatti-Bresnahan (BBB) score of 4. Once the 46 rats were subjected to contusion SCI, they were divided randomly into the two groups. Using a 25-gauge syringe (Hamilton, Reno, NV) fixed in a stoelting stereotaxic frame (Dae Jong) at 7 days after injury, hUCB-MSCs were transplanted into the spinal cord (0.5 mm from the midline, 1.5 mm down from the dura, and 5 mm rostral from the contusion epicenter). Each rat received a 5 μL injection in the contusion site over a 10 min period each time. The cannula of the Hamilton syringe was left in place after injection for an additional 5 min. All animal received antibiotics (Gentamicin sulfate, 30 mg/kg/day) during the first week after transplantation.

2.4. 5-Bromo-2-Deoxyuridine (BrdU) Administration. Sprague-Dawley rats ($n = 12$) were injected with 50 mg/kg BrdU (Sigma-Aldrich, St. Louis, MO, USA) intraperitoneally each day for 7 days to label the newly generating cells after transplantation. The examined proliferative response focused on cell genesis occurring within 7 days after transplantation.

2.5. Behavioral Testing. The motor function restoration after spinal cord contusion was observed by open-field BBB locomotor ratio scale [20]. The scale used for measuring hind-limb function with these procedures ranges from a score of 0, indicating no spontaneous movement, to a maximum score of 21, with an increasing score indicating the use of individual joints, coordinated joint movement, coordinated limb movement, weight-bearing, and other functions. Behavioral testing was performed weekly on each hindlimb from the postoperative day to 7 weeks after SCI. Spinal cord contusion and cell transplantation were separately performed in double-blinded experiments by different investigators.

2.6. Tissue of Harvest. To study functional recovery and differentiation of transplanted hUCB-MSCs, rats from each group were sacrificed at 1 and 2 weeks (PBS, $n = 3$; Transplantation, $n = 3$) after transplantation and the others were

examined by the BBB locomotor test 6 weeks after transplantation (PBS, $n = 7$; transplantation, $n = 7$). Also, to study endogenous cell proliferation after transplantation, rats from each group were sacrificed at 2 h and 1 week after the last BrdU injection ($n = 5$). All the rats were deeply anesthetized with a ketamine-xylazine cocktail (80 mg/kg of ketamine, 10 mg/kg of xylazine) and then perfused transcardially with 0.01 M PBS (pH 7.4), followed by 4% paraformaldehyde (PFA) in 0.01 M PBS. The spinal cord was removed from each rat and postfixed in 4% PFA for 4 hours. Postfixed tissue was cryoprotected in 0.1 M phosphate buffer (pH 7.4) containing 15% and 30% sucrose solution at 4°C. The spinal cords were embedded in OCT compound and stored at −70°C. To examine the cavity volume, 14 μm thick serial transverse sections were prepared from 20 mm long spinal cord stumps (1 mm each for rostral and caudal to the lesion epicenter). Also, to compare the coexpression of various cell-type-specific markers and $BrdU^+$ cells, 10 μm thick serial coronal sections were prepared as described above. Coronal sections were collected from cell transplantation site to the injury epicenter sites and mounted on gelatin-coated slides.

2.7. Histology and Immunohistofluorescence. Single and double fluorescent staining was used. Single staining was used to identify newly generated cells after transplantation. For BrdU immunohistochemistry, the sections were warmed for 20 min and washed with 0.01 M PBS for 10 min. Sections were incubated in 50% formamide-2X standard saline citrate at 60°C for 2 h, subsequently treated with 2 N HCL at 37°C for 30 min to denature deoxyribonucleic acid, and then incubated in 0.1 mol/L boric acid at room temperature for 10 min to neutralize residual acid. The sections were incubated with rat anti-BrdU (1 : 100; Abcam, Cambridge, UK) or mouse anti-BrdU (1 : 100; DakoCytomation, Glostrup, Denmark). Subsequently, sections were incubated for 1 h at room temperature with fluorescence-conjugated secondary antibody or biotinylated antibody; the latter was reacted with avidin peroxidase for 30 min (ABC-kit; Vectastain Elite; Vector Laboratories, Burlingame, CA) followed by detection solution (0.25 mg/mL diaminobenzidine, 0.03% H_2O_2, 0.04% NiCl).

To determine the fate of newly generated cells after transplantation, double-fluorescent immunolabeling was performed, combining BrdU labeling with one of cell-specific phenotypic markers listed below. We used mouse anti-Nestin (1 : 100; Millipore, Billerica, MA) to identify neural stem progenitor; mouse anti-NG-2 chondroitin sulfate proteoglycan (anti-NG-2; 1 : 100; Millipore) to identify oligodendrocyte progenitor; mouse anti-2′,3′-cyclic nucleotide 3′-phosphodiesterase (anti-CNPase; 1 : 100; Millipore), mouse antimyelin basic protein (anti-MBP, 1 : 100; Millipore), rabbit anti-glial fibrillary acidic protein (anti-GFAP; 1 : 500; Millipore) to identify astrocytes. After washing, samples were incubated in Alexa 488-conjugated goat anti-rat IgG (1 : 200; Vector Laboratories), Alexa 546-conjugated goat anti-mouse IgG (1 : 200; Vector Laboratories), or Alexa 546-conjugated goat anti-rabbit IgG (1 : 200; Vector Laboratories) for 1 h. Fluorescently stained slides were stored at −20°C and observed using a fluorescence microscope equipped with a spot digital camera or a model LSM 510 confocal scanning laser microscope (Zeiss, Jena, Gemany). Apoptosis was detected by the terminal deoxynucleotidyl-transferase-mediated d-UTP-biotin nick end (TUNEL) assay using the in situ cell death detection kit (Roche, Indianapolis, IN) developed using the Cy2-conjugated streptavidin (Jackson Laboratories, West Grove, PA). The slides were observed using the aforementioned confocal scanning laser microscope.

2.8. Cell Counts. The counting of $BrdU^+$ cells was done by previously described [21]. $BrdU^+$ cells were counted within a reticule of a specified area (0.0682 mm^2) positioned in the ependymal and parenchymal region (dorsal (above the corticospinal tract), lateral, and ventromedial region of the residual white matter) in sections. White matter regions were counted in six randomly chosen sections per 1 mm^2 length of spinal cord, and the numbers were averaged.

2.9. Measurement of the Cavity Volume. For measurement of the cavity volume, rats at 6 weeks after transplantation were used. The transverse sections were stained with hematoxylin-eosin (HE). The area of the cavity in the damaged spinal cord was measured in images of the sections using ImageJ version 1.38 image analyzer software (National Institutes of Health, Bethesda, MD) on consecutive sections at an interval of 70 μm. The volume of the cavity was then calculated by multiplying the average area by the depth of the spinal cord.

2.10. Statistical Analysis. The BBB score and cell counts were subjected to the paired t-test or one-way ANOVA for transplantation and PBS-treated groups of rats. Data are presented as mean ± SE. Value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Behavioral Assessment and Measurement of the Cavity Volume. We assessed the recovery of hindlimb function with the BBB locomotor scale from 1 day to 6 weeks after SCI. In the case of SCI rats, BBB scores were low (<9). The motor function scores of MSCs-injected rats (11.07 ± 0.3) were significantly higher than the PBS-injected rats (9.25 ± 0.3) at 7 weeks after SCI. The behavioral data from the BBB locomotor scores demonstrated that MSCs-treated rats were dramatically improved in neurological function ($P < 0.005$, Figure 1). In addition, the spinal cords of MSCs-injected rats had cavities much smaller than those of the PBS-injected rats. The cavity volume of MSCs-treated rat was 0.82 ± 0.14 mm^3 on average, whereas the PBS-treated rats showed a volume of 2.12 ± 0.28 mm^3. These results for cavity volume were significantly different between the MSCs-treated and PBS-treated rats. Thus, MSC transplantation led to a significant improvement of behavior as well as reduction of cavity volume after SCI.

3.2. Proliferation of Endogenous Generated Cells. MSCs promoted the functional recovery and reduced the cavity volume following transplantation in SCI (Figure 1). Since an effect

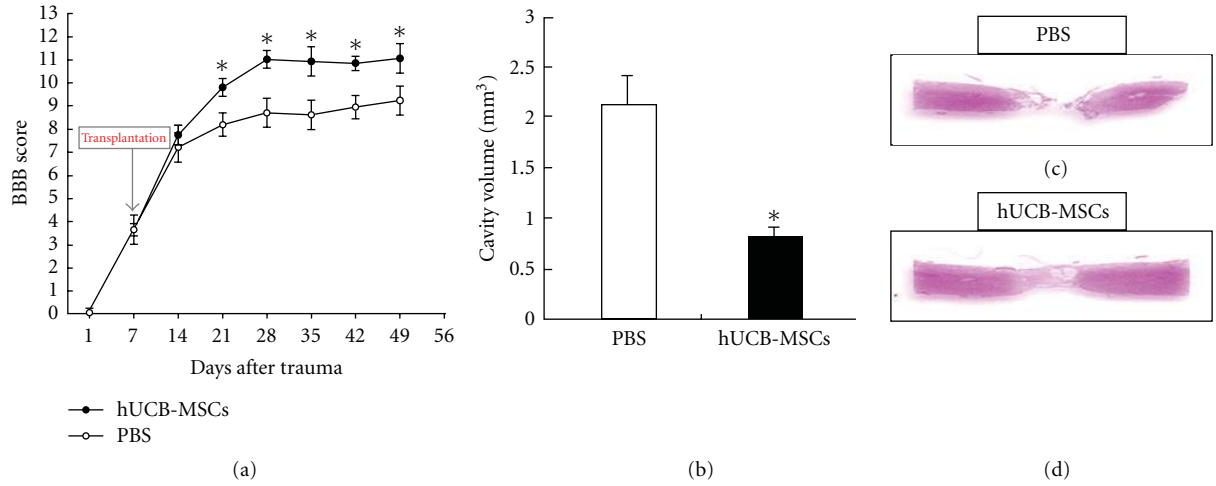

Figure 1: BBB scores of rats with SCI before and after hUCB-MSCs transplantation at 7 days after SCI. (a) hUCB-MSC transplantation group displayed significantly improved scores compared with control at 6 weeks after transplantation. (b) Cavity volume between the hUCB-MSC and control groups at 6 weeks after transplantation. The values of the cavity volume of the hUCB-MSC group were lower than those of the control group. (c) and (d) HE-stained sections of transplantation group and control group, $P < 0.05$.

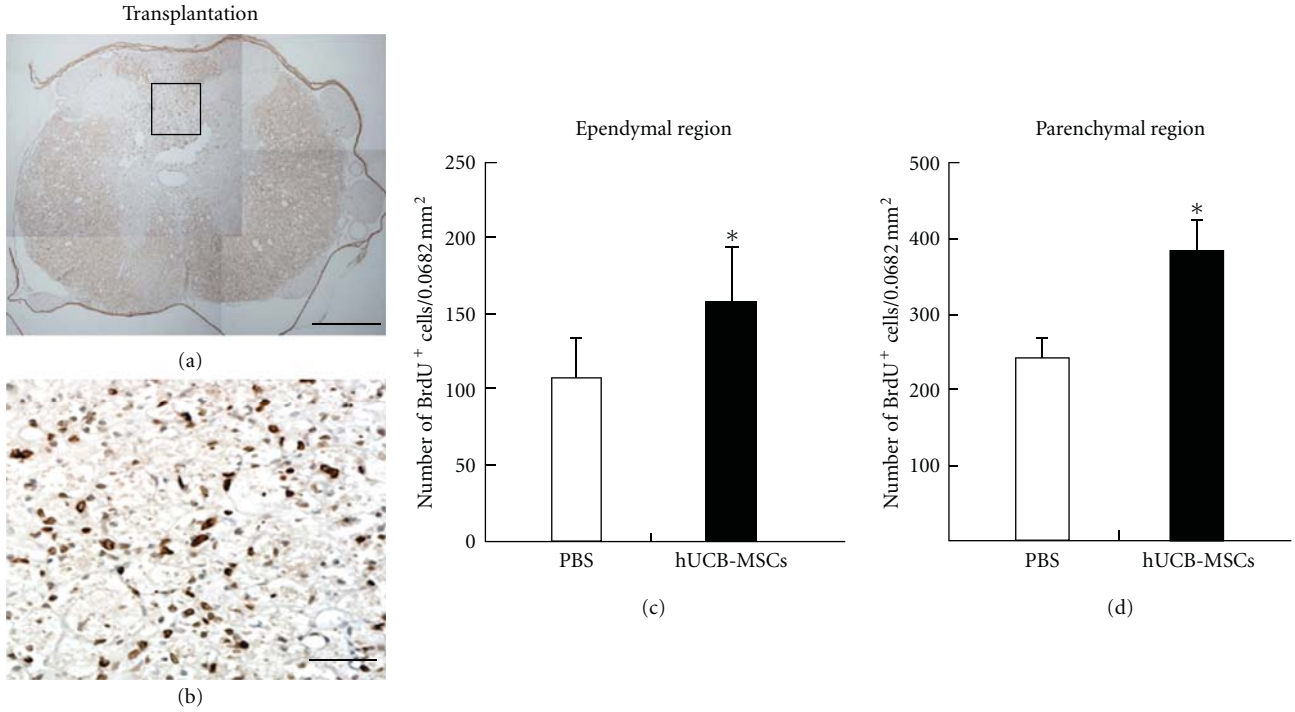

Figure 2: Quantitative analysis of BrdU-labeled cells in the ependymal and parenchymal regions. (a) Result of immunohistochemistry using anti-BrdU antibody. (b) Enlargement of the boxed region in (a), showing BrdU-labeled cells in the parenchymal region. (c) Average number of BrdU-labeled cells per white matter area from all five white matter areas in the parenchymal region. (d) Average number of BrdU-labeled cells per ependymal region in grey matter. At 14 days after transplantation, proliferation of endogenous cells was significantly increased from injury site to cell transplantation site in hUCB-MSCs-transplanted group compared with control group, $^*P < 0.05$.

of hUCB-MSCs was evident, we investigated whether newly generated cells were enhanced by the transplanted cells [22]. It has been suggested that oligogenesis [23] by endogenous OPCs and survival of these cells can contribute to self-repair after myelin loss [24]. With the thought that these processes might be stimulated recovery to CNS injury, an experiment was done to investigate the proliferation endogenous generated cells by daily injection of BrdU during the 7 days after transplantation. BrdU-positive cells were counted in the ependymal and parenchymal regions (Figures 2(a) and 2(b)) as previously described [23, 25]. Proliferation of the newly generated cells increased greatly in hUCB-MSCs-treated rats

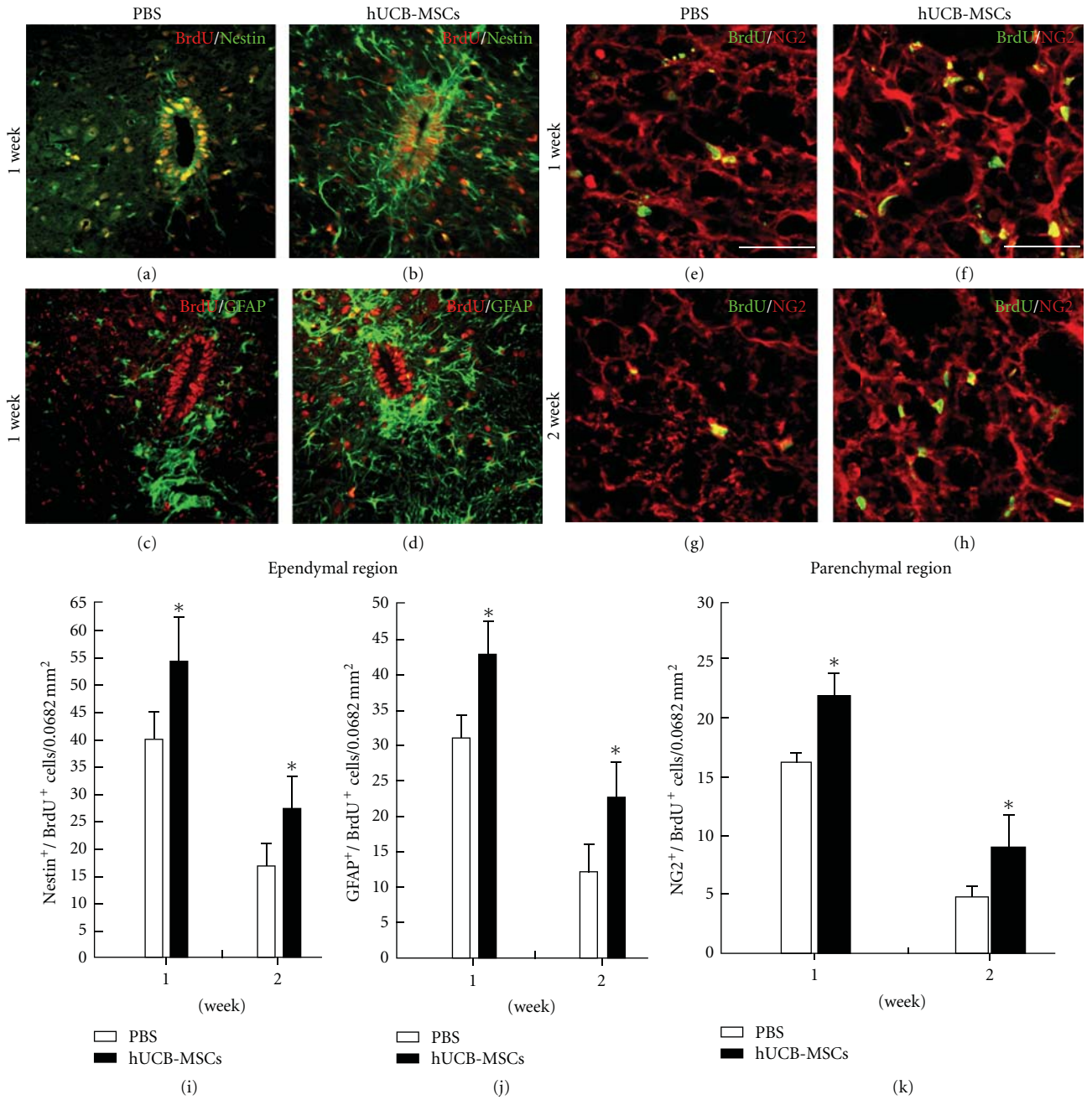

Figure 3: Endogenous neurogenesis induced by transplantation. Endogenous stem cells were assessed quantitatively by double staining of BrdU with nestin, GFAP, and NG2 at 1 and 2 weeks after transplantation in both the ependymal and parenchymal regions. (a)–(d) At 1 and 2 weeks following transplantation, BrdU/nestin-labeled cells as well as BrdU/GFAP-labeled astrocytes were present in ependyma. (e) and (h) BrdU-labeled NG2 cells were coexpressed at 1 and 2 weeks in the parenchyma. (i) and (j) The numbers of BrdU-labeled ependyma coexpressing GFAP/nestin were quantified at 1 and 2 weeks after transplantation. (k) The numbers of BrdU-labeled parenchyma coexpressing NG2 were quantified at 1 and 2 weeks after transplantation. $^*P < 0.05$, scale bars = 10 μm in (a)–(d); 20 μm in (e)–(h).

as compared with PBS-treated rats (Figure 2(c)). This data demonstrated that hUCB-MSCs could enhance proliferation of endogenous cells within the spinal cord.

3.3. Characterization of Endogenous Stem Cells. Functional recovery in response to therapeutic grafting of stem cells after SCI is related to the differentiation of grafted cells into glial cells, including astrocytes or oligodendrocytes [24]. Appropriately, an experiment was done to examine if the transplantation of MSCs could enhance the differentiation of endogenous OPCs into astrocytes or oligodendrocytes by performing immunostaining for BrdU and several phenotype markers including differentiating oligodencrocyte markers NG2, CNPase, the mature oligodendrocyte marker

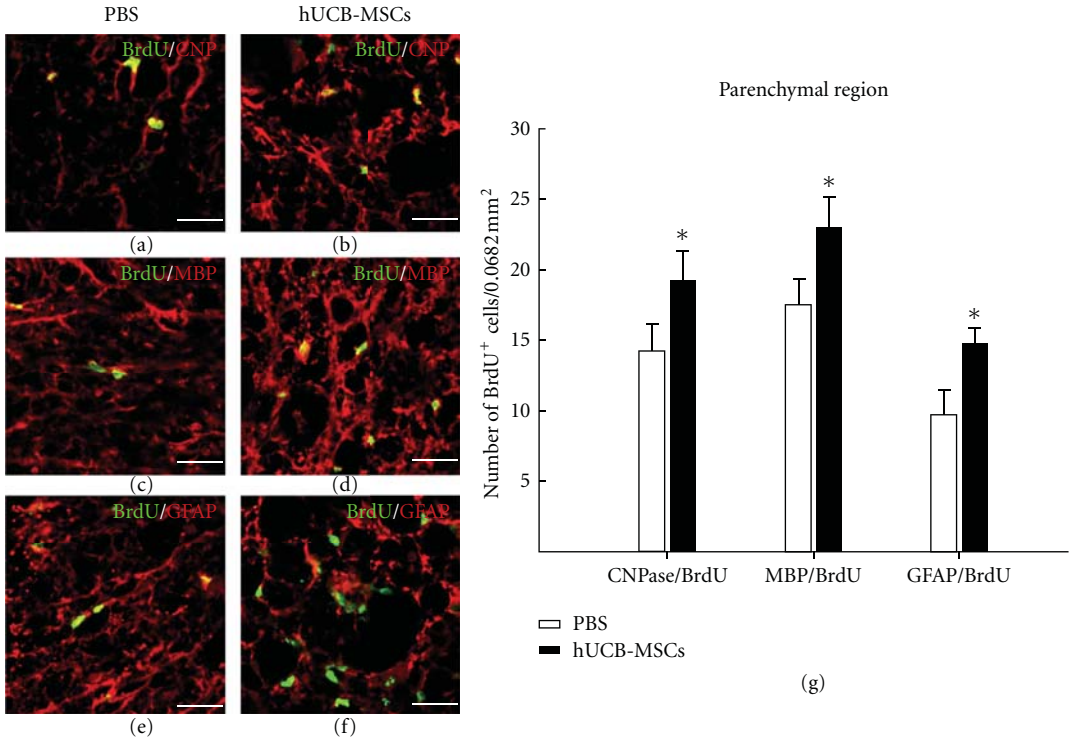

Figure 4: Quantitative analysis of endogenous oligogenesis by hUCB-MSCs. At 2 weeks after cell transplantation, BrdU and cell-specific markers were observed up to the edge of the SCI region. (a) and (b) BrdU/CNPase-labeled cells were present. (c) and (d) BrdU/MBP-labeled cells. (e) and (f) BrdU/GFAP-labeled cells. (g) Quantity of BrdU/CNPase, MBP, and GFAP-labeled cells. $^*P < 0.05$. Scale bars: 10 μm.

MBP, GFAP typical of astrocytes, and the neural stem cell marker nestin. Cells in the ependymal and parenchymal region were counted in sections from the injury epicenter to cell transplantation site. One and 2 weeks after cell transplantation, the numbers of BrdU positive cells were significantly increased compared with the PBS group (Figure 3). In the ependymal region, BrdU-labeled nestin and GFAP cells were increased compared with the PBS group at 1 and 2 weeks (Figure 3). The numbers of BrdU-labeled NG2 positive cells were also significantly increased compared with the PBS group in the parenchymal region (Figure 3). Also, BrdU-labeled cells displaying strong immunoreactivities for CNPase, MBP, or GFAP in the cell transplantation group were evident. But these immunoreactivities were weak for those rats treated with PBS (Figure 4). These data suggest that hUCB-MSCs are an influential microenvironment within the spinal cord.

3.4. Apoptotic Phenomena of Endogenous Cells. To investigate whether transplantation of MSCs have a protected injured spinal cord cells from apoptosis, a TUNEL assay was performed on sections obtained from the injury site on 2 weeks after transplantation. Numerous TUNEL-positive (green) cells were observed at the injury site in PBS-treated rats. The number of TUNEL positive cells was significantly lower in MSC-treated rats than in PBS-treated rats (Figure 5(b)). Taken together, these results indicate that hUCB-MSCs not only promote oligogenesis in the spinal cord but also have a neuroprotective effect relative with cavity volume (Figure 1(c)).

4. Discussion

In this study, hUCB-MSCs that were transplanted after SCI survived in and around the injured site and were able to ameliorate some of the behavior effects of SCI, as measured by spontaneous limb movement in an open-field test, hind limb extension, and toe spread. In addition, the cavities of MSC-treated rats were much smaller than PBS-injected rats. Cavity formation is a characteristic of progressive tissue necrosis, which follows the initial primary cell destruction in SCI. Therefore, reduction of the cavity volume means that transplanted MSCs after SCI have a neuroprotective effect. The presently indicated therapeutic effect of hUCB-MSCs in SCI agrees with previous data [26], but the exact mechanisms to improve the functional deficits remain to be elucidated.

A prior study showed that transplanted cells ameliorated the functional recovery through the integration into spinal cord tissue and establishment of some connections within the injured area of the spinal cord [27]. However, the transplantation of hUCB-MSCs could not solely account for functional recovery after SCI. Other possibility may be various beneficial actions of endogenous neurogenesis or oligogenesis within the adult spinal cord which is largely mediated via trophic influences. Previous studies have indicated that MSCs could produce trophic factors, cytokines, and other neuroprotective factors in stroke or traumatic brain injury [28, 29]. These factors and cytokines can then promote the regrowth of interrupted nerve fiber tract. BMS cells secrete more than 20 cytokines *in vitro*, and hUCB-MSCs can secrete a number of cytokines and

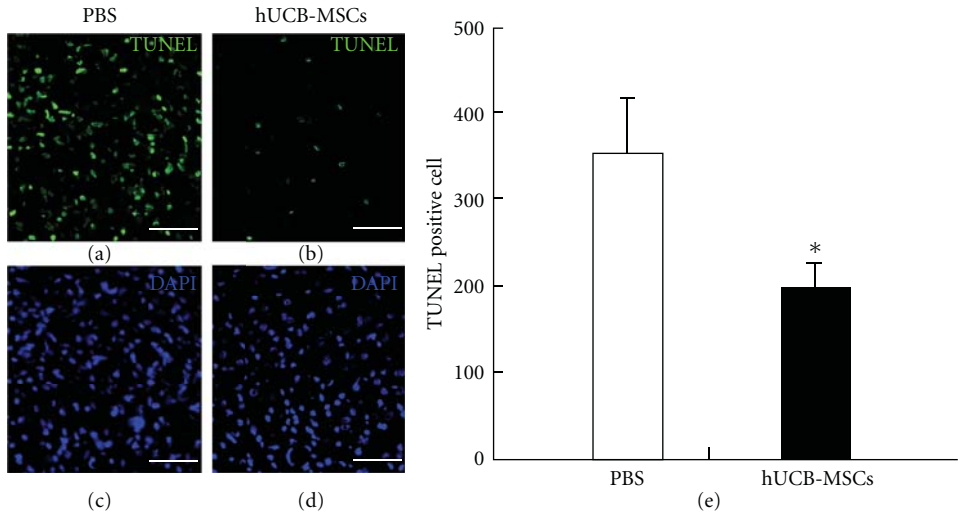

Figure 5: Protection of apoptosis by hUCB-MSCs as revealed by TUNEL assay in the injury site at 2 weeks after transplantation. (a)–(d) TUNEL staining (green) and staining with 4′,6-diamidino-2-phenylindole (blue) indicate undergoing apoptotic cell death. (e) Quantity of TUNEL positive cells. The number of TUNEL positive cells was significantly reduced in cell transplantation group than in control group. $^{*}P < 0.05$, scale bars denote: 10 μm.

chemokines [30]. Therefore, these factors and some of the other cytokines secreted by hUCB-MSCs may function as survival and differentiation factors for neural progenitor cells and then play an important role in the proliferation and differentiation of neural tissue and in the increase of central nerve system plasticity [31, 32].

To understand whether the transplanted hUCB-MSCs are capable of restoring the production of endogenous cells, we studied the mechanisms that contributed to functional recovery by determining the endogenous cell proliferation and differentiation into glial cells following transplantation. Compared to the control group, transplanted cells increased endogenous cell division within the SCI area and a subpopulation of newly dividing cells. Also, in the received, the transplanted cells, immature and mature oligodendrocytes, and astrocytes were stimulated. These observations support the possibility that factors produced by hUCB-MSCs activate nearby oligogenesis, and that activation of the astrocytes increases in oligogenesis, since astrocytes are located in close proximity to neural stem cells and express several factors that independently increase oligogenesis. In addition, some of transplanted cells were BrdU-positive cell. It has been shown that transplanted cells might proliferate in the spinal cord. But, these cells are not differentiated neural lineage markers. In agreement with the present findings, a previous study reported not only extensive oligogenesis of newly born cells after SCI but also that MSCs promote oligogenesis in neural stem cells *in vitro* [24, 33].

Presently, the majority of hUCB-MSCs progressed to apoptotic cell death. However, MSC-treated rats displayed markedly reduced apoptotic cell death in the injured site. These results suggest that functional recovery might result in endogenous oligogenesis and neuroprotection stimulated by trophic factors secreted into transplanted cells.

The collective results support the view that hUCB-MSCs transplantation is beneficial in SCI by virtue of their growth factor secretion and ability to provide physical support to growing axons. Further studies are needed to confirm that the benefit obtained from hUCB-MSCs persists at later time points and/or to improve the efficacy of the transplanted hUCB-MCSs. Also, the mechanisms underlying functional recovery after transplantation of hUCB-MSCs remain to be further investigated.

5. Conclusion

We have shown that stem cell therapy of hUCB-MSCs may provide more of functional recovery in spinal cord injury such as reduction of cavity volume, increasing of cell proliferation and endogenous oligogenesis, and decreasing of apoptosis. Therefore, the author suggests that promotion of oligogenesis by hUCB-MSCs may provide a scientific basis for the potential use of these cells as a therapeutic tool for the treatment of other disease.

Conflict of Interests

The authors declare that there is no conflict of interests.

Acknowledgments

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Research Article

Potential Stemness of Frozen-Thawed Testicular Biopsies without Sperm in Infertile Men Included into the *In Vitro* Fertilization Programme

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We describe the potential stemness of a small amount of frozen-thawed testicular tissue without sperm obtained by biopsy from six patients undergoing assisted reproductive treatment. The patients were diagnosed with Sertoli Cell-Only Syndrome alone or combined with maturation arrest. Trying to provide the natural stem cell niche for cultured stem cells, all isolated cells from enzymatically degraded biopsies where cultured together in different culture media and the presence of putative mesenchymal and putative pluripotent ES-like stem cells was indicated using different methods. High throughput real-time quantitative PCR followed by multivariate analysis revealed the formation of distinct cell clusters reflecting high degree of similarity and some of these cell clusters expressed the genes characteristic for pluripotent stem cells. In the presence of the follicular fluid, prepared as serum, putative testicular stem cells showed a certain degree of plasticity, and spontaneously differentiated into adipose-like and neuronal-like cells. Additionally, using differentiation protocols putative testicular stem cells were differentiated into neuronal- and pancreatic-like cells. This study shows that in assisted reproduction programmes, testicular tissue with no sperm might be an important source of stem cells, although it is discarded in daily medical practice; this requires further research.

1. Introduction

Stem cells derived from adult human tissues are of great scientific interest to provide potential individual cell-based therapy without ethical and immunological problems associated with human embryonic stem cells. Testicular tissue retrieved in the assisted reproduction programme might be an important source of stem cells. In infertile men with azoospermia (no sperm in the ejaculate), a diagnostic testicular biopsy is usually performed to find sperm and to cryopreserve it until use for *in vitro* fertilization. In a certain number of these patients, there is no sperm in the testicular tissue due to Sertoli Cell-Only Syndrome (SCOS) or maturation arrest (MA) of germ cells at different stages of development. In these patients, fresh or frozen-thawed testicular tissue is thrown away in daily medical practice, but could be used for the personalized cell therapy in the future. The majority of studies on testicular stem cells have been performed with the whole animal or human testicles or large biopsies [1–14], but in a clinical practice only small testicular biopsies of infertile men are available.

Most of the earlier studies concerning testicular stem cells were performed in the mouse model, mostly to study spermatogonial stem cells and their *in vitro* reprogramming [1–10]. This work was followed by studies on human testicular tissue [11–14], and the results have shown that germinal stem

cells derived from the human and mouse testes have cellular and molecular characteristics comparable to pluripotent embryonic stem cells. Due to these properties, germinal stem cells seem to have a great potential for autologous cell-based therapies respecting their unstable imprinting patterns and potential teratoma formation [15]. Another possibility are multipotent mesenchymal stem cells with some advantages, such as immunomodulatory effects [16], keeping the functionality of organs, and regeneration of damaged tissues [17–19]. Gonzalez et al. have isolated putative mesenchymal stem cells from adult human testes that they named gonadal stem cells [20]; these cells expressed markers characteristic of mesenchymal stem cells (CD105, CD73, CD90, CD166, and STRO-1), some markers characteristic of pluripotent stem cells (OCT4, NANOG, and SSEA-4), and were capable to differentiate into chondrogenic, adipogenic, and osteogenic lineages.

When talking about gonadal stem cell cultures and potential cell-based therapies, the role of the stem cell niche needs to be considered seriously since it may improve the conditions for cell growth, proliferation, and maintenance of natural properties [21]. It is known that stem cells isolated from the gonadal tissue (i.e., spermatogonial stem cells) change their properties when isolated from the testicular niche and that it is difficult to propagate and maintain them *in vitro* for a longer period [1]. Therefore, the aim of this study was to culture putative testicular stem cells with potentially pluripotent/multipotent character in the presence of other testicular cells, including Sertoli cells, which are known to have an important role in the regulation of spermatogonial fate and support of other testicular cells [22]. Moreover, we were trying to evaluate the stemness of small testicular biopsies of infertile men with no sperm. Putative testicular stem cells were differentiated into different types of cells by differentiation protocols and by heterologous follicular fluid retrieved from patients undergoing *in vitro* fertilization and rich in different substances important for germ cell growth, differentiation, and maturation, added to the conventional culture media.

2. Materials and Methods

2.1. Testicular Tissue Retrieval. Into this study six infertile men from the assisted reproduction programme were included. They were aged from 21 to 41 years (mean age: 34.3 years). In each patient, an approximately 5 mm^3 volume of testicular tissue was retrieved at diagnostic biopsy to obtain sperm before the potential *in vitro* fertilization procedure. In all patients the observation under an inverted microscope revealed no sperm in the tissue. A part of the tissue was sent to the Unit of Pathology, where the hematoxylin-eosin (HE) staining of testicular tissue sections was performed and observed by the very experienced pathologist. HE staining revealed SCOS in two patients and SCOS combined with MA in remaining patients. The testicular tissue was used for research purposes after the patient's written consent according to the research approval of the National Medical Ethical Committee (Ministry of Health, Republic of Slovenia).

2.2. Testicular Tissue Cryopreservation. Testicular tissue retrieved at the diagnostic biopsy was cut into smaller pieces with a sterile surgical blade. It was diluted in a freezing medium: Flushing medium (Origio, Denmark) containing 20% of cryoprotectant glycerol (1v/v of tissue versus 2v/v of freezing solution) in two 2 mL vials. The tissue was cooled in a liquid nitrogen vapour in a L'Air Liquide machine (France) by the slow-freezing programme: from 20°C to −6°C at 5°C/minute, from −6°C to −30°C at 10°C/minute, and from −30°C to −140°C at 20°C/minute. After cooling, vials with the testicular tissue were transferred into the liquid nitrogen at −196°C and stored until use.

2.3. Follicular Fluid Retrieval. In the *in vitro* fertilization programme, the follicular fluid was retrieved at the oocyte aspiration and after a written consent was donated by two young patients with a normal ovarian reserve and normal response to the hormonal ovarian stimulation. Previous testing on HIV and hepatitis viruses revealed that they were healthy. Follicular fluid was used immediately after the removal of the oocytes so as not to coagulate. To prepare the follicular fluid, it was centrifugated for 10 minutes at 2,500 rpm. The supernatant was filtered through a sterile Sartorius Minisart 0.45 μm filter to remove all possible cells (i.e., granulosa cells, theca cells, blood cells, and cells from the immunological system). The filtered supernatant was heat inactivated at 56°C for 45 minutes. Then it was aliquoted and stored at −20°C until use.

2.4. Testicular Tissue Thawing, Isolation, and Culture of Testicular Cells. Two vials of frozen testicular tissue of each patient were thawed in a water bath (37°C), and the content of the vials was transferred into a warm Dulbecco's Modified Eagle's Medium (DMEM)/Nutrient Mixture F12 Ham with L-glutamine and 15 mM HEPES (Sigma, cat.no. D8900). This medium was supplemented with 3.7 g/L $NaHCO_3$, 1% penicillin/streptomycin (Sigma), and the pH was adjusted to 7.4 with 1 M NaOH. The whole frozen-thawed testicular tissue was enzymatically degraded in two steps according to the modified protocol of Kanatsu-Shinohara et al. [1]. We did not isolate any special type of testicular cells but handled the whole population of testicular cells to enable the testicular niche to potential stem cells, if present. After enzymatic degradation at least 100.000 testicular cells were retrieved, as counted in a Neubauer counting chamber. Approximately 60% of cells survived the freeze-thawing procedures, as revealed by Trypan Blue staining. Then testicular tissue was centrifuged for 8 minutes at 1,500 rpm, and after centrifugation the supernatant was removed. The pellet was resuspended in collagenase type XI (0.5 mg/mL), incubated for 10 minutes at 37°C, and centrifuged for 8 minutes at 1,500 rpm again. After centrifugation, the supernatant was removed and the pellet resuspended in an enzyme mixture of collagenase type XI (0.5 mg/mL) and hyaluronidase (SynVitro Hydase, Origio). After 10 minutes of incubation at 37°C, 20% fetal bovine serum (FBS) was added to inactivate enzymes, and the suspension of cells was vigorously stirred and left for 5 minutes to separate the bigger pieces of tissue from

the suspension of cells by gravity. The supernatant was then collected and centrifuged for 8 minutes at 1,500 rpm. This gravity separation was done in first three isolations of testicular cells. In further isolations the suspension of cells was passed through a 70 μm cell strainer (BD Falcon) and centrifuged for 8 minutes at 1,500 rpm. After centrifugation the supernatant was removed, and the pellets were resuspended in culture media.

3. The Plasticity of Cell Cultures Was Tested on Two Different Ways

3.1. By Culturing of Isolated Cells in Conventional Media with or without Follicular Fluid. Cells were cultured in the following culture media: (1) DMEM/F12 with 20% FBS, (2) DMEM/F12 with 20% follicular fluid (FF), and (3) medium, which is usually used to culture human embryonic stem cells—hESC medium: DMEM/F12, 20% KnockOut Serum Replacement (Gibco), 1 mM L-glutamine (PAA), 1% nonessential amino acids (PAA), 0.1 mM 2-mercaptoethanol (Invitrogen), 13 mM HEPES, and 4 ng/mL human basic FGF (Sigma). For each biopsy one gelatin-coated 4-well culture dish (Nunc) was used to establish the primary cell culture. Subculturing of cells was performed when necessary with collagenase type IV (Sigma) or with trypsin (Sigma). The cells were cultured in a CO_2 incubator at 37°C and 6% CO_2 in air and daily monitored at the heat-staged inverted microscope (Nikon, Japan) under 40x, 100x, and 200x magnifications (Hoffman illumination). All cell cultures were performed at the University Medical Centre Ljubljana.

3.2. Or By In Vitro Differentiation of Cell Cultures. Neural differentiation was performed as described previously [23], with some modifications. Briefly, the cells from approximately 10-day-old cultures were cultured on matrigel in DMEM/F12 culture medium supplemented with 1% HSA—human serum albumin, 80 ng/mL human basic FGF, 30 μM forskolin, 1% nonessential amino acids, 0.1 mM 2-mercaptoethanol, and 1% of Insulin-Transferrin-Selenium (ITS). The cells were daily monitored, and after first morphological changes (approximately 2 days) they were stained using immunocytochemistry.

To initiate pancreatic differentiation cells from approximately 10-day-old cultures were cultured for 7 days on gelatine in pancreatic proliferation medium (DMEM/F12 supplemented with 1% of N2, 2% of B27, 1% of penicillin/streptomycin, and 25 ng/mL bFGF) and then for 15 days in pancreatic differentiation medium (DMEM/F12 supplemented with 1% of N2, 2% of B27, 1% of penicillin/streptomycin and 10 mM nicotinamide) [24–26].

4. Cell Analyses

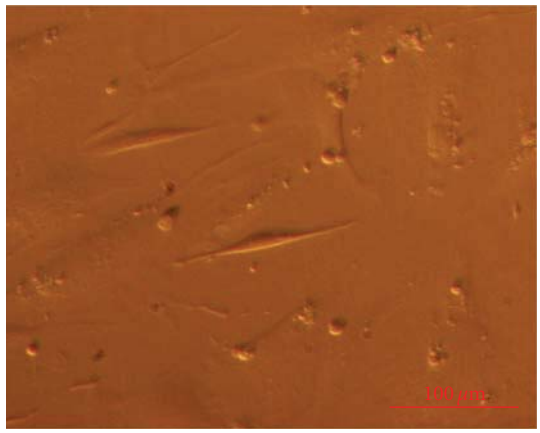
4.1. Alkaline Phosphatase Staining. An alkaline phosphatase detection kit (Millipore) was used for alkaline phosphatase (AP) staining for the presence of pluripotent and mesenchymal stem cells. Briefly, the cells were fixed in 4% paraformaldehyde for 3 minutes, permeabilized with 0.2% Tween-20 for 10 minutes and incubated for 30 minutes in a working solution of reagents, which consisted of Fast Red Violet, Naphtol AS-BI phosphate solution and water in a 2 : 1 : 1 ratio. The culture was observed under an inverted microscope (Hoffman illumination) to confirm AP activity. The cells or cell colonies expressing AP activity were stained from pink to a red colour.

4.2. Oil Red O Staining. Oil Red O staining was used to confirm the adipogenic differentiation of cultured cells. The cells were fixed in 4% paraformaldehyde for 30 minutes and incubated for 10 minutes in an Oil Red O working solution. After staining, the cells were washed 2 times with PBS and observed under an inverted microscope (Hoffman illumination) to detect the red staining of the lipid droplets.

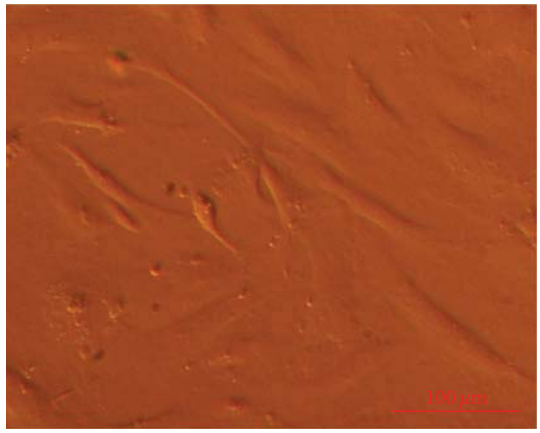
4.3. Flow Cytometry. The cells were analyzed by using FITC- (fluorescein isothiocyanate-) conjugated antibodies against CD105 (EuroClone) and PE- (phycoerythrin-) conjugated antibodies against SSEA-4 (BD Pharmingen). Mouse IgG3 conjugated with PE (BD Pharmingen) and mouse IgG1 conjugated with FITC antibodies (BD Pharmingen) were used as isotype controls. The analyzed testicular cell culture was previously cultured in a DMEM/F12 culture medium with 20% of follicular fluid, prepared as serum, on a gelatine-coated plate for 84 days (8 passages) before analysis. The whole cell culture was collected, including the cell clusters, which were treated with trypsin to achieve single-cell suspension. The sample was analyzed by using FACSCalibur (BD) and the data by using BD CellQuest Pro Software. A proportion of stained cells was monitored under a fluorescent microscope to evaluate the morphology of the CD105- and SSEA-4-positive cells.

4.4. Dithizone Staining. Dithizone staining of cell cultures was performed as described previously [27]. Briefly, the stock solution of dithizone was prepared by dissolving 10 mg of dithizone in 1 mL of dimethyl sulfoxide (DMSO). Then 10 μl of stock solution was added to 1 mL of DMEM/F12, filtered through a 0,4 μm filter, and cells were incubated in this working solution for 15 minutes at 37°C. After incubation cells were washed 4 times with PBS and observed under an inverted microscope.

4.4.1. Immunocytochemistry. Cells were fixed in 4% paraformaldehyde and permeabilized with 0.2% Triton and then incubated with 3% H_2O_2 for 10 minutes to block the endogenous peroxidase activity and for 20 minutes with 10% FBS to block the nonspecific binding sites. Then the cells were incubated for 1 hour at room temperature with following primary antibodies: mouse antinestin monoclonal antibodies (clone 10C2, 1 : 200, Millipore), rabbit anti-S100 polyclonal antibodies (1 : 500, Dako), rabbit anti-insulin (H-86) polyclonal antibodies (1 : 200, Santa Cruz Biotechnology), mouse anti-C-peptide monoclonal antibodies (1 : 100, BioVendor), rabbit anti-NSE polyclonal antibodies (1 : 70, Abcam), and mouse anti-NeuN monoclonal antibodies (1 : 200, Millipore). After washing with PBS, the cells were incubated with

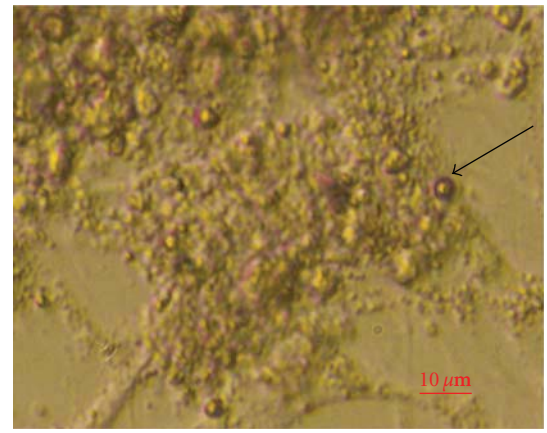


(a)

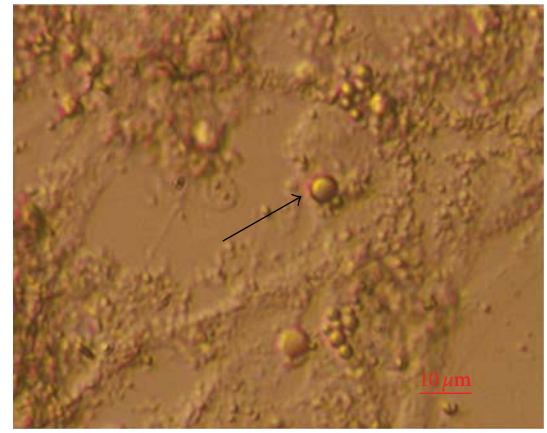


(b)

Figure 1: Cell culture grown in a DMEM/F12 medium supplemented with FBS. (a) The second passage of cell culture on day 76. (inverted microscope, Hoffman). *Scale Bar*: 100 μm.



(a)



(b)

Figure 2: Presence of small round cells (arrow) grown in an ESC medium (day 6). (inverted microscope, Hoffman). *Scale Bar*: 10 μm.

biotinylated secondary antibodies (polyclonal rabbit anti-mouse Immunoglobulins (1 : 400) or polyclonal goat anti-rabbit Immunoglobulins (1 : 600), both DakoCytomation) for 30 minutes and then with an ABC reagent (Vectastain ABC Kit-Standard) for 30 minutes. Finally, the cells were incubated in a DAB substrate (Sigma) until the brown staining appeared (usually about 5 minutes), washed with PBS and observed under an inverted microscope (Hoffman illumination) to detect positive brown-stained cells or cell colonies. For a negative control, the primary antibodies were omitted from the procedure and replaced with 1% FBS.

4.4.2. Gene Expression Analyses. Gene expression analyses of putative stem cells cultured in different media were performed using the Biomark Real-Time quantitative PCR (qPCR) system (Fluidigm). In all samples, expressions of 19 genes: *OCT4A*, *OCT4B*, *LIN28*, *GDF3*, *NANOG*, *MYC*, *KLF4*, *SOX-2*, *UTF1*, *TDGF1*, *DNMT3B*, *LIN28B*, *TERT*, *CD9*, *NANOS*, *CDH1*, *STAT3*, *REX01*, *DNMT1* mostly related to pluripotency, and of the housekeeping gene *GAPDH*, which was used for normalization, were analyzed. The inventoried TaqMan assays (20x, Applied Biosystem) were pooled to a final concentration of 0.2x for each of the 20 assays. Testicular cell clusters, human embryonic stem cells (hESC; positive control), and human fibroblast samples (F161; negative control) were harvested directly into 9 μL RT-PreAmp Master Mix (5.0 μL CellsDirect 2x Reaction Mix (Invitrogen); 2.5 μL 0.2x assay pool; 0.2 μL RT/Taq Superscript III [Invitrogen]; 1.3 μL TE buffer). The harvested cells were immediately frozen and stored at -80°C. Cell lysis and sequence-specific reverse transcription were performed at 50°C for 15 min. The reverse transcriptase was inactivated by heating to 95°C for 2 min. Subsequently, in the same tube, cDNA went through limited sequence-specific amplification by denaturing at 95°C for 15 s, and annealing and amplification at 60°C for 4 min for 14 cycles. These preamplified products were diluted 5-fold prior to analysis with Universal PCR Master Mix and inventoried TaqMan gene expression assays (ABI) in 96.96 Dynamic Arrays on a BioMark System. Each sample was analyzed in two technical replicates. Ct values were obtained from the BioMark System and were transferred to the GenEx software (MultiD). Missing data in the Biomark system were given a Ct of 999. These were removed in GenEx. Also Ct's larger than 30 were removed, since samples with such high Ct's in the Biomark 96 $\times$ 96 microfluidic card were expected

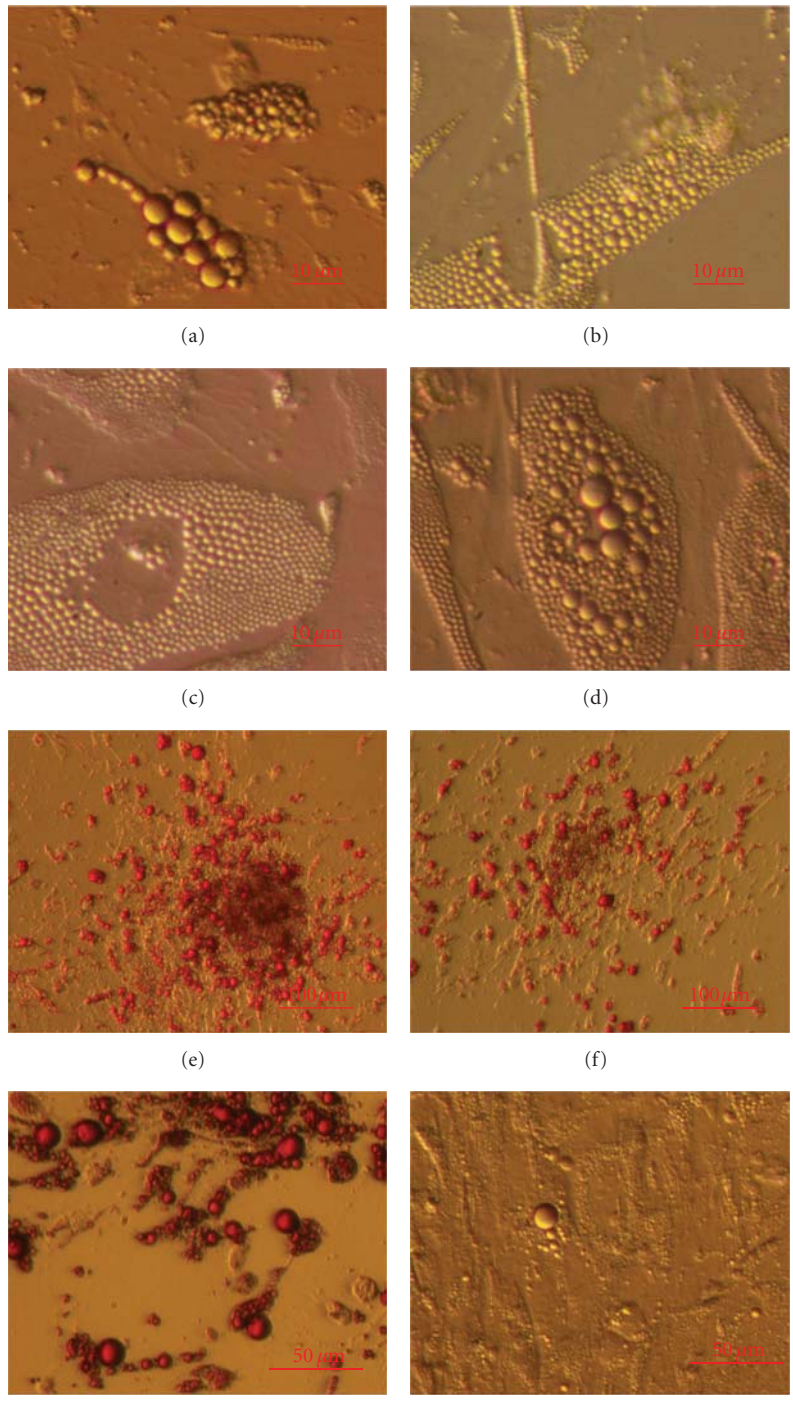
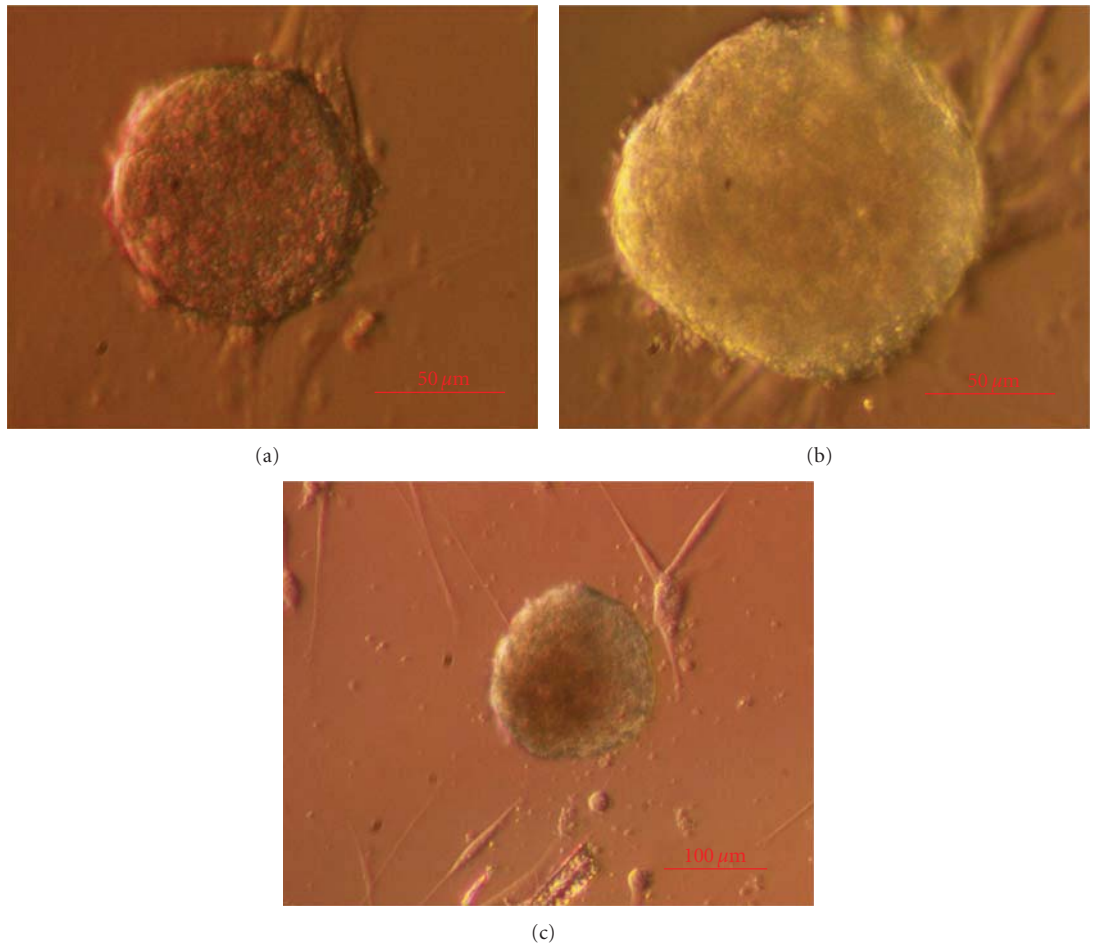


Figure 3: Accumulation of lipid droplets in cells cultured in an ESC medium with added follicular fluid. (a, b, c, d) Cell culture before staining with Oil Red O. (e, f, g) Cell culture stained with Oil Red O (passage 3, day 68). (h) Negative control. (inverted microscope, Hoffman). *Scale Bar*: (a–d) 10 μm. (e, f) 100 μm. (g, h) 50 μm.

to be negative, and these readings were unreliable. Technical repeats were then averaged. Missing data were then replaced by the highest Cq+1 for each gene. This corresponded to assigning a concentration to these samples that was half of the lowest concentration measured and was motivated by sampling ambiguity. There was also a need to handle missing data for downstream classification with multivariate tools. Linear quantities were calculated relative to the sample having lowest expression, and data were converted to $\log_2$ scale.



(a) (b)

(c)

Figure 4: Cell colonies grown in a DMEM/F12 culture medium with added follicular fluid (passage 3, day 33) on native testicular fibroblasts. (inverted microscope, Hoffman). *Scale Bar*: (a, b) 50 μm and for (c) is 100 μm.

The data were now prepared for multivariate analysis to classify the samples based on the combined expression of all the genes. Heatmap, hierarchical clustering (Ward's Algorithm, Euclidean Distance Measure) and principal component analysis (PCA) were performed. In addition, descriptive statistics was calculated individually for the genes using 0.95% confidence level and groups were compared using 1-way ANOVA and unpaired 2-tailed *t*-Test. Statistical significance was set at $P < 0.00269$ (Bonferroni correction) to account for false positives due to multiple testing. The groups of samples compared were: putative testicular stem cells (TSC), human embryonic stem cells (hESC), and human fibroblasts (F161).

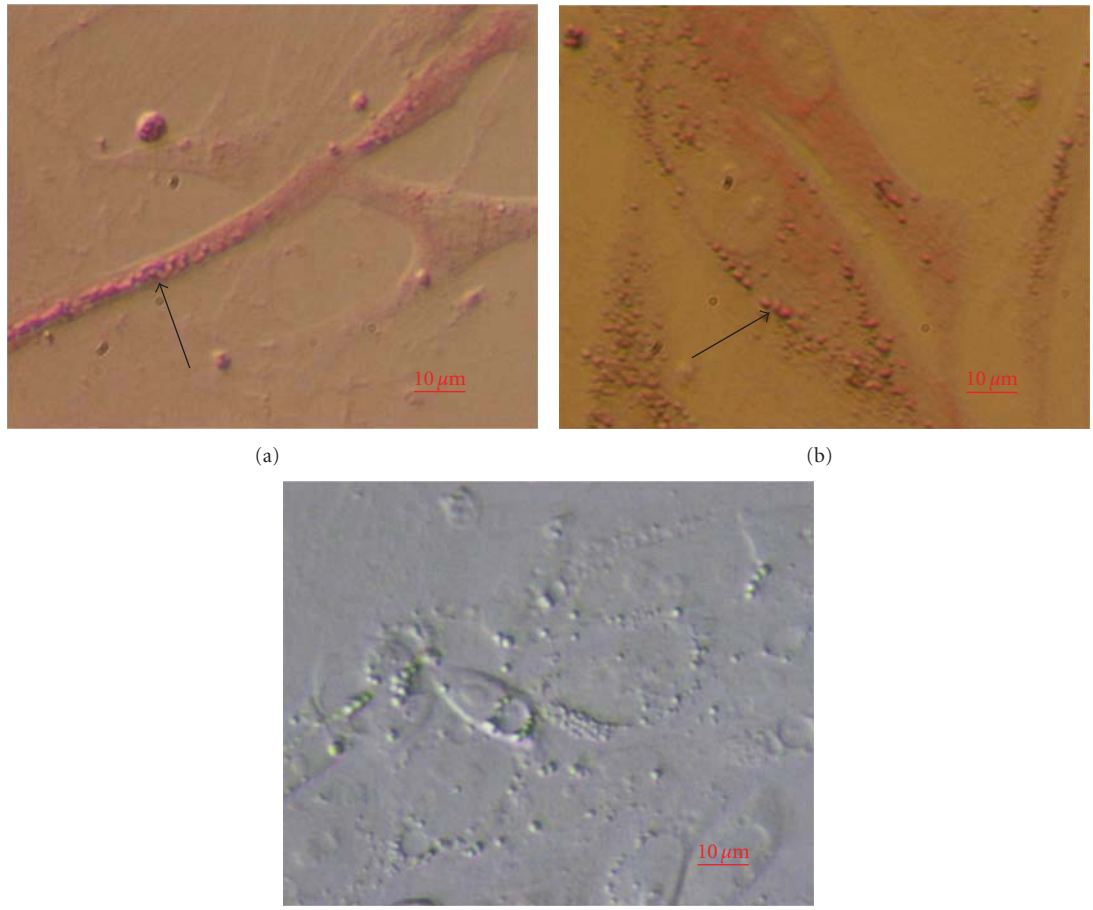
5. Results and Discussion

Testicular cell culture, forming cell colonies and persisting in a condition *in vitro*, was successfully established from testicular biopsies of 5 from 6 infertile men.

5.1. Plasticity of Cell Cultures in Conventional Culture Media with or without Added Follicular Fluid. This is the first report on the use of heterologous follicular fluid retrieved in the *in vitro* fertilization programme as a media supplement to culture cells isolated from the frozen-thawed testicular tissue. In the *in vitro* fertilization programme, follicular fluid retrieved in infertile women after hormonal ovarian stimulation is normally discarded after oocyte removal and could be used as an interesting supplement to culture media, while it contains several components important for cell growth, differentiation, and maturation, such as estrogens, progesterone, FSH, and androgens [28], proteins [29], amino acids [30], a high concentration of lipids-free cholesterol and meiosis-activating sterol (FF-MAS) [30, 31], growth factors [32], stem cell factor (SCF) [33], and other substances important for cell growth, differentiation, and maturation.

At the beginning of this experiment, three different culture media were used to establish a primary cell cultures: DMEM/F12 with 20% FBS, DMEM/F12 with 20% follicular fluid (FF), and a culture medium which is usually used to culture human embryonic stem cells (hESC medium). The primary cell cultures were successfully established in all three culture conditions. After 2 weeks of culture, there were distinguishable morphological differences between them.

5.1.1. Culture 1: DMEM/F12 Culture Medium with Added Fetal Bovine Serum (FBS). The cell cultures consisted of



(a) (b)

(c)

Figure 5: Alkaline phosphatase staining of cells grown in a DMEM/F12 culture medium with added follicular fluid (passage 3, day 44). (a, b) Some attached cells with a phenotype comparable to mesenchymal stem cells and small round cells with diameters of up to 5 μm (arrow), weakly positive for alkaline phosphatase activity. (c) Negative control. (inverted microscope, Hoffman). *Scale Bar*: 10 μm.

adherent fibroblasts or fibroblast-like cells. During passages, these cell cultures showed very little change. After passages up to 114 days, the cell cultures were morphologically similar to what they were at the beginning (Figure 1).

5.1.2. Culture 2: Human Embryonic Stem Cell (hESC) Culture Medium with or without Added Follicular Fluid (FF). These cell cultures morphologically looked different from the cell cultures grown in the DMEM/F12 medium with added FBS, and they were more similar to the cell cultures grown in the DMEM/F12 medium with added follicular fluid. Structures which morphologically resembled embryoid bodies were observed. They usually developed on the native testicular fibroblasts. At this culture condition, a proliferation of small, yellow-coloured round cells with different diameters of up to 5 μm was found (Figure 2). These cells appeared as single cells, small clusters of cells, or cells attached to other types of cells (i.e., fibroblasts). A similar type of Oct4A- positive cells has been previously identified in histological sections and cell cultures of adult human testes by Bhartiya et al. [38] and adult human ovaries [39]. Very comparable small cells were also found in other adult human tissues and organs as reported by Ratajczak and his group in more publications [36–38]. They named the cells they found as very small embryonic-like (VSEL) stem cells.

The cell cultures were passaged and were transferred to matrigel-coated plates. Around day 20, 5% follicular fluid was added to the culture medium. Seven days later, the cell cultures were passaged into the hESC medium without the follicular fluid. Around day 40, small lipid droplets were observed in the cell cultures (Figures 3(a)–3(d)), and 3 weeks later cell cultures were stained with Oil Red O to confirm lipids. A proportion of cells and cell colonies stained positively for the presence of lipids (Figures 3(e)–3(h)). There were also cells which did not accumulate lipid droplets. This phenomenon was not observed, when cells were cultured in the same way on the gelatine instead of matrigel.

5.1.3. Culture 3: DMEM/F12 Culture Medium with an Added Follicular Fluid (FF). The cell cultures were morphologically similar to the cell cultures grown in the hESC medium, and some round structures morphologically resembling embryoid bodies developed (Figure 4). These cell cultures were

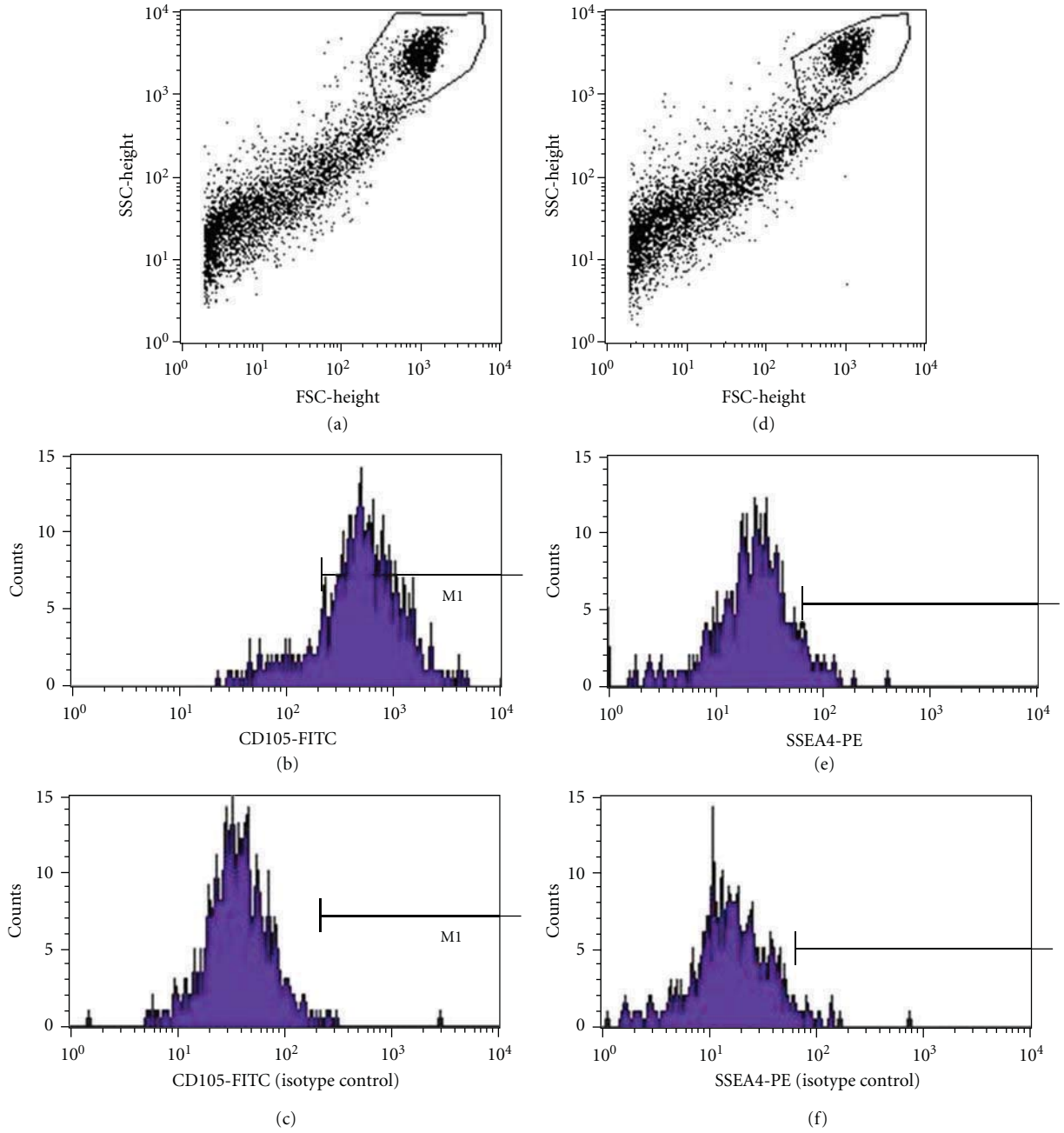


Figure 6: Flow cytometry analysis of cells grown in a DMEM/F-12 culture medium with added follicular fluid. (a, b) A subpopulation of cells expressing a mesenchymal stem cell marker—CD105-FITC. (c) Isotype control. (d, e) A subpopulation of cells expressing a stem cell marker—SSEA-4-PE. (f) Isotype control.

passaged, and at passage 2 they were transferred to the gelatine- and matrigel-coated plates. There were no cells accumulating lipid droplets. The cells formed clusters, and at passage 3 the cell cultures were stained for the presence of alkaline phosphatase activity. Some single cells with mesenchymal stem cell-like morphology attached to the dish bottom and some small round cells with diameters up to 5 μm attached to other types of cells were weakly positive for alkaline phosphatase activity (Figure 5) thus indicating the possible presence of mesenchymal or pluripotent stem cells in the cell culture. This assumption was also supported by the flow cytometry analyses, which confirmed the presence of a subpopulation of CD105-positive cells (87.0%) (Figures 6(a)–6(c)) and a small proportion of SSEA-4-positive cells (2.0%) (Figures 6(d)–6(f)) around 80 days of cell culture. The relatively low proportion of SSEA-4-positive cells may reflect the fact that many cells were attached to other types of cells, and the whole cell culture consisted of different types of cells, including fibroblasts. CD105-positive cells were round and with diameters of approximately 10 μm, whereas SSEA-4-positive cells were smaller—with diameters of up to 5 μm but with a quite strong expression of SSEA-4 surface

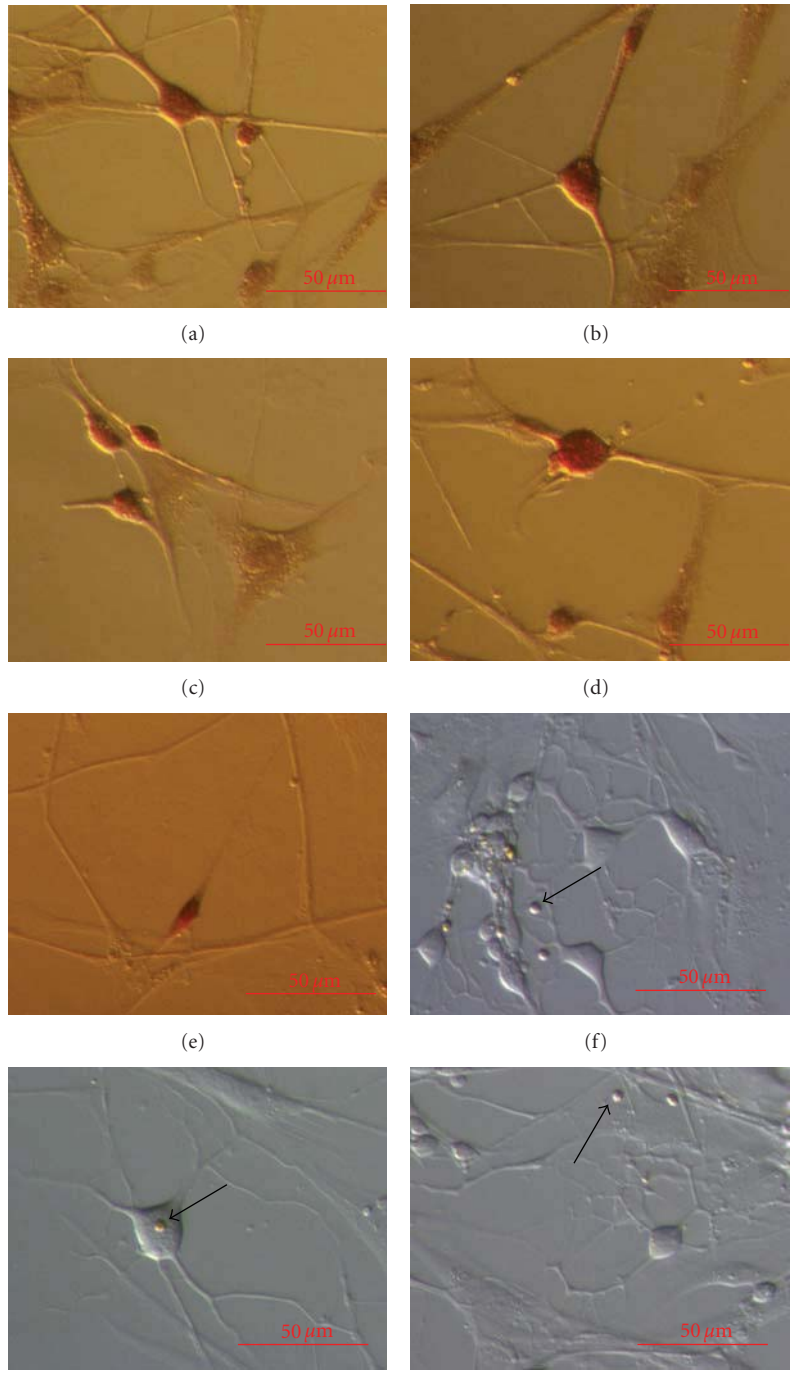


Figure 7: Differentiation of neuronal-like cells. (a–d) S100-positive cells. (e) Single nestin-positive elongated cell (arrow). (f–h) Negative controls with present small round cells (arrows) with diameters of up to 5 μm. (inverted microscope, Hoffman). *Scale Bar*: 50 μm.

antigen and nuclear staining by DAPI, as revealed by fluorescent microscopy. Besides pluripotent germinal stem cells derived from otherwise unipotent spermatogonia by reprogramming, as published before [11–14], there might be still another (maybe native) source of pluripotency in the adult human testes.

Around day 90, some of the cells spontaneously began to differentiate into neuronal-like cells. The cell cultures were stained for the expression of nestin, and a few elongated cells were indeed positive for nestin (Figure 7(e)). We supposed that these cells differentiated *in vitro*, because they were not present in the primary cell culture and earlier passages of this cell culture.

5.2. Plasticity of Cell Cultures Tested by Different Differentiation Protocols. When cell cultures were cultured in the media

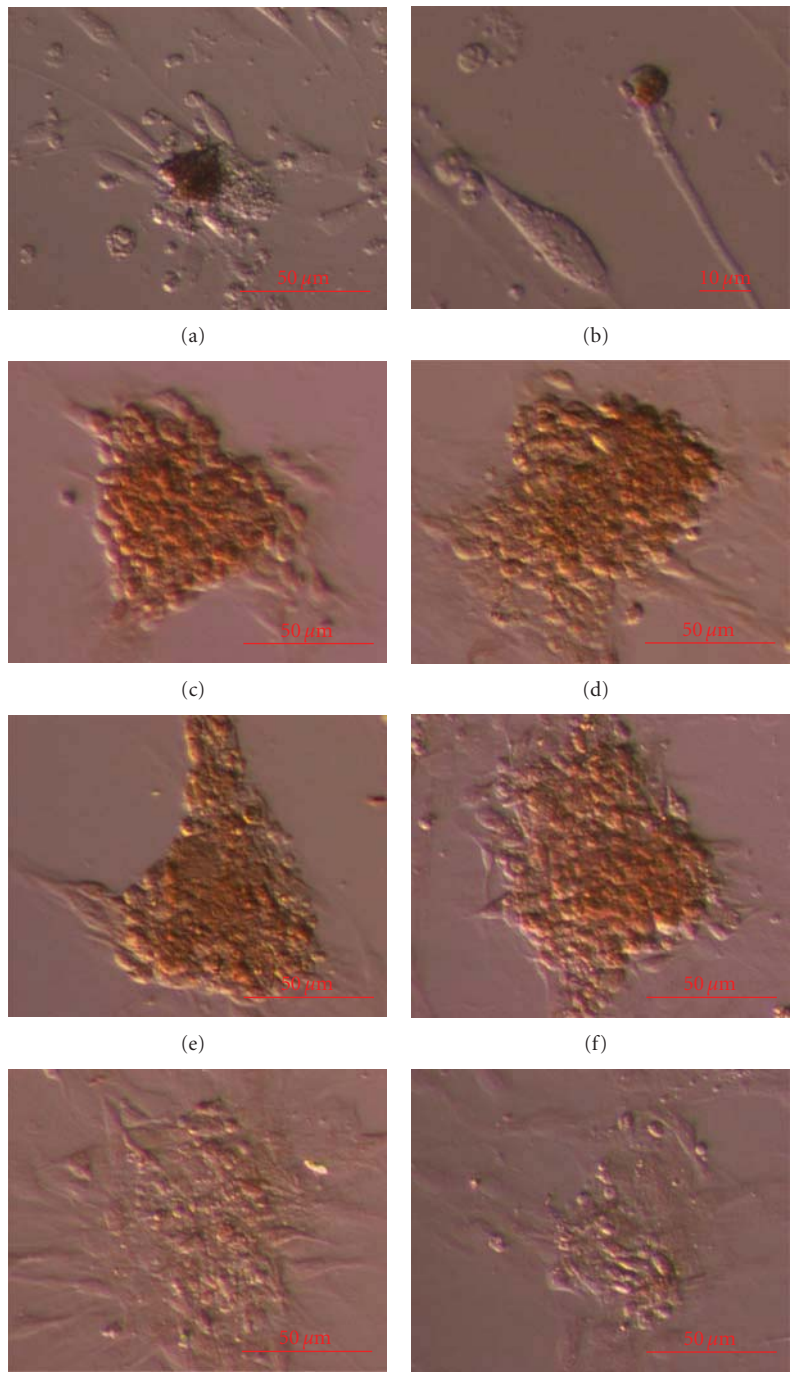
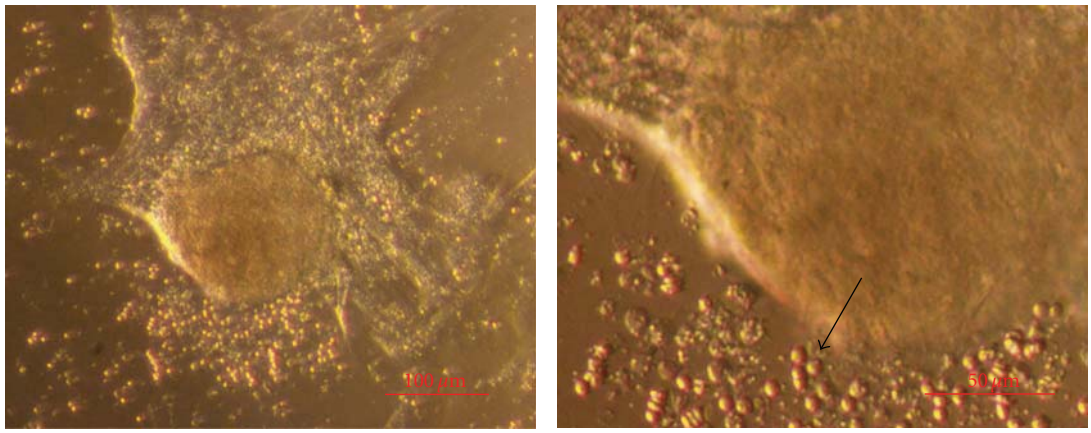


Figure 8: Differentiation of pancreatic-like cells. (a) Dithizone-positive (red) cell cluster. (b) Dithizone-positive (red) single cell. (c, d) C-peptide-positive cell clusters. (e, f) Insulin-positive cell clusters. (g, h) Negative controls. (inverted microscope, Hoffman). *Scale Bar*: (a) 50 μm, (b) 10 μm, (a–h) 50 μm.

for neuronal differentiation, development of neuronal-like cells was found. Neuronal-like cells were appearing as single cells, or they formed some kind of nets. Neuronal-like cells were positively stained on S-100 marker (Figures 7(a)–7(d)), whereas they did not stain on some other markers, such as NSE and NeuN (data not shown). This indicated the potential glia cell-like character of neuronal-like cells rather than the real neuronal character.

Additionally, when cell cultures were exposed to the media for pancreatic differentiation, the cell morphology was changed, and development of typical colonies was observed (Figure 8). Cell cultures (some colonies and single cells) were



(a) (b)

Figure 9: Testicular cell cluster (TSC1) cultured for 14 days in DMEM/F-12 culture medium with follicular fluid and expressing a variety of genes related to pluripotency and germ cells. (a) Morphology. (b) Small round and yellow cells with diameters of up to 5 μm (arrow) in the close surrounding. (inverted microscope, Hoffman). *Scale Bar*: (a) 100 μm. (b) 50 μm.

positively stained on dithizone (Figures 8(a), and 8(b)), c-peptide (Figures 8(c), and 8(d)), and insulin (Figures 8(e), and 8(f)), as revealed by immunocytochemistry.

5.3. Gene Expressions of Cell Clusters. Four samples of 6 testicular cell clusters were isolated from one cell culture: TSC1—one cluster cultured for 14 days in DMEM/F-12 medium with follicular fluid, TSC2—one cluster cultured for 14 days in hESC medium with follicular fluid, TSC3—two clusters cultured for 140 days in DMEM/F-12 medium with follicular fluid, and TSC4—two clusters cultured for 140 days in DMEM/F-12 medium with follicular fluid. Gene expression in these samples was compared with control groups based on human embryonic stem cells—H1 line (150 and 200 cells) and human fibroblasts (150 and 200 cells). Cluster TSC1 (Figure 9(a)) strongly expressed a variety of genes related to pluripotency. In culture, small round cells with yellow colour and a diameter of up to 5 μm appeared close to this cluster (Figure 9(b)). Also the other cell clusters (Figures 10(a)–10(e)) expressed some of the genes characteristic of pluripotency, but to lower extent than TSC1 (Figure 10(f)). Gene expression in the putative testicular stem cells (TSCs) was comparable to that in the human embryonic stem cells (hESC), but was quite different from the expression in the human fibroblasts, as reflected by the heatmap (Figure 10(f)), corresponding dendrogram (Figure 11(a)), PCA clustering (Figure 11(b)), and univariate analysis of the genes summarized by descriptive statistics (Figure 12); TSCs clustered with the hESCs and in the PCA they have similar PC1 score; they differ, however, in the PC2 (Figure 11(b)). The fibroblasts were clearly different. Comparing genes' expression between putative TSCs and hESCs and using Bonferroni correction for multiple testing, we found significantly lower expression of *DNMT3B* in TSC's ($P = 0.00026$). Genes *DAZL*, *NANOS*, *KLF4*, *DNMT1*, *STELLA*, *NANOG*, *STAT3*, *OCT4A*, and *GPR125* were differentially expressed at $P < 0.05$ but cannot be considered significant without validation because of the large number of genes compared (Figure 12(a)). On the other hand, fibroblasts showed quite different expression; in particular they did not express many of the genes related to pluripotency. Comparing with TSCs they underexpress *OCT4A* ($P = 0.00187$), *NANOG* ($P = 0.00206$), *SOX-2* ($P = 0.00145$), and *NANOS* ($P = 0.00247$) (Figure 12(b)). Comparing with hESCs they underexpress *OCT4A* ($P = 0.00108$), *OCT4B* ($P = 0.00045$), *NANOG* ($P = 0.00151$), *SOX-2* ($P = 0.00014$), *DNMT3B* ($P = 0.00106$), and *CDH1* ($P = 0.00066$). One-way ANOVA confirmed that the variation in genes' expression among the groups TSCs, hESCs, and fibroblasts was larger than expected by chance for *OCT4A* ($P = 0.00040$), *LIN28* ($P = 0.00170$), *GDF3* ($P = 0.00012$), *NANOG* (0.00044), *SOX*-2 ($P = 0.00044$), *TDGF1* ($P = 0.00154$), *DNMT3B* ($P = 0.00011$), *TERT* ($P = 6.068E-5$), *NANOS* ($P = 0.00126$), and *CDH1* ($P = 2E-8$). These results were consistent with the observations by flow cytometry and immunocytochemistry.

In the researched testicular biopsies differentiation of cell cultures into cells of all three germ layers (adipogenic cells—mesoderm, pancreatic-like cells—endoderm, and neuronal-like cells—ectoderm) was found in spite of relatively low amount of processed testicular tissue. Additionally, there was some experimental evidence about the possible presence of putative mesenchymal and putative pluripotent stem cells forming clusters and their differentiation *in vitro* into adipose-like and neuronal-like cells, as induced by the follicular fluid addition. The population of putative stem cells found in this study seems to be comparable to the previously found population of putative stem cells from adult human testes expressing most of the mesenchymal stem cell markers (including CD105) and also some pluripotent stem cell markers (OCT4, SOX2, NANOG) and differentiating *in vitro* into adipogenic, osteogenic, and chondrogenic cells, as published by Gonzalez et al. [20]. Our study has shown that we are dealing with two different populations of stem cells in adult testes, mesenchymal and pluripotent, possibly. More advanced characterisation of putative stem cells from adult human testes is needed, respecting the nonclear distinction between

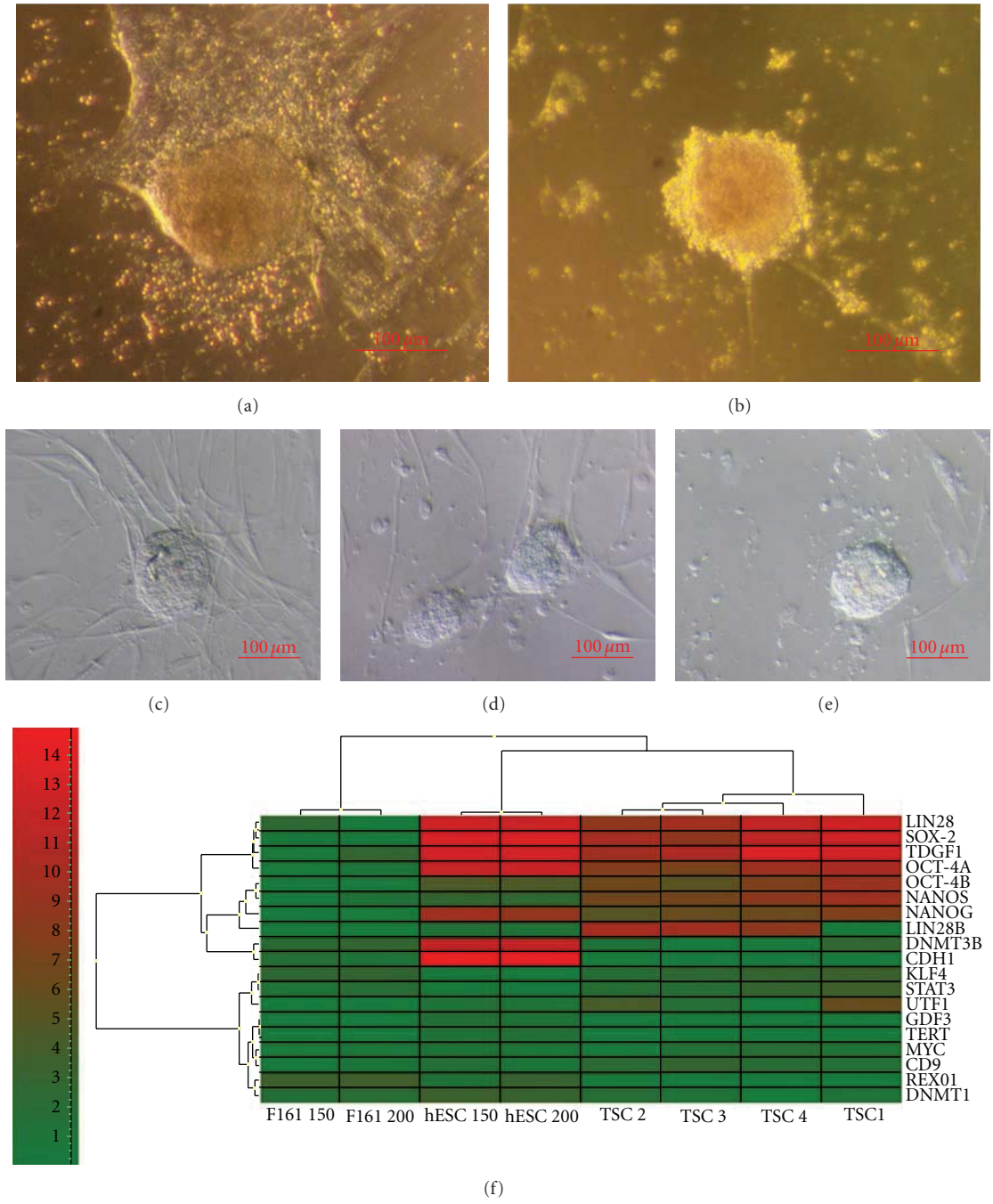
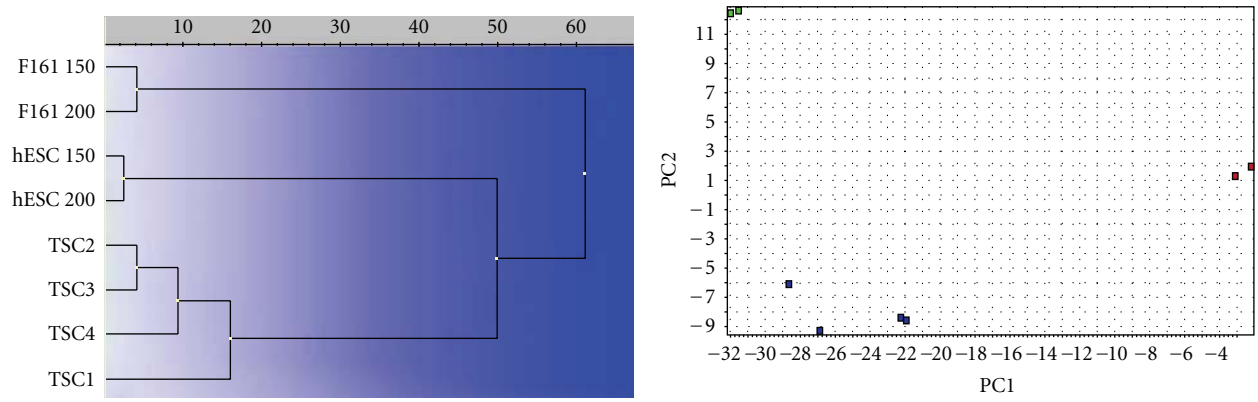


Figure 10: Testicular cell clusters and their gene expression analyses. (a) TSC1. (b) TSC2. (c) TSC3. (d, e) TSC4. (inverted microscope, Hoffman, with/without Autowhite). (f) Heatmap comparing genes' expressions in testicular cell clusters with human embryonic stem cells and fibroblasts. *Scale Bar*: (a–e) is 100 μm.

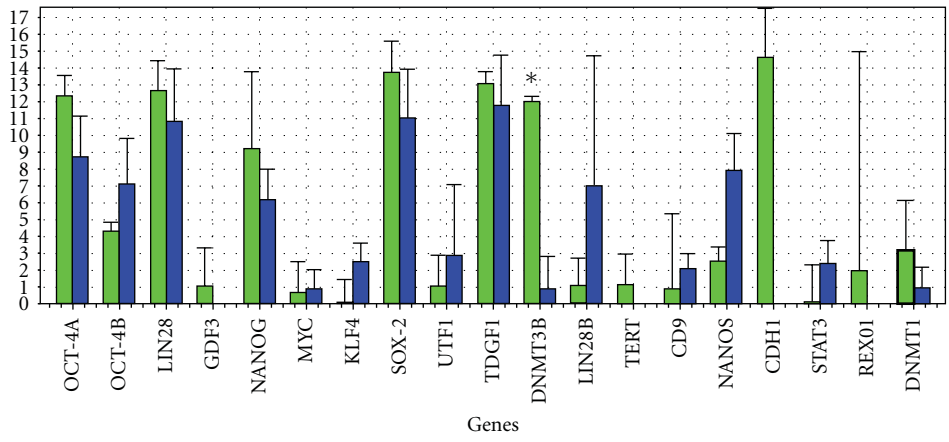
mesenchymal and embryonic-like pluripotent stem cells [39–41].

The results of this study have shown that a relatively small amount of frozen-thawed testicular tissue without sperm and with an early germ cell maturation arrest expressed some level of stemness induced *in vitro* by heterologous follicular fluid added to the culture medium or by differentiation media and cultured in a testicular niche provided by the presence of other testicular cells after enzymatic degradation. This observation definitely needs to be further studied to solve the important clinical problem about the testicular tissue without sperm retrieved in the assisted reproduction programmes, possibly. Frozen-thawed or fresh testicular tissue of azoospermics without sperm is thrown away in daily medical practice, but could possibly be useful for different autologous cell therapies in the future.



(a) (b)

Figure 11: Testicular cell clusters and their gene expression analyses. (a) Dendrogram from hierarchical clustering. (b) Principal component analysis (green-hESCs, blue-testicular stem cells TSCs, red-fibroblasts).



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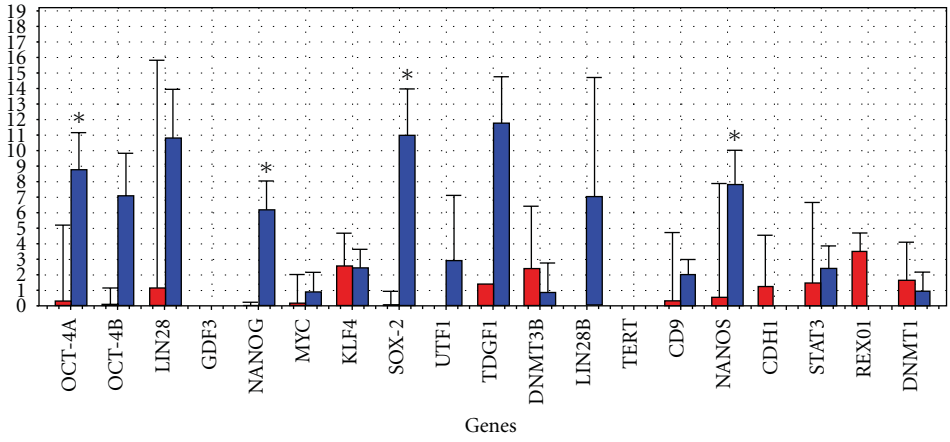

(b)

Figure 12: Descriptive statistics of genes' expressions. (a) Putative testicular stem cells (TSC1,2,3,4) compared to human embryonic stem cells hESC (150 and 200). (b) Putative testicular stem cells (TSC1,2,3,4) compared to human fibroblasts (150 and 100) *statistically significant difference based on t-Test with Bonferroni correction ($P = 0.00270$).

Acknowledgments

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Research Article

Rapamycin Conditioning of Dendritic Cells Differentiated from Human ES Cells Promotes a Tolerogenic Phenotype

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While human embryonic stem cells (hESCs) may one day facilitate the treatment of degenerative diseases requiring cell replacement therapy, the success of regenerative medicine is predicated on overcoming the rejection of replacement tissues. Given the role played by dendritic cells (DCs) in the establishment of immunological tolerance, we have proposed that DC, rendered tolerogenic during their differentiation from hESC, might predispose recipients to accept replacement tissues. As a first step towards this goal, we demonstrate that DC differentiated from H1 hESCs (H1-DCs) are particularly responsive to the immunosuppressive agent rapamycin compared to monocyte-derived DC (moDC). While rapamycin had only modest impact on the phenotype and function of moDC, H1-DC failed to upregulate CD40 upon maturation and displayed reduced immunostimulatory capacity. Furthermore, coculture of naïve allogeneic T cells with rapamycin-treated H1-DC promoted an increased appearance of $CD25^{hi}$ $Foxp3^{+}$ regulatory T cells, compared to moDC. Our findings suggest that conditioning of hESC-derived DC with rapamycin favours a tolerogenic phenotype.

1. Introduction

Human embryonic stem cells (hESCs) derived under conditions compliant with their downstream clinical application, serve as a renewable source of cell types that may one day enable the replacement of tissues whose function has become compromised by chronic or degenerative disease [1]. Nevertheless, the routine implementation of cell replacement therapy (CRT) requires strategies to address the immunological barriers encountered by the use of hESC of allogeneic origin [2]. While conventional immunosuppression offers a potential solution to the immunogenicity of hESC-derived tissues, the risks inherent in its protracted use make the induction of transplantation tolerance an attractive alternative.

Dendritic cells (DCs) play a critical role in determining the outcome of antigen presentation to naive T cells, either promoting their activation and subsequent immunity, or favouring the induction of tolerance [3]. The delivery of foreign antigen to DC in the steady state by conjugation to monoclonal antibodies (mAbs) specific for the surface receptor CD205, was, for instance, found to render recipient mice specifically tolerant to the antigen upon subsequent immunization [4]. Such findings have been extended to a transplantation setting by demonstrating how administration of immature donor DC to mice across a minor histocompatibility barrier is sufficient to secure the indefinite survival of donor skin grafts. In this model, the resulting tolerance could be attributed to the polarisation of responding T cells towards a regulatory phenotype, characterised by upregulation of the transcription factor Foxp3 [5]. Such findings, together with early success at inducing tolerance in healthy human volunteers by the administration of immature antigen-pulsed monocyte-derived DC (moDC) [6], augur well for the future use of DC as a conditioning regime in the context of CRT. Indeed, the recent description of protocols for the differentiation of DC from hESC under conditions substantially free of animal products paves the way for such an approach: given that this source of DC would share with

the replacement tissue the very alloantigens to which tolerance must be established, their administration in advance of CRT might be anticipated to condition the recipient to accept the transplanted tissue, providing the DC have first been rendered stably tolerogenic [7]. Accordingly, Senju et al. generated DC expressing the inhibitory receptor programmed death ligand 1 (PD-L1) by genetic modification of the parent hESC line [8], a similar approach in the mouse having successfully yielded DC capable of preventing the onset of experimental autoimmune encephalomyelitis by induction of tolerance to myelin antigens [9]. While such a strategy is clearly promising, the administration of genetically modified cells to patients poses additional regulatory barriers, suggesting that exposure of DC to pharmacological agents, known to promote a tolerogenic phenotype, may prove to be a more pragmatic approach [10].

The macrocyclic triene antibiotic, rapamycin, displays potent immunosuppressive properties that are routinely employed to facilitate whole-organ transplantation. In addition to its systemic use, however, rapamycin has been shown to render DC profoundly protolerogenic through inhibition of mammalian target of rapamycin (mTOR) signalling pathways. In the mouse, rapamycin-treated DC display profoundly suppressed allostimulatory capacity *in vitro* and enhanced propensity for the induction of $Foxp3^+$ regulatory T (Treg) cells [11]. Furthermore, exposure to rapamycin, unlike other immunosuppressive agents, leads to the upregulation of CCR7 by both mouse and human DC and a commensurate increase in responsiveness to CCL19, compatible with their trafficking *in vivo* to regional lymph nodes [12, 13]. Furthermore, the administration of rapamycin-treated recipient DC pulsed with donor alloantigens has secured the indefinite survival of tissue allografts in various animal models [14–16], the resulting tolerance having been demonstrated to rely on the expansion of antigen-specific Treg cells [17]. Nevertheless, despite its compelling credentials, rapamycin has been reported to exert quite distinct effects on human DC, depending on the source and subset involved [18]. We have, therefore, investigated the compatibility of protocols for the differentiation of DC from the H1 hESC line (H1-DC) with the use of rapamycin. Here we report that H1-DCs are peculiarly sensitive to the immunomodulatory effects of rapamycin, compared with conventional moDC, as evidenced by the specific loss of immunogenicity and enhanced capacity to polarise responding T cells towards a regulatory phenotype. Our findings provide an important first step towards the use of DC differentiated from hESC in the establishment of tolerance to replacement tissues, providing a proof of concept for their future application in regenerative medicine.

2. Materials and Methods

2.1. Isolation of Primary Cells. Monocytes and naïve T cells were isolated from peripheral blood mononuclear cells (PBMCs) of buffy coats (NHS Blood Transfusion Service) or from blood provided by volunteers under informed consent using CD14-coated beads or naïve $CD4^+$ T cell selection kit (Miltenyi Biotec). Cell populations were positively selected or depleted from PBMC using AutoMACS separation according to the manufacturer's instructions.

2.2. Culture of hESC. H1 ESCs were cultured in X-VIVO-10 medium (without gentamycin or phenol red, Lonza) supplemented with nonessential amino acids (PAA Laboratories GmbH), 2 mM L-glutamine (PAA Laboratories GmbH), 50 μM 2-mercaptoethanol (Sigma), 0.5 ng/mL recombinant human transforming growth factor β (TGF-β, R&D Systems), and 80 ng/mL recombinant human basic fibroblast growth factor (bFGF, R&D Systems) on 6-well plates, previously coated with Matrigel (phenol red-free, growth factor reduced, BD Biosciences) diluted 1 : 30 using ice-cold knockout Dulbecco's Modified Eagle's Medium (KO-DMEM, Invitrogen). Supplemented X-VIVO-10 medium was replaced daily except the day following passaging.

Human ESCs were routinely passaged as cell clusters of about 0.5 mm diameter every 4–6 days. For passaging, colonies were incubated in filter-sterilised warm collagenase IV (Invitrogen) until detachment of the stromal cells. Stromal cells were removed by washing with Dulbecco's Phosphate-Buffered Saline (DPBS) and hESC were scraped off into supplemented X-VIVO-10 Medium for 1 : 5 passaging. All cell cultures were incubated in a humidified incubator at 37°C and 5% CO_2.

2.3. Differentiation of hESC. H1 hESCs were plated at 3×10^6 per well of 6-well ultralow attachment (ULA) plates (Costar) in a total volume of 4 mL of X-VIVO-15 medium (Lonza), supplemented with 1 mM sodium pyruvate, nonessential amino acids, 2 mM L-glutamine (all PAA Laboratories GmbH) and 5 μM 2-mercaptoethanol (Sigma). The following growth factors were added: 50 ng/mL recombinant human bone morphogenetic protein-4 (BMP-4, R&D Systems), 50 ng/mL recombinant human vascular endothelial growth factor (VEGF, R&D Systems), 20 ng/mL recombinant human stem cell factor (SCF, R&D Systems), and 50 ng/mL recombinant human granulocyte macrophage-colony stimulating factor (GM-CSF, R&D Systems). After 2-3 days, the medium was topped up with 2 mL of fresh supplemented X-VIVO-15 medium to produce a total volume of 6 mL. Subsequent feeding was performed every 2-3 days by replacing 2-3 mL of old medium with new supplemented X-VIVO-15 medium from which every 5 days a growth factor was removed starting with BMP-4 at day 5, followed by VEGF at day 10 and SCF at day 15 of differentiation [19]. Once macrophage-like cells were observed, 25 ng/mL of IL-4 (Peprotech) was added, which was increased stepwise to 100 ng/mL.

On days 30–35, monocytes were harvested by gentle pipetting, leaving adherent macrophages in the culture dish. The cell suspension was passed through a 70 μm cell strainer (BD Falcon) to remove cellular debris, washed with DPBS and plated at $1-1.5 \times 10^6$ monocytes per well of a 6-well Cellbind plate (Corning) in X-VIVO-15 supplemented with 50 ng/mL GM-CSF and 100 ng/mL IL-4.

2.4. Derivation of DC from Human Monocytes. Monocytes were cultured in RPMI 1640 (Invitrogen) supplemented with

2 mM L-glutamine (PAA laboratories GmbH), 50 U/mL penicillin (PAA laboratories GmbH), 50 μg/mL streptomycin (PAA laboratories GmbH), 10% heat-inactivated and filter-sterilised fetal bovine serum (FBS), 50 ng/mL GM-CSF, and 100 ng/mL IL-4 on 6-well Cellbind plates for 6–8 days.

2.5. DC Maturation and Rapamycin Treatment. Two days after monocytes were plated, monocyte-derived and hESC-derived immature DC were treated with 10 ng/mL and 5–7 ng/mL of rapamycin (Sigma), respectively. On day 5, DCs were matured for 48 hr using a maturation cocktail consisting of 50 ng/mL of GM-CSF (R&D Systems), 100 ng/mL IL-4 (R&D Systems), 20 ng/mL IFNγ (R&D Systems), 50 ng/mL TNFα (R&D Systems), 10 ng/mL of IL-1β (R&D Systems), and 1 μg/mL PGE_2 (Sigma). On day 6-7, DCs were harvested by gentle pipetting, passed through a 70 μm cell strainer, centrifuged, and resuspended prior to their use in experiments.

2.6. Allogeneic Mixed Leukocyte Reaction (MLR). DCs were incubated in 10 μg/mL mitomycin C (Sigma) in supplemented RPMI 1640 at 37°C for 30 minutes. Cells were washed, resuspended in supplemented RPMI 1640, and plated in triplicate to give either 2.5×10^3 cells, 5×10^3 cells, or 1×10^4 cells in a total volume of 100 μl per well using 96-well round-bottom plates (Corning). Naïve $CD4^+$ T cells were plated at 5×10^4 cells per well to yield a stimulator to responder ratio of 1 : 5, 1 : 10, and 1 : 20 and a total volume of 200 μl/well. Wells containing T cells and mitomycin C-treated DC alone were included as controls for background proliferation of either cell type. Cells were incubated for 5 days at 37°C, after which T cells were pulsed with 0.5 μCi of [^{3}H]-thymidine per well for 18 hr before harvesting.

2.7. DC-T-Cell Cocultures. DC (2×10^5) and 1×10^6 T cells were cocultured in supplemented RPMI 1640 using 24-well Cellbind plates (Corning). After 7 days of coculture, cells were harvested and stained for CD4, CD25, and Foxp3 and analysed by flow cytometry as described below.

2.8. Flow Cytometry. Cells were incubated for 15 min in blocking solution (5% normal rat serum, 0.5% bovine serum albumin, and 0.1% NaN_3 in DPBS) on ice. Cells were washed with DPBS containing 1% FBS and 0.1% NaN_3 and resuspended in this solution together with one or several of the following fluorescently labelled antibodies: SSEA-4 (clone: MC-813-70, R&D Systems), eZFluor anti-human CD4-FITC and either CD25-APC or CD25-AF488 Cocktail (eBioscience), CD83 (HB15e, AbD Serotec), CD86 (BU63, AbD Serotec), CD40 (LOB7/6, AbD Serotec), PD-L1 (AbD Serotec), CD127 (40131, R&D Systems), CTLA-4 (BNI3, BD Pharmingen), MHC II HLA-DR/DQ/DP (WR18, AbD Serotec), CD80 (MEM-233, AbD Serotec), CD45 (15.2, AbD Serotec), CD14 (MEM18, AbD Serotec), CD11c (BU15, AbD Serotec), and CD13 (AbD Serotec). Cells were incubated at 4°C in the dark for 30–60 minutes. For the last 10 minutes, 250 ng/mL 7-AAD was added. Cells were washed, fixed in 2% formaldehyde, and analysed by flow cytometry.

Intracellular staining was performed according to the manufacturer's instructions using permeabilisation and fixation buffers (eBioscience) and antibodies specific for Oct-4 (240408, R&DSystems) or Foxp3 (eBioscience).

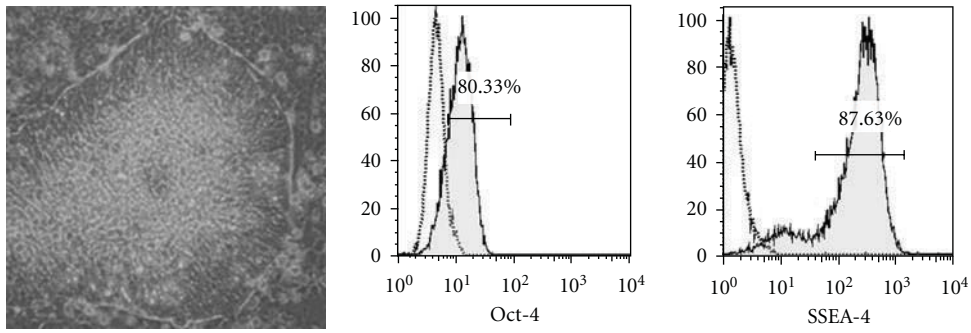
3. Results

3.1. Differentiation and Characterisation of DC from the H1 hESC Line. In order to investigate whether protocols we have established previously for the differentiation of DC from hESC might be compatible with the use of rapamycin, we made use of the well-characterised H1 hESC line. In keeping with its downstream clinical application, H1 was maintained in serum-free medium devoid of animal products and feeder cells, as described previously [20, 21]. Under these conditions, H1 formed compact colonies with clearly defined boarders (Figure 1(a)), the individual cells displaying a high nucleus : cytoplasm ratio and prominent heterochromatin. Flow cytometric analysis revealed expression of the transcription factor Oct-4 and stage-specific embryonic antigen 4 (SSEA-4), both of which are known to strongly correlate with pluripotency (Figure 1(b)).

The differentiation of H1 was directed along the DC lineage in ultralow attachment plates by exposure to a cocktail of growth factors consisting of BMP-4, VEGF, SCF, and GM-CSF, as described previously [19]. The initiation of hematopoiesis was apparent by day 20 of culture, as evidenced by the appearance of $CD45^+$ cells, although the lack of expression of CD13, CD14, and CD11c suggested that commitment to the myeloid lineage had yet to occur (Figure 2(a)). In contrast, by day 27 of culture, a small proportion of cells, residing within a population expressing intermediate levels of CD45, had upregulated these markers, consistent with their progressive commitment to the myeloid lineage (Figure 2(b)). Indeed, from day 28 of culture onwards, cells with the characteristic morphology of human DC could be identified within cultures, either as clusters with prominent veils of cytoplasm or individual cells with long dendrites (Figure 2(c)).

By day 33 of culture, up to 21% of cells had adopted a $CD45^{hi}$ phenotype, the majority of which were $CD11c^+$ (Figure 2(d)). Whereas these cells predominantly expressed MHC class I and CD86, CD83, and MHC class II expression were low, consistent with the phenotype of immature DC. Culture of H1-DC for 2 days in a cocktail of cytokines consisting of GM-CSF, IL-4, IFNγ, TNFα, IL-1β, and PGE_2 induced their maturation, as evidenced by the upregulation of CD83, similar to moDC (Figure 2(e)). Although, as previously described, MHC class II was not upregulated by H1-DC to the same extent as their monocyte-derived counterparts [19], surface expression of the costimulatory molecules CD40, CD80, and CD86 was consistent with our previous reports of the ability of this novel source of DC to stimulate proliferative responses among naïve allogeneic T cells [19].

3.2. Rapamycin Reduces the Immunogenicity of H1-DC. We next investigated whether the exposure of H1-DC to rapamycin could promote the acquisition of a protolerogenic phenotype, similar to that described for other populations



(a) (b)

Figure 1: Maintenance of the H1 hESC line. (a) Colony of H1 hESC showing the morphology typical of pluripotent stem cells, including prominent boarders (×20 magnification). (b) Expression by H1 hESC of the transcription factor Oct-4 and the surface marker SSEA-4, both of which correlate with pluripotency. Dead cells were removed from flow cytometric analysis using 7-AAD staining. Open histograms represent appropriate isotype controls.

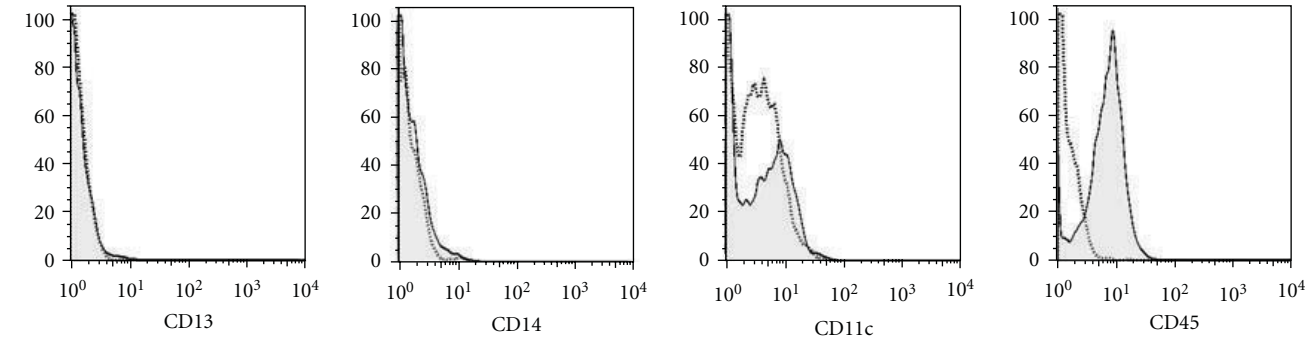
of mouse and human DC [11–13]. Accordingly, we cultured H1-DC with rapamycin for 3 days prior to inducing their maturation with proinflammatory cytokines and assessed their surface phenotype and immunostimulatory capacity in the allogeneic MLR. Whereas the addition of 10 ng/mL of rapamycin to moDC had only a modest impact on their viability, H1-DC proved especially sensitive to its toxicity, undergoing significant levels of apoptosis at concentrations greater than 7 ng/mL, as described in other studies [22]. Nevertheless, careful titration of the compound revealed that exposure of H1-DC to concentrations between 5 and 7 ng/mL exerted immunomodulatory effects without compromising their viability. Interestingly, conditioning of H1-DC with rapamycin did not appear to inhibit their maturation since they upregulated CD83 and CD86 and maintained surface expression of MHC class II and the inhibitory receptor PD-L1 (Figure 3(a)), strongly implicated in the polarisation of naïve T cells towards a Treg phenotype [23]. Significantly, however, H1-DC consistently failed to up-regulate CD40 following exposure to rapamycin, even though higher concentrations of the pharmacological agent had little impact on CD40 expression by moDC (Figure 3(b)). Consistent with their reduced levels of CD40 expression, the immunostimulatory capacity of rapamycin-treated H1-DC was significantly reduced in cocultures with naïve allogeneic T cells (Figure 3(c)). In contrast, 10 ng/mL of rapamycin exerted only modest inhibitory effects on the capacity of moDC to stimulate proliferative responses among naïve allogeneic T cells (Figure 3(c)).

3.3. Rapamycin-Treated H1-DC Polarise Naïve T Cells towards a Regulatory Phenotype. Given the reduced immunostimulatory capacity of rapamycin-treated H1-DC and their acquisition of a $CD40^{lo}$ PD-$L1^{+}$ phenotype, we next investigated whether their coculture with naïve $CD4^{+}$ T cells might favour the induction of Treg cells, defined as $CD4^{+}CD25^{hi}$ cells with persistent expression of Foxp3. Although at the outset, T cells enriched for $CD4^{+}$ cells were predominantly $Foxp3^{-}$ (Figure 4(a)), coculture with immature H1-DC for 7 days, resulted in up to 8.5% of $CD4^{+}CD25^{hi}$ cells retaining Foxp3 expression by the end of the culture period (Figure 4(b)). When the H1-DC had been matured prior to coculture with naïve allogeneic T cells, the proportion of cells committed to the Treg cell lineage increased marginally to 12.5%. However, the use of H1-DC, which had been induced to mature following exposure to rapamycin, consistently resulted in a significant increase in the induction of Treg cells which represented approximately 26.5% of $CD4^{+}CD25^{hi}$ cells, similar results being obtained in four independent experiments. By contrast, rapamycin conditioning of moDC exerted only a marginal effect on the ability of the cells to polarise responding T cells towards a regulatory phenotype (Figure 4(b)).

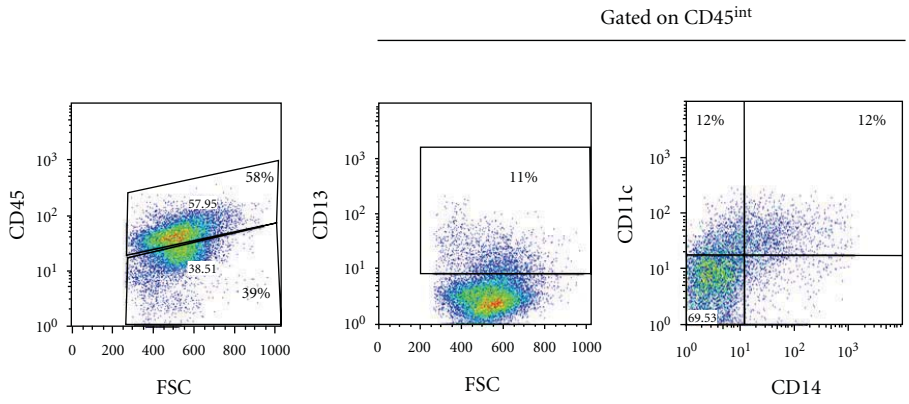
Given that the identification of *bona fide* human Treg cells is confounded by the universal upregulation of CD25 by activated T cells and their transient expression of Foxp3, irrespective of final lineage commitment, we investigated whether $CD25^{hi}$ $Foxp3^{+}$ cells appearing in such cultures displayed other known phenotypic features of Treg cells. Cells co-expressing CD25 and Foxp3 were found to express CTLA-4 (Figure 5(a)), while lacking expression of the α subunit of the IL-7R, CD127 (Figure 5(b)), such a phenotype being strongly suggestive of a regulatory function [24, 25].

4. Discussion

The development of robust protocols for the differentiation of DC from hESC lines, derived under cGMP conditions, offers a potentially unlimited source of cells with little variability between batches, which may be subjected to rigorous quality control. The potent immunostimulatory capacity of DC differentiated in this way has suggested that they will find a likely application in the presentation of tumour associated antigens to the T-cell repertoire, thereby overcoming many of the limitations inherent in the use of moDC for cancer immunotherapy [19]. Nevertheless, given the accumulation of evidence in favour of an additional role played by DC in the establishment and ongoing maintenance of immunological tolerance [3], the availability of DC differentiated from hESC suggests they may enjoy a broader remit. We have, for instance, proposed that hESC-derived DC might be exploited



(a)



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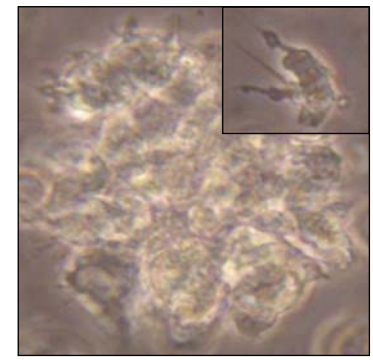

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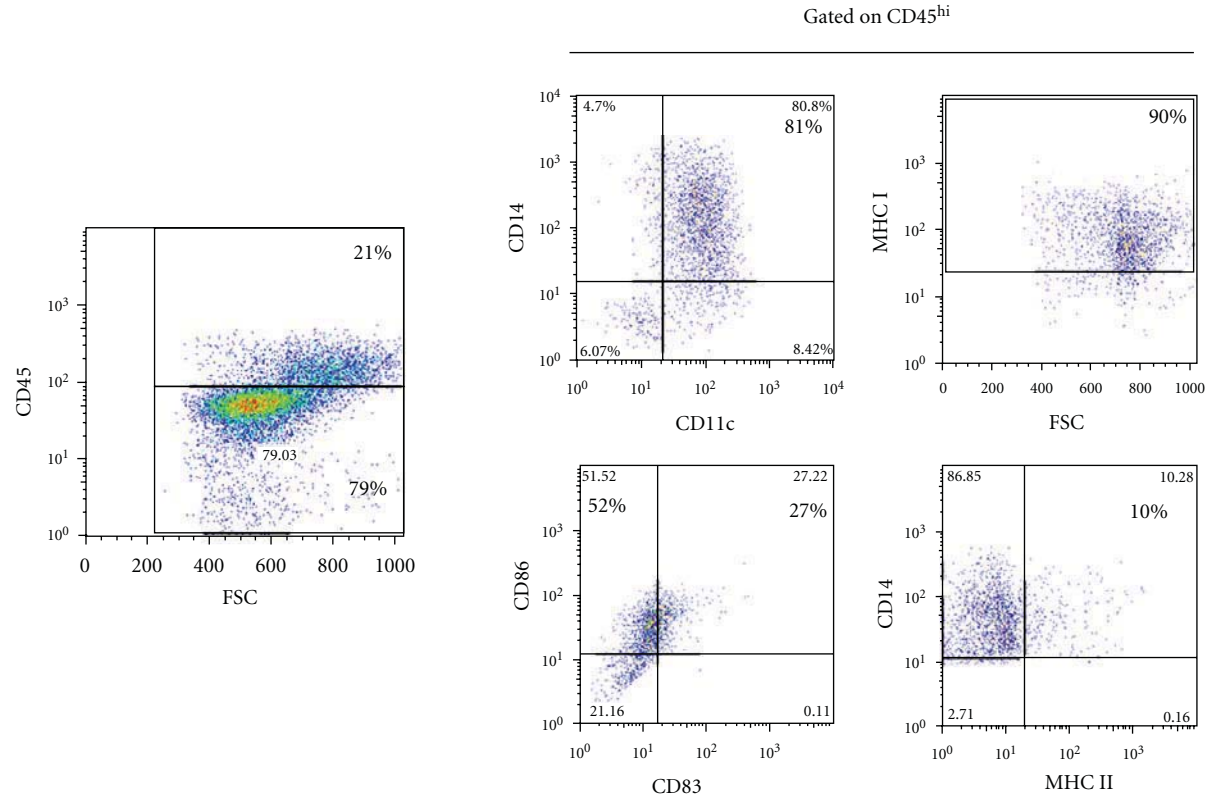
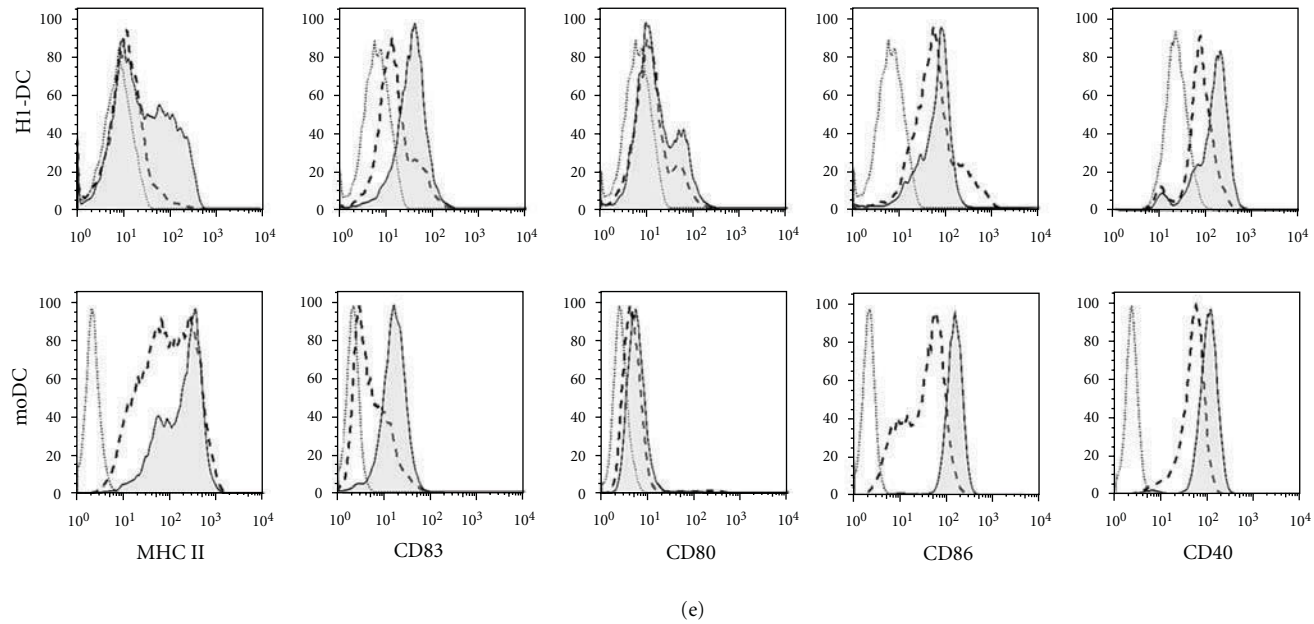


(d)

Figure 2: Continued.



(e)

Figure 2: Time course of DC differentiation from H1 hESC. Cells were harvested from cultures at various time points and analysed by flow cytometry for the onset of hematopoiesis and the appearance of DC. (a) Cells harvested at day 20 of culture showing expressing of CD45 but lack of myeloid commitment, as evidenced by staining for CD13, CD14, and CD11c. Open histograms show levels of background staining using isotype-matched control antibodies. (b) Appearance of $CD45^{int}$ cells at day 27 of culture, accompanied by the upregulation of myeloid-specific markers. (c) Photomicrograph, taken at day 28 of culture, showing the morphology of DC, including veils of cytoplasm and long dendrites (inset) (×40 magnification). (d) Cells harvested at day 33 of culture, showing the appearance of a $CD45^{hi}$ population containing predominantly DC progenitors expressing CD14, CD11c, CD86 and MHC class I. (e) Phenotype of immature and mature H1-DCs compared with human moDC. DCs were cultured either in medium alone or medium supplemented with the maturation cocktail and stained for MHC class II, the maturation marker CD83 and classical costimulatory molecules. Dead cells were excluded from the analysis using 7-AAD. Dashed histograms show the phenotype of immature DCs while the filled histograms represent mature DCs. Open histograms depict background staining using isotype-matched controls.

to induce tolerance to the alloantigens they express, thereby conditioning recipients to accept replacement tissues differentiated from the same parent cell line [7]. This prospect is, however, contingent on the development of clinically compliant strategies to ensure the stable tolerogenicity of DC generated in this way. While the introduction of transgenes, such as PD-L1, at the ESC stage might confer on the resulting DC an immunomodulatory function [8], the additional regulatory hurdles encountered by the administration to patients of genetically modified cells, has fuelled attempts to identify approved pharmacological agents that coerce DC to adopt a protolerogenic phenotype [10].

Rapamycin is one such agent routinely exploited for its immunosuppressive properties in the treatment of allograft rejection but which has been shown to exert a profound effect on the function of individual components of the immune system, including DC. Indeed, treatment of DC with rapamycin *in vitro* has been demonstrated to arrest them in an immature or semimature state rendering them tolerogenic [11–13]. Accordingly, in various preclinical transplantation models, administration of rapamycin-treated recipient DC, pulsed with a source of donor alloantigens, secured the long-term survival of organ allografts [14–16]. If such a conditioning regime could be applied to DC differentiated from hESC, it may prove feasible to establish operational tolerance to the alloantigens they endogenously express, in advance of CRT. As a first step towards this goal, we have demonstrated the sensitivity of H1-DC to rapamycin which significantly reduces their immunostimulatory properties in the allogeneic MLR (Figure 3(b)), an *in vitro* correlate of the direct pathway of alloantigen presentation. Furthermore, rapamycin substantially augments their ability to polarise responding $CD4^+$ T cells towards a regulatory phenotype (Figure 4(b)), as determined by their sustained expression of Foxp3 and adoption of a $CTLA4^+CD127^-$ phenotype. Furthermore, our preliminary results indicate that, while maturation of H1-DC induces secretion of high levels of the inflammatory cytokine IL-6 [19], prior exposure to rapamycin significantly reduces IL-6 production, possibly guiding responding T cells away from Th1/Th17 commitment towards a Treg phenotype. These results strongly suggest, therefore, that rapamycin may have the desirable properties of preventing activation of alloreactive T cells through both the direct and indirect pathways of alloantigen presentation, the induction of Treg cells potentially modulating responsiveness to indirectly presented alloantigens that have been reprocessed by endogenous recipient DC. In contrast to our findings with H1-DC, rapamycin-treatment of moDC had only a modest impact on their immunostimulatory capacity and little effect on their surface phenotype. Although our results are contrary to

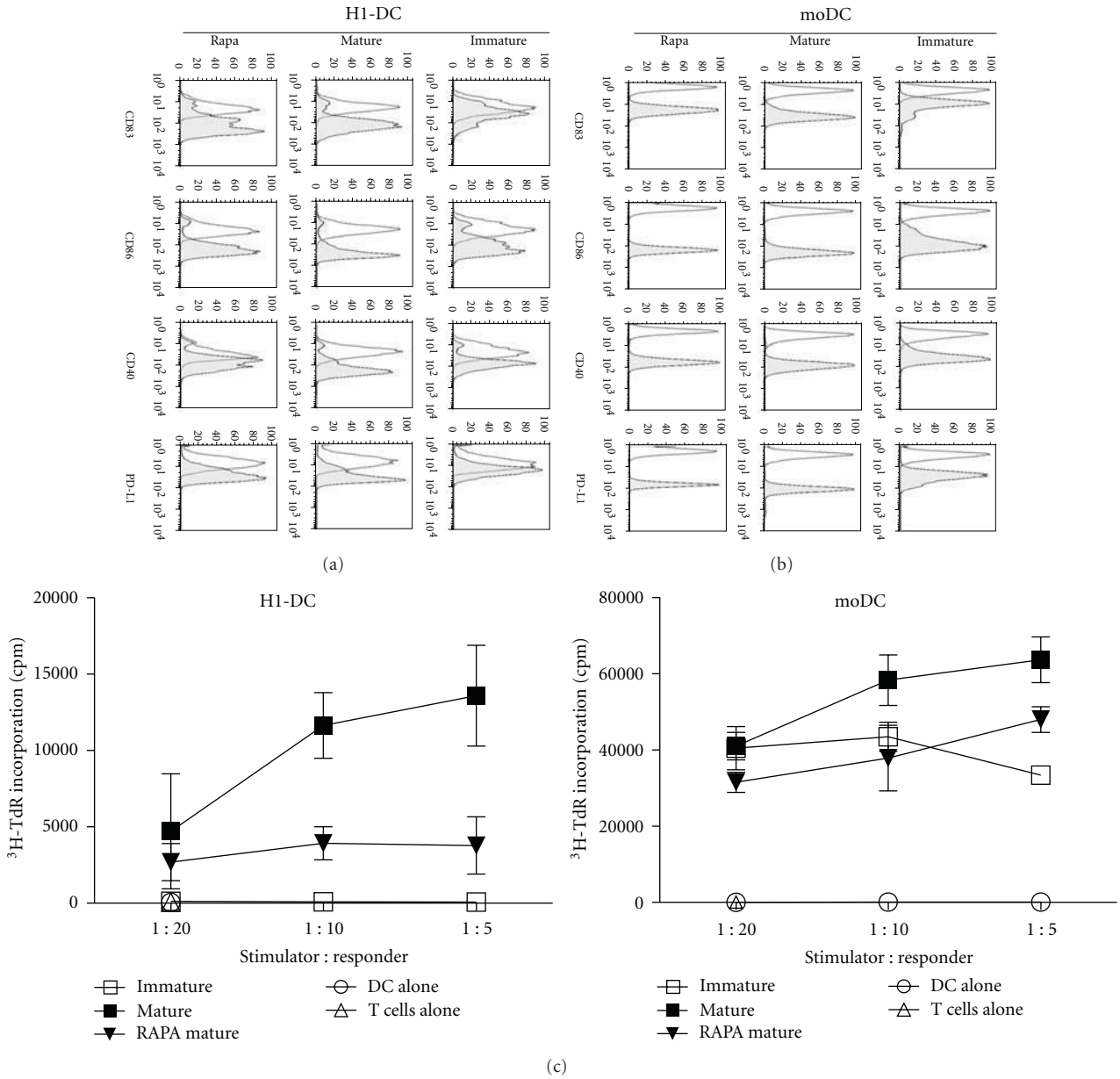


Figure 3: Effect of rapamycin (Rapa) on the phenotype and function of H1-DC. DCs were either untreated, matured in response to the maturation cocktail or treated with Rapa for 3 days prior to maturation. (a) H1-DC stained for the expression of the maturation marker CD83, the costimulatory molecules CD86 and CD40, as well as the inhibitory receptor PD-L1. Dead cells were excluded from analysis using 7-AAD. Open histograms represent the level of background staining using appropriate isotype-matched controls. Data from one of 3 independent experiments are shown. (b) Phenotypic analysis of control populations of moDC treated and stained in parallel with rapamycin. (c) Effect of rapamycin on the allostimulatory capacity of DC in the allogeneic MLR. DCs were mitotically-inactivated using mitomycin C and plated in triplicate at a top dose of 10^4 cells per well of a 96-well round-bottomed plate; naïve $CD4^+$ T cells were plated at 5×10^4 cells/well. Cells were incubated for 5 days before pulsing with ^{3}H-thymidine overnight. Graphs show the mean of triplicate cultures $\pm$S.D. Data are shown from one experiment, representative of 3 independent experiments.

some other reports [12, 18], many studies have typically used higher concentrations of rapamycin and regimes for the maturation of moDC involving exposure to bacterial products, such as lipopolysaccharide, which target different intracellular signalling pathways from those solicited upon culture with the cocktail of proinflammatory cytokines used in these studies.

Despite the profound effect that rapamycin exerts on the functional potential of H1-DC, phenotypic analysis of cells treated with the compound was largely unremarkable, with the exception that upregulation of the costimulatory molecule CD40 upon maturation was prevented by prior exposure to the compound. The significance of these findings may lie in the growing appreciation of the role played by CD40 as

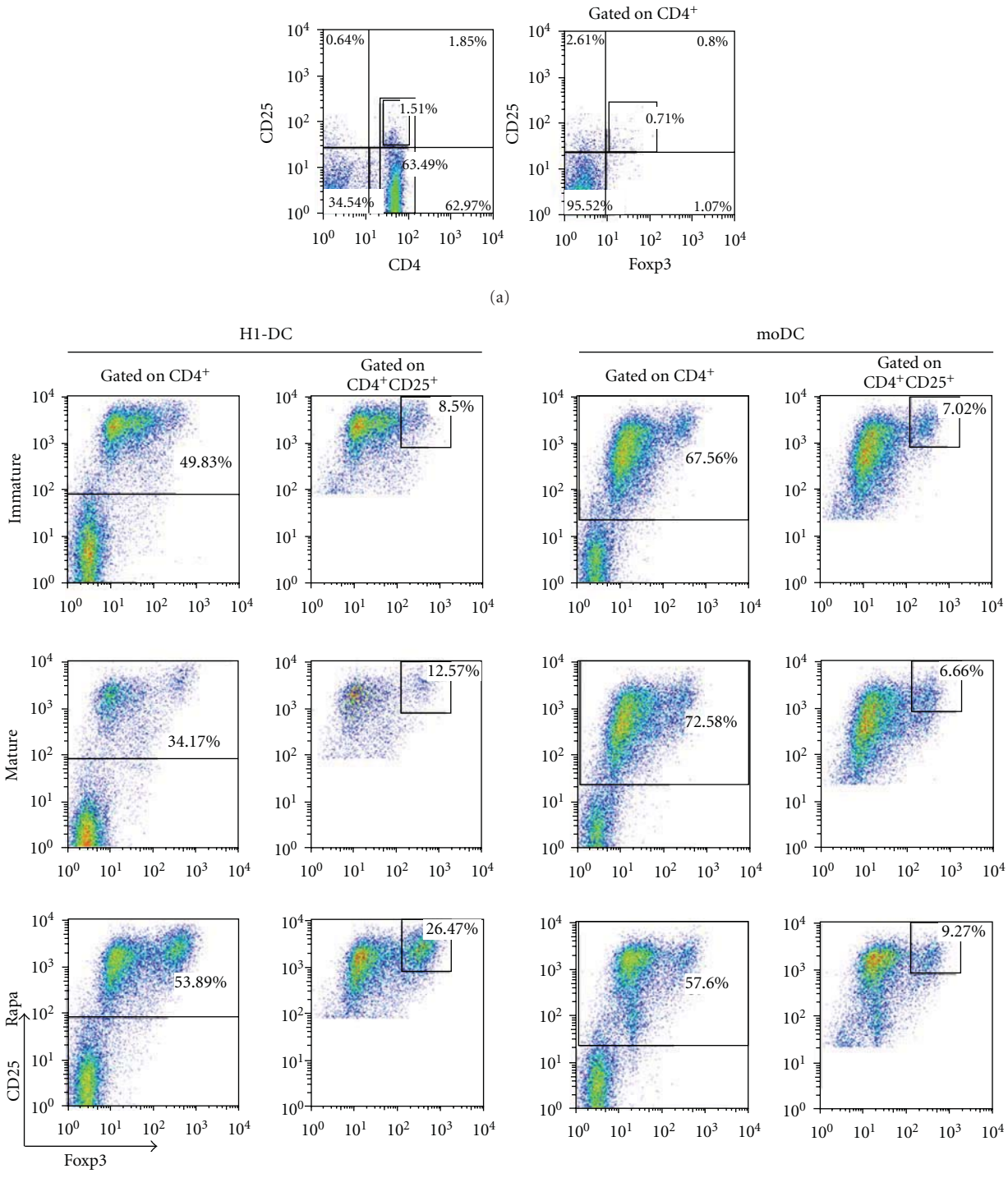


Figure 4: Enhanced capacity of rapamycin-treated H1-DC to promote Treg induction. (a) The starting population of naive $CD4^+$ T cells was analysed by flow cytometry for the expression of CD25 and Foxp3, markers associated with commitment of T cells to the regulatory T cell lineage. (b) Rapamycin enhances the capacity of H1-DC to induce Treg cells compared to moDC. DCs were either untreated, matured with the maturation cocktail or treated with rapamycin for 3 days prior to maturation. DCs were harvested, washed, and plated at 2×10^5 per well with 10^6 naive $CD4^+$ T cells per well of a 24-well plate to yield a ratio of DC : T cells of 1 : 5. On day 7, cocultures were stained for CD4, CD25, and Foxp3 and analysed. Dead cells were excluded from the analysis using 7-AAD staining. Data from one experiment representative of 4 independent experiments are shown.

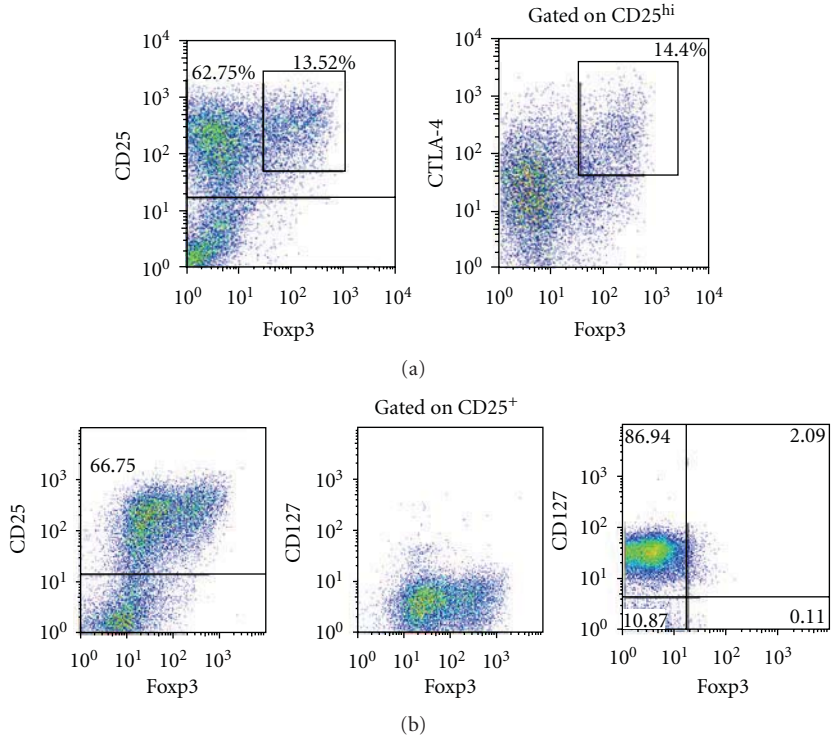


Figure 5: Phenotypic characterisation of putative CD25^{hi} Foxp3^{+} Treg cells from cocultures of DC and naïve T cells. The CD25^{hi} Foxp3^{+} population expresses CTLA-4 (a) but lacks expression of CD127 in comparison with control Foxp3^{-} T cells (b), consistent with the reported phenotype of *bona fide* Treg cells.

the fulcrum on which the balance between tolerance and immunity has been shown to pivot. For instance, the administration to mice of CD40$^{-/-}$ DC laden with foreign antigen was shown to induce profound antigen-specific tolerance upon subsequent immunization, results which are consistent with the induction of a repertoire of Treg cells [26]. Furthermore, in mice receiving foreign antigen chemically conjugated to CD205-specific mAb as a way of delivering antigen to DC in the steady state, the induction of tolerance could be abrogated in favour of systemic immunity by the concomitant administration of agonistic antibodies specific for CD40 [4].

It is the central role played by CD40 in a transplantation setting that underlies the success of strategies for intervening in allograft rejection based on the blockade of CD40-CD154 interactions [27]. Although unanticipated complications associated with the use of mAb specific for CD154 have hindered the application of such a strategy to the clinic; the long-term acceptance of allografts in mice was found to bear the distinctive features of regulation, including linked suppression and infectious tolerance [28, 29]. A conditioning regime that limits the delivery of CD40 signalling by donor DC might, therefore, be anticipated to predispose recipients towards tolerance based on the generation of a repertoire of alloantigen-specific Treg cells. Indeed, the level of expression of CD40 has been shown to be critical in determining the outcome of antigen recognition in a model of *Leishmania donovani* infection, high levels of expression inducing effector T cells, low levels favouring polarisation towards a Treg phenotype [30]. Furthermore, blockade of the CD40-CD154 axis in combination with rapamycin was shown to achieve tolerance even across a xenogeneic barrier [31]. Although the mechanisms involved were not specifically elucidated in this study, it may be significant in our own experiments that PD-L1 expression by H1-DC was unaffected by rapamycin treatment, being upregulated in response to maturation stimuli, irrespective of prior exposure to the compound. Given the essential role described for PD-L1 in polarisation of naïve T cells towards a Treg cell phenotype [23], it is tempting to speculate that it is by altering the critical balance between costimulatory and inhibitory signals delivered by H1-DC, that rapamycin treatment strongly favours a tolerogenic profile.

5. Conclusions

We have demonstrated previously that hESC may be differentiated into populations of immunogenic DC whose properties may be exploited in regimes of cancer immunotherapy. Here, we extend this paradigm by showing that our protocols are fully compliant with the use of rapamycin which favours a protolerogenic phenotype of the resulting DC. Our results pave the way for the future use of rapamycin-conditioned hESC-derived DC in regimes for the induction of tolerance, as a prelude to CRT.

Conflict of Interests

A. Reddy and K. P. Nishimoto declare a potential financial conflict of interests as employees of Geron Corporation.

Acknowledgments

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Review Article

Human Blood-Vessel-Derived Stem Cells for Tissue Repair and Regeneration

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Multipotent stem/progenitor cells with similar developmental potentials have been independently identified from diverse human tissue/organ cultures. The increasing recognition of the vascular/perivascular origin of mesenchymal precursors suggested blood vessels being a systemic source of adult stem/progenitor cells. Our group and other laboratories recently isolated multiple stem/progenitor cell subsets from blood vessels of adult human tissues. Each of the three structural layers of blood vessels: intima, media, and adventitia has been found to include at least one precursor population, that is, myogenic endothelial cells (MECs), pericytes, and adventitial cells (ACs), respectively. MECs and pericytes efficiently regenerate myofibers in injured and dystrophic skeletal muscles as well as improve cardiac function after myocardial infarction. The applications of ACs in vascular remodeling and angiogenesis/vasculogenesis have been examined. Our recent finding that MECs and pericytes can be purified from cryogenically banked human primary muscle cell culture further indicates their potential applications in personalized regenerative medicine.

1. Introduction

Multipotent adult stem/progenitor cells have been identified in nearly all human organs and extensively investigated to date [1–6]. For example, the human bone marrow (BM) functions as a diverse reservoir for several stem/progenitor cell populations, including hematopoietic stem cells (HSCs), multipotent mesenchymal stem/stromal cells (MSCs), and endothelial progenitor cells (EPCs) [7, 8]. The human skeletal muscle contains committed myogenic precursors, skeletal myoblasts, primitive myogenic stem cells, and satellite cells [1]. On the other hand, the human fat harbors adipose progenitor cells and adipose-derived stem cells (ADSCs) which are functionally and phenotypically resembling the BM-MSCs [9, 10].

However, many of these stem/progenitor cell populations have been identified retrospectively in *ex vivo* tissue and organ cultures, such as multipotent adult progenitor cells (MAPCs), mesoangioblasts, and MSCs [10–13]. This obscures the origin and the native identity of these stem/progenitor cells *in vivo*. In order to fully utilize the developmental potentials and therapeutic potencies of the adult stem/progenitor cells, it is deemed important to understand whether these different populations of adult stem/progenitor cells are developmentally and/or hierarchically connected. Due to the phenotypical and functional similarities of several of these stem/progenitor cell populations, we hypothesized the existence of a common source in the human body. In this paper, we will discuss this newly emerged concept: blood-vessel-derived mesodermal

stem/progenitor cells and their therapeutic applications in the futuristic personalized regenerative medicine.

2. Stem/Progenitor Cell-Based Therapy

The use of stem/progenitor cells for cell-based therapy is deemed promising owing to not only their high proliferative capacity and multilineage differentiation potential but also their functionality in secretion of trophic molecules and antienvironmental stress to promote cell survival. Specifically, adult stem/progenitor cells from an abundant autologous origin have the additional advantages over other stem cell types: high availability, no immunogenicity, low tumorigenicity, and with no associated ethical issues [14]. To efficiently repair/regenerate defective organs, the donor stem/progenitor cells are expected to possess desirable therapeutic properties, for example, minimal side effects, ability to integrate into host tissue, differentiation into desired cell lineages, paracrine effect, immunomodulation, regulation of tissue remodeling, and activation of endogenous repair/regeneration mechanisms [15–18].

The therapeutic potentials of adult stem/progenitor cells have been extensively investigated in the preclinical and clinical studies. For instance, MSCs have been widely explored in a number of phase I, II, and phase III clinical trials for several indications such as cardiovascular disease, stroke, multiple sclerosis/amyotrophic lateral sclerosis, Crohn's disease, and osteogenesis imperfecta [19–21]. Many other recent and ongoing human studies have chosen bone marrow mononuclear cells (BM-MNCs), skeletal myoblasts, EPCs, or ADSCs as their donor cell populations [16, 22–24]. Unfortunately, in some cases, donor stem/progenitor cells initially demonstrated encouraging outcome but later on showed uneven success in clinical trials, especially in the treatment of cardiovascular disease [16, 23]. Nevertheless, BM-MSCs, ADSCs, and other MSC-like mesodermal stem/progenitor cells still hold great promise for tissue repair and regeneration because of their multipotency, autologous availability, immune tolerance and regulation and robust paracrine secretion of trophic effectors.

3. Blood Vessels as a Source of Stem/Progenitor Cells

The evidence of the vascular wall as a source of stem/progenitor cells has been demonstrated in the emerging hematopoietic system in the early development of human embryo and fetus [25]. The hematopoietic cells emerge in close vicinity to vascular endothelial cells in both intra and extraembryonic hematopoiesis. Specifically, a population of angiohematopoietic stem cells expressing flk-1 and angiotensin-converting enzyme (ACE) migrates from the para-aortic splanchnopleura into the ventral part of the aorta, where they give rise to hemogenic endothelial cells and subsequently hematopoietic cells [25]. Furthermore, hematopoietic cells also appear to develop from endothelium in the embryonic liver and fetal bone marrow, albeit at a much lower frequency.

The similarity between many human mesodermal stem/progenitor cell populations that are retrospectively discovered in tissue/organ cultures has made us hypothesize that there exists a common, systemic source of stem/progenitor cells in the adult human body [26]. Blood vessels, which consist of three structural layers: tunica intima, tunica media, and tunica adventitia distribute throughout nearly all human organs where adult mesodermal stem/progenitor cells can be identified [27]. Apart from tunica intima, in which the subendothelial zone has been suggested as one of the sources of endothelial progenitor cells (EPCs), the possibility that other structural layers of the blood vessels harbor stem/progenitor cells was recently demonstrated in tunicae media and adventitia [26, 28, 29].

Perivascular cells, often known as vascular mural cells, are the cells that surround tunica intima of the blood vessels and constitute a major component of the vascular wall [27, 30]. Microvascular pericytes, though similar but distinct from vascular smooth muscle cells, closely encircle endothelial cells in capillaries and microvessels (arterioles and venules) in most human tissues [30–32]. Pericytes are commonly regarded as a structural element of blood vessels that regulate vascular contractility and support the stability of blood vessels [33–35]. Intimate interactions between pericytes and endothelial cells tightly relate to the vascular growth, maturation, and remodeling [30, 34–36]. In addition, pericytes have been implicated in a number of pathological conditions, making them the potential targets for therapeutic interventions [36, 37].

Historically, the outmost layer of arteries and veins, the tunica adventitia, has been considered as a mere structural bystander constituted by collagen and fibroblasts. A number of recent findings have led to the reevaluation of the active role of tunica adventitia in cell trafficking, immune response mediation, and vascular remodeling [38]. The importance of the tunica adventitia in regenerative vascular medicine is highlighted by the numerous reports describing the presence of multipotent progenitors within the wall of arteries and veins [29, 39–42]. In a vascular remodeling setting following an injury, it has been shown that adventitial cells (ACs) start a process of proliferation, migration, into the tunicae media and intima, and differentiation into smooth muscle cells [41, 43, 44].

We have previously investigated whether the blood vessels contribute to stem/progenitor cell lineages other than hematopoietic cells. Through immunohistochemistry and flow cytometry analyses, we documented evidence showing the existence of rare subsets of human blood vessel derived stem cells (hBVSCs) in multiple human tissues, including skeletal muscle, fat, and placenta.

4. The Three Musketeers: Myogenic Endothelial Cells, Pericytes, and Adventitial Cells

We and other laboratories recently reported the existence of three distinct subpopulations of mesodermal precursors within the blood vessel walls through anatomic and molecular identifications. At least one precursor subset, that is,

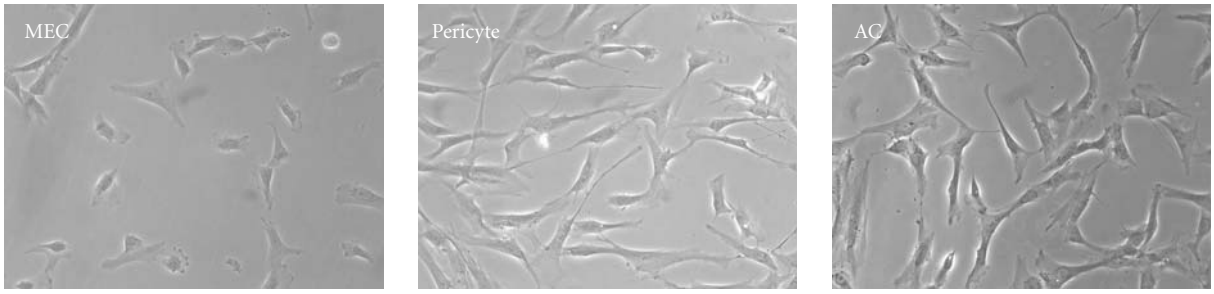

Figure 1: Distinct morphology of the three purified subpopulations of human blood-vessel-derived stem cells (hBVSCs) in culture: myogenic endothelial cell (MEC), pericyte, and adventitial cell (AC) (100x).

myogenic endothelial cells (MECs), pericytes, and adventitial cells (ACs) (Figure 1), was contained in each of the three tunicae of blood vessels: intima, media, and adventitia, respectively [26, 40, 45–47].

Myogenic endothelial cells (MECs), which suggest a developmental relationship between endothelial and myogenic cells, have been identified within the vasculatures of human skeletal muscle at a very low frequency (<0.5%) [45]. MECs uniquely coexpressed myogenic (CD56) and endothelial cell markers (CD34 and CD144) and were identified by immunohistochemistry and flow cytometry. Using fluorescent-activated cell sorting (FACS), we were able to purify these cells to homogeny [45]. MECs (CD56+CD34+CD144+CD45−) proliferate long term, retain a normal karyotype, survive better under oxidative stress than CD56+ myogenic cells, and are not tumorigenic [45]. Cultured MECs displayed mesenchymal developmental capacities, including myogenesis, osteogenesis, chondrogenesis, and adipogenesis, under appropriate inductive conditions *in vitro* [45]. Their stem cell characteristics were further confirmed by the expression of classic MSC markers and the mesodermal differentiations in culture from clonally derived MECs (Zheng et al., in revision). However, it is not clear yet whether MECs give rise to authentic MSCs in culture. Based on the phenotypic and functional similarities between MECs and the previously reported murine-muscle-derived stem cells (mMDSCs), we believe that MECs represent the human counterpart of mMDSCs. In addition to MECs, which are primarily located in the intimal compartment of the blood vessels within human skeletal muscle, other distinct subsets of multipotent stem/progenitor cells were recently found in the perivascular compartment of the vasculature (tunicae media and adventitia), not only within the skeletal muscle but throughout the human body [26, 40, 46, 47].

Though microvascular pericytes have long been considered to possess mesenchymal plasticity, the lack of a proper purification method undermined the characterization of this potential precursor population [48–50]. Recently, our group identified the native expression of classic MSC markers by microvascular pericytes and further discovered a collection of cell surface markers, that is, CD146+CD34−CD45−CD56− that enabled us to prospectively isolate homogenous pericyte populations by FACS from multiple human organs [26]. Purified pericytes proliferate long term and express CD146, NG2, PDGFR-β, alkaline phosphatase (ALP), and α-smooth muscle actin (α-SMA), with the absence of endothelial cell markers, including von Willebrand factor (vWF), CD31, CD34, and CD144 [26, 46]. These cells can be efficiently expanded *in vitro* and demonstrate robust mesodermal developmental potentials, at the clonal level, by differentiating into osteogenic, chondrogenic, adipogenic, and myogenic lineages under suitable inductive conditions *in vitro* [26]. The MSC characteristics of these CD146+CD34−CD45−CD56− pericytes can be maintained for the long-term in culture. Their myogenic and osteogenic capacities were further displayed by transplantation into the muscle pocket of immunodeficient mice. To date, no tumorigenicity of pericytes has been reported [26, 46]. We hypothesized that these cells are one of the developmental origins of MSCs [26].

In the past, fibroblasts that are capable of differentiating into myofibroblasts/smooth muscle cells (SMCs) following vascular injury have been regarded as the primary cellular component of the tunica adventitia [38, 51]. Recent studies have gradually uncovered the true identity of the cells residing in this outmost layer of the blood vessels [42]. Cells located at the interface between the tunica adventitia and media, the so-called "vasculogenic zone", have been identified as CD34+CD31− and described as progenitors endowed with the ability to differentiate into endothelial cells and participate in the blood vessel formation as well as the pathogenesis of atherosclerosis [29, 41, 51]. The concept that the tunica adventitia functions as a reservoir for stem/progenitor cells is highlighted by a recent study in which a population of CD34+CD31− progenitors residing in human saphenous vein was described [40]. These cells were localized in the tunica adventitia *in situ* and could be isolated and expanded at the clonal level *in vitro*. Our study also showed the stem cell characteristics of a CD34+CD31−CD146− nonpericyte perivascular cell population in the vasculature of human adipose and other tissues [47]. The FACS-purified CD34+CD31−CD146− population exhibited the phenotype and developmental potentials of MSCs. Furthermore, immunohistochemistry revealed that CD34+CD31−CD146− cells reside in tunica adventitia of blood vessels in multiple human organs and similar to pericytes, natively express classic MSC surface markers.

5. Myogenic Endothelial Cells for Skeletal Muscle Regeneration and Cardiac Repair

The therapeutic potential of MECs was first tested in the severe combined immunodeficiency (SCID) mouse model of cardiotoxin-injured skeletal muscle [45]. MECs (CD56+CD34+CD144+CD45−) isolated from fresh human muscle biopsies were shown to regenerate skeletal myofibers more effectively than skeletal myoblasts (SkMs, CD56+/CD34−/CD144−/CD45−), endothelial cells (ECs, CD34+/CD144+/CD56−/CD45−), and unpurified primary muscle cells, with or without *in vitro* expansion. Injection of MECs into skeletal muscle of mdx/SCID mice, a disease model of Duchenne muscular dystrophy (DMD), also displayed an efficient regeneration of human skeletal myofibers, indicated by positive staining of both dystrophin and human lamin A/C [45]. Furthermore, after expansion in culture, clonal MECs exhibited robust chondrogenesis and osteogenesis *in vivo* after implantation into the hindlimb muscle pocket of SCID mice (Zheng et al., in revision). These results suggest the feasibility of utilizing MECs to treat various musculoskeletal disorders.

The application of MECs in the cardiovascular disease was examined in an immune-deficient mouse model of acute myocardial infarction (AMI) [52]. Myocardial infarction was induced in SCID-nonobese diabetic (NOD/SCID) mice by ligation of left anterior descending coronary artery (LAD), and cells were immediately injected into the ischemic myocardium. Cardiac function was assessed by echocardiography. The results demonstrated a significant improvement in cardiac contractility after intramyocardial injection of MECs when compared with injections of SkMs and ECs [52]. Transplanted MECs not only displayed robust engraftment within the infarcted myocardium but also stimulated angiogenesis, attenuated scar tissue formation, and promoted proliferation and survival of endogenous cardiomyocytes more effectively than the other two cell types [52]. This is presumably attributed to higher secretion of vascular endothelial growth factor (VEGF), a potent angiogenic factor, by MECs under hypoxia. Similar to mMDSCs, MECs regenerated significantly more fast-skeletal MHC-positive myofibers in the ischemic heart. A minor fraction of engrafted MECs differentiated into and/or fused with cardiomyocytes by expressing cardiomyocyte markers, cardiac troponin-T, and -I [52]. These findings suggest that MECs represent a promising stem cell subset within human skeletal muscle, an accessible autologous tissue source, for cardiac repair and regeneration.

6. Pericytes for Tissue Repair and Regeneration

The application of pericytes in regenerative medicine was first examined in SCID mouse models of injured and dystrophic skeletal muscle [26]. Microvascular pericytes freshly sorted by FACS from human skeletal muscle were injected into the hindlimb muscles of SCID-nonobese diabetic (NOD/SCID) mice that had been injured by intramuscular injection of cardiotoxin. The presence of regenerating human spectrin-positive myofibers was detected by immunohistochemistry and confirmed by fluorescence *in situ* hybridization of central human nuclei [26]. Quantification showed that freshly sorted or long-term-cultured pericytes produced more human myofibers than purified SkMs and unpurified muscle cells, ruling out the possibility that the myogenic potential observed in pericytes resulted from a consequence of the contamination by myoblasts. Most importantly, this myogenic potential can be generalized to pericytes residing in nonmuscle tissues. Placenta-, white adipose tissue-, and pancreas-derived pericytes also exhibited high myogenic potential in culture and *in vivo*, yielding human dystrophin- or spectrin-positive myofibers upon transplanted into mdx/SCID or cardiotoxin-treated NOD/SCID mouse muscles [26]. Dellavalle et al. also showed that pericytes sorted from healthy and dystrophic human skeletal muscles by ALP expression regenerate human myofibers in muscles of dystrophic immunodeficient mice [46]. Very recently, the same group demonstrated that pericytes residing in the postnatal skeletal muscle natively participate in the skeletal myofiber development, and the satellite cell compartment and further contribute to the regeneration of injured/dystrophic skeletal muscle, using a transgenic label of inducible Alkaline Phosphatase CreERT2 [53]. These results indicate that pericytes play a role in muscle ontogeny and are endowed with robust myogenic potential that can be applied to skeletal muscle repair and regeneration.

With their inherent functions in the vascular physiology, pericytes seem to match the criteria of the ideal donor cell population for cardiovascular repair. Recently, we investigated the hypothesis that transplantation of cultured pericytes benefits the ischemic heart [54]. Briefly, long-term-cultured human muscle pericytes were injected into acutely infarcted hearts of NOD/SCID mice. Echocardiography revealed a significant improvement of cardiac function in pericyte-injected hearts. Pericytes exhibited cardioprotective effects such as promotion of angiogenesis, reduction of scar formation, and inhibition of chronic inflammation (Chen et al., submitted). Moreover, a human pericyte-based small-diameter vascular graft has been successfully engineered with high patency after long-term transplantation [55]. These data suggest that pericytes serve as a promising donor cell source for stem-cell-based cardiovascular therapy.

7. Adventitial Cells Exhibit Multilineage Potential for Blood Vessel and Tissue Regeneration

The potential application of ACs in the clinical settings has thus far been focused on the cardiovascular repair and regeneration. Campagnolo et al. recently demonstrated that CD34+CD31− ACs interact with endothelial cells and promote the formation and stabilization of the capillary-like structures [40]. Most importantly, injection of adventitial in a hindlimb ischemia mouse model cells showed a significant proangiogenic effect as demonstrated by a full blood flow recovery as early as 7 days after-injection, indicating the

therapeutic potential of ACs in angiogenesis/vasculogenesis [40]. Very recently, Katare et al. further reported that transplantation of ACs improves the repair of infarcted hearts through angiogenesis involving microRNA-132 [56]. Together, these data indicate the therapeutic potential of ACs in ischemic tissue repair. Interestingly, the use of ACs derived from the umbilical artery, in conjunction with SMCs of the same source, in tissue-engineered small-caliber vessel constructs resulted in superior mechanical properties than the same constructs using cells derived from the umbilical vein, suggesting that ACs and SMCs originating from different sources may lead to distinct tissue characteristics in regenerative medicine [57].

The application of ACs is not limited to postnatal angiogenesis/vasculogenesis or cardiovascular repair. Though the myogenic potential of ACs remains to be determined, we and other authors have demonstrated that adventitial cells, regardless of their tissue of origin, display features typical of MSCs [40, 42, 47]. The ability of ACs to differentiate into major mesodermal cell lineages, including osteogenesis and chondrogenesis, suggests a likely contribution of these cells to the formation/regeneration of surrounding mesenchymal tissues after injury and further expands the potential clinical applications of this unique hBVSC subset into the field of musculoskeletal diseases [47]. Altogether, these reports suggest that rather than a passive constituent of the vascular wall, the adventitia is a dynamic reservoir of stem/progenitor cells that participate in vascular remodeling and regeneration of surrounding tissues.

8. Cryopreserved Human Skeletal Muscle Culture as a Source of Myogenic hBVSCs

Despite the successful isolations of various lineages of hBVSCs from fresh tissue biopsies, the prolonged procedure of FACS purification and subsequent culture expansion complicate the usage of these promising adult stem/progenitor cell populations. Specifically, this represents a major hurdle to the clinical translation of hBVSCs. We recently reported that long-term cryopreserved human primary skeletal muscle cell cultures (cryo-hPSMCs) include diverse cell populations; two subpopulations of hBVSCs, myogenic endothelial cells (MECs) and pericytes/perivascular stem cells (PSCs), can be purified from cryo-hPSMCs by FACS [58]. Due to the frequent loss of CD34 and CD144 expression in culture, another endothelial cell marker, UEA-1 receptor (UEA-1R), was used for purification in this case. Cryo-MECs express all three cell lineage markers (CD56+UEA-1R+CD146+CD45−), and cryo-PSCs express only CD146 (CD146+CD56−UEA-1R−CD45−). The preservation of the myogenic capacity of cryo-MECs and cryo-PSCs was demonstrated by the regeneration of human spectrin-positive myofibers after injections of newly sorted cells (with or without culture expansion) into the cardiotoxin-injured skeletal muscles of immunodeficient mice [58]. MECs showed the highest regenerative capacity in the injured mouse muscles, better than the unsorted muscle cells, CD56+ myoblasts, and cryo-PSCs. Cryo-PSCs remained myogenically superior to the unsorted cells and myoblasts. These findings suggest the feasibility to further purify subpopulations of the hBVSCs from banked human skeletal muscle cells, highlighting a new approach to extract therapeutic stem/progenitor cells from a cryogenically banked source for personalized regenerative medicine.

9. Ontogeny and Heterogeneity, Innate Factors Affecting the Therapeutic Potency of Stem/Progenitor Cells?

MSCs represent a highly heterogeneous population of widely studied but poorly defined multipotent stem/progenitor cells [59]. Indeed, Guilak et al. have shown that only 52% of the clones obtained from cultured ADSCs retain the ability to differentiate into two or more mesodermal cell lineages [60]. The presence of subsets of cells with limited or no differentiation potential within the conventional stromal cultures may therefore hamper the clinical efficacy of these promising stem/progenitor cells. This raised the possibility that clinical trials based on the transplantations of the total SVF or unfractionated MNCs often showed uneven success because of the variable frequency of progenitors within the total stroma [16, 23]. Additionally, mesenchymal progenitors have mostly been selected by their plastic adherence and expanded long-term in culture with reagents originated from animals, which ultimately limits their clinical use due to FDA regulations.

The identification of pericytes as, at least in part, the ancestors of MSCs has represented a breakthrough in the search for the true identity of MSCs [26]. Though this has raised the possibility that most MSCs, if not all, are derived from pericytes, other subsets of stem/progenitor cells residing in the blood vessel walls may constitute part of the MSC entity as described above [61]. The question of whether all MSCs originate from microvascular pericytes is partially answered by Tormin et al., who reported that, in bone marrow, MSCs can be derived not only from the subendothelial sinusoidal CD146+ cells, as previously demonstrated by Sacchetti et al., but also from the bone-lining CD146− cells [62, 63]. Furthermore, using the genetic lineage tracing, Feng et al. recently reported MSCs from both pericyte and nonpericyte origins differentiate into odontoblasts and participate in tooth growth and repair in mice, suggesting that the pericyte contribution to MSCs may vary in different tissues and possibly depend on the density of the local vascularity [64].

Another good example that different subsets of stem/progenitor cells contributing to the MSC entity is from the observation of the concurrent presence of two distinct subpopulations of perivascular multipotent progenitor cells, namely, CD34−CD31−CD146+ microvascular pericytes and CD34+CD31−CD146− adventitial cells, in the adipose tissue [39, 47]. These two subpopulations of hBVSCs share *in situ* and *in vitro* expression of typical MSC surface markers, CD44, CD73, CD90, and CD105, but they are phenotypically and anatomically distinct [39, 47]. Pericytes are indeed defined as CD45−CD34−CD31−CD146+ cells

Table 1: Comparison of hBVSC subpopulations and bone marrow MSCs.

Native location	MEC Intima	Pericyte Media	AC Adventitia	BM-MSC Bone marrow
Cell surface marker profile for cell sorting	CD34+ CD45− CD56+ CD144+	CD34− CD45− CD56− CD146+	CD31− CD34+ CD45− CD146−	N/A
Classic MSC marker expression in culture	CD29+ CD44+ CD90+ CD105+	CD44+ CD73+ CD90+ CD105+	CD44+ CD73+ CD90+ CD105+	CD29+ CD44+ CD73+ CD90+ CD105+
Differentiation *in vitro*	Osteogenic (+) Chondrogenic (+) Adipogenic (ND) Myogenic (+)	Osteogenic (+) Chondrogenic (+) Adipogenic (+) Myogenic (+)	Osteogenic (+) Chondrogenic (+) Adipogenic (+) Myogenic (ND)	Osteogenic (+) Chondrogenic (+) Adipogenic (+) Myogenic (+)
Differentiation *in vivo*	Myogenesis	Myogenesis Osteogenesis	Vasculogenesis	Osteogenesis Chondrogenesis Adipogenesis Myogenesis Cardiomyogenesis Vasculogenesis
Potential clinical application	Skeletal musclere-pair/regeneration; Cardiac repair	Skeletal muscle repair/regeneration; Vascular repair/regeneration	Vascular repair/regeneration; Cardiac repair	Bone repair; Cartilage repair; Tendon/ligament repair; skeletal muscle repair; Vascular repair; Cardiac repair; Wound healing; Immunoregulation

MEC: myogenic endothelial cell; AC: adventitial cell; BM-MSC: bone marrow mesenchymal stem/stromal cells; N/A: not available; ND: not determined.

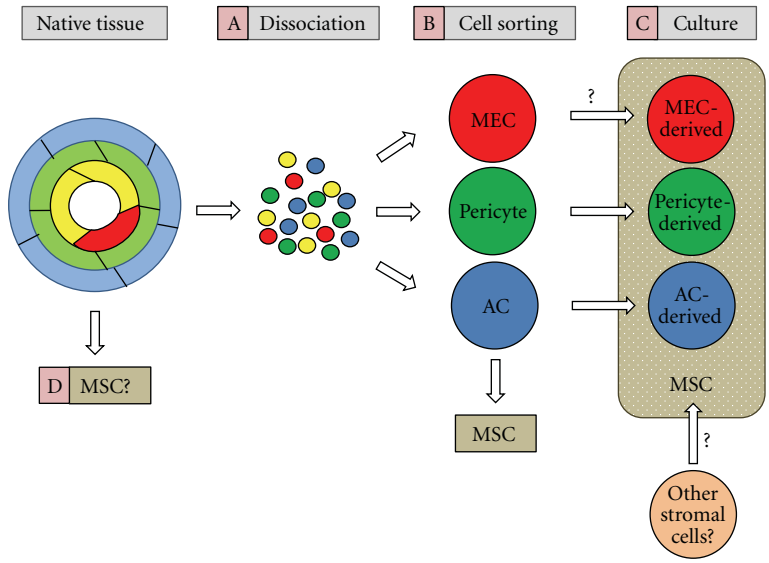

Figure 2: Schematic depiction of hBVSCs at the origin of mesenchymal stem/stromal cells (MSCs). (A) hBVSCs, including myogenic endothelial cells (MECs, red), pericytes (green), and adventitial cells (AC, blue), are dissociated from fresh muscle biopsy and separated from endothelial cells (yellow) and other cell types. (B) Dissociated cells are purified to homogeneity by fluorescence-activated cell sorting (FACS) and newly sorted MECs, pericytes, and ACs already exhibit multilineage developmental potentials. (C) FACS-purified pericytes, ACs, and possibly MECs give rise to authentic MSCs in long-term culture. (D) Nevertheless, whether native hBVSCs serve as a source of MSCs *in situ* and participate in tissue repair and regeneration remains an open question.

tightly surrounding microvessels, whereas CD45−CD34+ CD31−CD146− adventitial cells are located in the outmost layer of larger blood vessels in the human adipose, a literally unlimited tissue source. Both subpopulations of cells are endowed with multilineage mesenchymal differentiation capacity at the clonal level and represent ideal candidates for the treatment of musculoskeletal and vascular diseases [39, 40, 65]. Above all, both subpopulations are abundant in lipoaspirate, and with the definitive phenotypes of each hBVSC fraction, we can readily enrich both fractions by cell sorting in order to improve their therapeutic efficiency and safety [47]. Table 1 summarizes the basic characteristics of hBVSC subpopulations and BM-MSCs as well as their potential clinical applications.

Nevertheless, while all, or at least part of, the three hBVSC subpopulations contributing to the MSC entity in culture are gradually becoming an accepted notion; whether the multilineage potentials are natively present within hBVSC subsets and subsequently responsive to pathological stimulations *in vivo* remains to be investigated (Figure 2). Ultimately, the current therapeutic strategy based on the transplantation of unfractionated stromal cells may in the near future be replaced by the purification, combination, and direct reinfusion of the distinct subsets of hBVSCs, devoid of cells with none or a restricted regenerative potential.

10. Conclusion

In this paper, we discussed the newly emerged concept of blood vessels as a systemic source of adult stem/progenitor cells. Three subpopulations of hBVSCs, that is, MECs, pericytes, and ACs have been respectively isolated from different layers of the blood vessels and examined in detail for their developmental capacities and therapeutic potentials in tissue repair and regeneration. Besides, the fresh tissue biopsy, the cryogenically banked primary human skeletal muscle culture was shown to be an alternative source of myogenic subsets of hBVSCs, shedding new lights on the future of the personalized regenerative medicine. Finally, the identification of the precise phenotypes of distinct hBVSC subpopulations represents not only an important milestone for understanding the nature and origin of MSCs but also a crucial step toward the improvement of stem/progenitor cell-based therapies.

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Research Article

Thin-Layer Hydroxyapatite Deposition on a Nanofiber Surface Stimulates Mesenchymal Stem Cell Proliferation and Their Differentiation into Osteoblasts

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Pulsed laser deposition was proved as a suitable method for hydroxyapatite (HA) coating of coaxial poly-ε-caprolactone/polyvinylalcohol (PCL/PVA) nanofibers. The fibrous morphology of PCL/PVA nanofibers was preserved, if the nanofiber scaffold was coated with thin layers of HA (200 nm and 400 nm). Increasing thickness of HA, however, resulted in a gradual loss of fibrous character. In addition, biomechanical properties were improved after HA deposition on PCL/PVA nanofibers as the value of Young's moduli of elasticity significantly increased. Clearly, thin-layer hydroxyapatite deposition on a nanofiber surface stimulated mesenchymal stem cell viability and their differentiation into osteoblasts. The optimal depth of HA was 800 nm.

1. Introduction

Stem cells have undoubtedly been at the center of interest and the object of intensive study in the last decade [1–3]. Clearly, multiple stem cells have, under suitable conditions, the potential to differentiate cell lineages and thus play a key role in tissue engineering and regenerative medicine. Several sources of stem cells have been described, including muscle [4, 5], synovium [6], periosteum [7], and bone marrow [8, 9]. Stem cells can be also isolated from adipose tissue, which can be obtained under local anesthesia with minimal discomfort [10, 11]. However, bone marrow is most widely utilized as a source of autologous MSCs. These cells can differentiate into osteogenic lineages when cultured in the presence of dexamethasone, ascorbic acid, and β-glycerophosphate [12] and potentially used for treating large bone defects. Autologous stem cells as the source of donor cells have numerous advantages for regenerative medicine. These include low donor site morbidity, a diminished or absent immune response, and a high proliferative potential [1, 2].

In fact, other growth factors such as transforming growth factor (TGF-β), insulin-like growth factor (IGF), and basic fibroblast growth factor (bFGF) have been described as stimulators of MSCs proliferation and osteogenic differentiation [13, 14]. The process of stem cell differentiation is undoubtedly complicated and time and concentration dependent. Thus, the main challenge of the successful application of MSCs in regenerative medicine seems to be the regulated release of a suitable concentration of differentiation factors, particularly under *in vivo* conditions. This is among the goals

of tissue engineering as a multidisciplinary field focusing on the reconstruction of biological tissues. Cells, especially autologous cells, and smart (functionalized) scaffolds enriched with growth factors, preferentially serving as a controlled delivery device, are fundamental components in the engineering of novel cell proliferation and differentiation systems [2].

Surface modification is one of the essential steps in constructing artificial cell-seeded systems. HA, which is similar to the apatite of living bone, can be used as a suitable material for improving cell proliferation and differentiation into osteoblasts. HA has been used in medicine and dentistry for over 20 years due to its biocompatibility and osteoconductivity and its excellent chemical and biological affinity with bone tissue [15, 16]. HA coatings of bone implants enable fast bony adaptation and reduced healing time [17–19]. There are a number of techniques used to produce thin HA films. Plasma-sprayed HA coatings, where HA is bound mechanically, have limited chemical bonding, and cracks, pores, and other impurities limit their mechanical strength in contact with a substrate and the stability of the layer [20, 21]. Another coating technique is ion beam sputtering, producing an amorphous coating. Subsequently, heat treatment is necessary to produce crystals [22, 23]. Very high temperatures, necessary for crystallization, are not favorable for nonmetallic materials such as polymers or bioactive molecules. Pulsed laser deposition (PLD) is mostly used as an alternative technique of HA coating [24, 25]. PLD employs an intense laser beam for material evaporation. Subsequent condensation on a mat can create a very thin layer (depth of several atoms only). The material surface properties are, consequently, directly dependent on the deposition conditions.

Aside from chemical and surface charge modification, the surface's physical properties are also vital for successful cell seeding on scaffolds. Nanotechnology is the term used to cover the design, construction, and utilization of functional structures with at least one characteristic dimension measured in nanometers and brings a new chance to stem cells research and development [26–28].

Electrospun nanofibers are novel materials characterized by an enormous surface to volume ratio, high porosity, and a structure resembling that of the extracellular matrix, thus facilitating their use in a broad range of applications [29, 30]. These properties predestined the use of nanofibers in various tissue engineering applications. In addition, nanofibers can also serve as drug delivery systems. Nanofibers have been utilized for the delivery of both water soluble and water insoluble substances [31, 32]. Due to their enormous surface area, nanofibers enable the adhesion of diverse bioactive agents, such as growth factors [33], enzymes [34], or nucleic acids [35]. The release kinetics of the content is determined by the form of the interaction between the fibers and the adhered drug. If the drug is noncovalently attached to the nanofiber surface, the interaction is weak, and a quick burst release occurs. For nonbiodegradable materials, the diffusion rate of the drug from the fibers depends strongly on the physiochemical properties of the delivered substances, such as the molecular weight, hydrophobicity, and charge of the molecule. For biodegradable materials, the process additionally depends also on the kinetics of the material's degradation, which for rapidly degradable materials is significantly hastened [29]. Clearly, drugs dissolved or dispersed in materials from which nanofibers are produced can be quickly released. However, healing processes often require a slower release on a scale of days or even weeks. This is especially important *in vivo*.

To overcome this obstacle, the incorporation of bioactive substances in the interior of the nanofiber has been employed. This can be achieved either by blend electrospinning or by coaxial electrospinning. Blend electrospinning is a single-step method enabling the incorporation of various bioactive substance [32]. The disadvantage of the process is its limitation by the compatibility of the delivered substances with the polymer solvent. Thus, blend electrospinning is not suitable for the delivery of proteins with polymers soluble only in organic solvents. Despite these constraints, blend electrospinning is a fast and convenient method for the microencapsulation of antibiotics [36, 37], anticancer drugs [38–42], proteins [43–45], DNA [46, 47], and siRNA [48]. Recently, coaxial electrospinning was introduced as a novel method for drug delivery resulting in the production of core-shell nanofibers [49]. The nanofiber core and shell could be prepared either from the same polymer solution or from different polymer solutions, thus combining the advantages of both polymer systems. Such systems could deliver highly susceptive substances in combination with various polymer systems without altering their structure or function. Electrospun coaxial fibers have been employed for the delivery of various bioactive substances, for example, proteins [50–52], DNA [46], and siRNA [48]. In addition, further drug encapsulation in the nanofiber core, such as in liposomes, can significantly prolong drug release from the nanofiber interior.

The aim of the present study was to introduce a modern system suitable for the treatment of bone defects. This system is based on MSCs and functionalized nanofibers. The nanofibers can be modified on their surface as well as enriched in their core with different drugs that could be slowly released over the course of days or weeks.

2. Methods

2.1. Materials and Reagents. Poly-ε-caprolactone (PCL, MW 45000), FITC-dextran, MTT, glycerol 2-phosphate disodium salt hydrate, BCECF-AM, and PCR primers were purchased from Sigma-Aldrich (Germany). Polyvinylalcohol sloviol (PVA) was purchased from Novacke Chemicke Zavody (Slovak republic). Hydroxyapatite was obtained in the form of a pressed powder (Lasak, Czech Republic). Gelofusine was purchased from B. Braun Melsungen (Germany). α-MEM cultivation medium and foetal bovine serum were purchased from PAA (Austria). Double-strand-specific dye for PCR analysis, SYBR Green I, was purchased from Roche (Roche Diagnostics, Mannheim, Germany) and an RNeasy Mini Kit for RNA isolation from Qiagen (Germany).

2.2. Coaxial Electrospinning of PCL/PVA Nanofibers. A 14% (w/v) PCL solution was prepared as the shell solution by dissolving 7 g PCL in 50 mL chloroform/ethanol (8 : 2) and

stirring at room temperature. The core solution consisted of 5% PVA (v/v). The coaxial spinneret apparatus consisted of two needles placed together coaxially [53]. Two syringe pumps were used to deliver the core and shell solutions, respectively. A high-voltage power supply was used to generate voltages of up to 60 kV, and a span bond was used as the receiving plate to collect the electrospun nanofibers. The distance between the tip of the syringe needle and the collecting plate was 12 cm. All electrospinning processes were performed at room temperature with 56% humidity. In case of the release study, the core solution consisted of FITC-dextran (2 mg/mL, 10,000 MW) dissolved either in 1%, 3%, or 5% (v/v) PVA. The process was performed on the apparatus described above at room temperature with 52% humidity.

2.3. HA Coatings of Nanofibers. Prepared nanofibers were coated by HA layers of different thickness. HA [$Ca_{10}(PO_4)_6(OH)_2$] films were created by a KrF excimer laser (COMPexPro 205 F) of 248 nm wavelength, frequency 10 Hz, and energy 600 mJ. The energy density of the laser beam was 2 Jcm^{-2}. The deposition proceeded in an H_2O + Ar atmosphere at a pressure of 40 Pa. The substrate was fixed at a distance of 5 cm from the target HA material (cake of pressed powder). The substrate was at room temperature. HA films of 200 (PCL/PVA200HA), 400 (PCL/PVA400HA), and 800 nm thickness (PCL/PVA800HA) were grown. Pure PCL/PVA core-shell nanofibers were used as a control (PCL/PVA).

2.4. Characterization of the Scaffolds. The prepared nanofibrous scaffolds were characterized by scanning electron microscopy. Air-dried samples of electrospun HA-coated nanofibers were mounted on aluminum stubs and sputter-coated with a layer of gold approximately 60 nm thick using a Polaron sputter-coater (SC510, Polaron, Now Quorum Technologies Ltd.). The samples were examined in an Aquasem (Tescan) scanning electron microscope in the secondary electron mode at 15 kV.

2.5. Biomechanical Characterization of Scaffolds. Young's moduli of elasticity, ultimate stresses, and ultimate strains of the scaffolds were obtained at room temperature using a Zwick/Roell traction machine equipped with a 1 kN load cell. Because of the difficulty to produce the layer of PCL/PVA nanofibers of uniform thickness, only the samples with the same thickness of the basic layer of PCL/PVA nanofibers of pure samples as well as with the layer of HA were used for mechanical testing. Thus, the samples without layer of HA were signed as type I ($n = 4$) and with the HA layer as type II ($n = 7$). The samples themselves were thin strips of the nanofibers. The initial length of all samples was 10 mm. The width of all samples was 10 mm. The thickness of individual samples was about 60 μm. The samples were prepared according to studies [54, 55]. The template of the paper 20 × 50 mm (height × width) with the centered rectangular hole 10 × 40 mm was cut, and lines marking 10 mm wide sample strips were drawn on its top and bottom stripes. Then it was glued to the sheet of the composite, and two other strips of paper 5 × 50 mm were glued to the back faces of the top and bottom stripes. Then the individual scaffolds were cut resulting in four 20 × 10 mm stripes consisting of 10 × 10 mm sample between two 5 × 10 mm grips of paper.

The tensile test with a loading velocity of 10 mm/min was applied to the samples. The load was applied until the scaffold ruptured. Young's moduli of elasticity were determined using linear regression of the stress-strain curves at a strain of approximately 1–6% (linear region depending on the shape of the curve). The ultimate stress and the ultimate strain were determined at the start of the rupture. The stress was defined as the force divided by the initial area, and the strain was defined as the elongation of the specimen divided by its initial length. Our own software written in Python programming language was used for evaluation [56].

2.6. Isolation and Cultivation of MSCs. Blood marrow aspirates were obtained from the os ilium (*tuber coxae Ala ossis ili*) of anesthetized miniature pigs (age 6–12 months). The bone marrow blood was aspirated into a 10 mL syringe with 5 mL Dulbecco's phosphate buffered saline (PBS), 2% fetal bovine serum (FBS), and 25 IU heparin/mL connected with a bioptic needle (15 G/70 mm). Under sterile conditions, the bone marrow blood (about 20 mL) was placed into 50 mL centrifuge tubes and 5 mL of gelofusine was added. After 30 min incubation at room temperature, the blood was centrifuged at 400 ×g for 15 min. Subsequently, the layer of mononuclear cells was removed and seeded into a culture flask, then cultured at 37°C in a humidified atmosphere with 5% CO_2. α-MEM medium with Earle's Salt and L-glutamine supplemented with 10% FBS and penicillin/streptomycin (100 IU/mL and 100 μg/mL, resp.) was used as the culture medium.

2.7. MSCs Seeding on the Scaffolds. Scaffolds were cut into a round shape with a diameter of 6 mm and sterilized using ethylenoxid. Cells were seeded on the scaffolds at a density of 70 × $10^3/cm^2$ in a 96-well plate. Scaffolds with seeded MSCs were cultivated in differentiation media: α-MEM supplemented with 10% FBS, penicillin/streptomycin (100 IU/mL and 100 μg/mL, resp.), 100 nM dexamethasone, 40 μg/mL ascorbic acid-2-phosphate and 10 nM glycerol 2-phosphate disodium salt hydrate. The medium was changed every 3 days.

2.8. Cell Proliferation Analysis by the MTT Test. 50 μL of [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), and 1 mg/mL in PBS (pH 7.4) were added to 150 μL of sample medium and incubated for 4 hours at 37°C. The MTT was reduced by the mitochondrial dehydrogenase of normally metabolizing cells to purple formazan. Formazan crystals were solubilized with 100 μL of 50% N,N-dimethylformamide in 20% sodium dodecyl sulfate (SDS) at pH 4.7. The results were examined by spectrophotometry in an ELISA reader at 570 nm (reference wavelength 690 nm).

2.9. Cell Proliferation Analysis by PicoGreen. The PicoGreen assay was carried out using the Invitrogen PicoGreen assay kit (Invitrogen Ltd., Paisley, UK). The proliferation of MSCs on scaffolds was tested on days 1, 7, and 14. To process

material for the analysis of DNA content, 250 μL of cell lysis solution (0.2% v/v Triton X-100, 10 mM Tris (pH 7.0), 1 mM EDTA) was added to each well containing a scaffold sample. To prepare the cell lysate, the samples were processed through a total of three freeze/thaw cycles, scaffold sample was first frozen at −70°C and thawed at room temperature. Between each freeze/thaw cycle scaffolds were roughly vortexed. Prepared samples were stored at −70°C until analysis. To quantify cell number on scaffolds, a cell-based standard curve was prepared using samples with known cell numbers (range 100–10^6 cells). The DNA content was determined by mixing of 100 μL PicoGreen reagent and 100 μL of DNA sample. Samples were loaded in triplicate and florescence intensity was measured on a multiplate fluorescence reader (Synergy HT, λ_{ex} = 480–500 nm, λ_{em} = 520–540 nm). Measured data were used for derivation of absorbance values measured by MTT assay to cell counts on the scaffolds.

2.10. Viability of Cells Seeded on Scaffolds. For determining cell viability, live/dead staining (BCECF-AM/propidium iodide) and visualization using confocal microscopy was performed. 2′, 7′-bis(2carboxyethyl)-5(6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM, diluted 1 : 100 in medium) was added to cell-seeded scaffolds and incubated for 45 min at 37°C and 5% CO_2 for live cell detection, then rinsed with PBS (pH 7.4); propidium iodide (5 μg/mL in PBS pH 7.4) was added for 10 min, rinsed with PBS (pH 7.4) again, and visualized using a Zeiss LSM 5 DUO confocal microscope (wavelengths: BCECF-AM λ_{exc} = 488 nm and λ_{em} = 505–535 nm; propidium iodide λ_{exc} = 543 nm and λ_{em} = 630–700 nm).

2.11. Quantitative Real-Time PCR Analysis. Total RNA was extracted using an RNeasy Mini Kit according to the manufacturer's protocol. Total RNA was stored at –20°C.

The cDNA from 1 μg of total RNA was used as a template. The synthesis of cDNA was performed by a standard procedure described in our previous work [57]. Bone sialoprotein (BS) and osteocalcin (OC) mRNA expression levels were quantified by means of a LightCycler 480 (Roche Diagnostics, Mannheim, Germany) using the double-strand-specific dye SYBR Green I according to the manufacturer's protocol. Primers used were as follows: BS, sense 5′-CGA CCA AGA GAG TGT CAC-3′, antisense 5′-GCC CAT TTC TTG TAG AAG C-3′ (498 bp); OC, sense 5′-TCA ACC CCG ACT GCG ACG AG-3′, antisense 5′-TTG GAG CAG CTG GGA TGA TGG-3′ (204 bp) and beta-actin, sense 5′-AGG CCA ACC GCG AGA AGA TGA CC-3′, antisense 5′-GAA GTC CAG GGC GAC GTA GCA C-3′ (332 bp). The PCR conditions were initial denaturation at 95°C for 10 min, followed by 45 cycles of denaturation at 95°C for 15 s, annealing at 57°C for 10 s, and extension at 72°C for 20 s. The expression levels of BS and OC mRNAs were normalized using the level of beta-actin mRNA as a housekeeping gene and expressed as the ratio to actin. Student's *t*-test was used to evaluate the statistical significance of the results. Differences with P values <0.05 were considered significant.

2.12. Measurement of FITC-Dextran Release Profile. In order to study the release profile of FITC-dextran, core-shell nanofiber meshes with either 1% PVA, 3% PVA, or 5% PVA were cut into round patches and incubated with 1 mL of TBS buffer at room temperature. At specific intervals, the TBS buffer was withdrawn and replaced with fresh buffer. The time interval was determined keeping in mind the balance between the release of a detectable amount of FITC-dextran and maintenance of the sink condition. Drug release was quantified using fluorescence spectroscopy. Briefly, 200 μL of samples and blank samples were measured on a multiplate fluorescence reader (Synergy HT, λ_{ex} = 480–500 nm, λ_{em} = 520–540 nm) and background subtraction was performed. The cumulative release profile of FITC-dextran was obtained, and the half time of release was determined as the time at which the initial fluorescence intensity I_0 decreased to $I = I_0 \cdot e^{-1}$.

2.13. Statistical Analysis. Quantitative data are presented as mean ± standard deviation (SD). For *in vitro* tests, average values were determined from at least three independently prepared samples. Results were evaluated statistically using one-way analysis of variance (ANOVA) and the Student-Newman-Keuls Method. The Shapiro-Wilk's W test was used to determine the normality of the Young's moduli of elasticity, ultimate strains, and ultimate stresses. The t-test was used to determine the differences between values of mechanical parameters obtained for pure PCL/PVA scaffolds (type I) and scaffolds covered by HA (type II).

3. Results and Discussion

3.1. Scaffold Characterization. Coaxial core-shell nanofibers were prepared from PCL as a shell material and PVA as a core material. PCL has good biocompatibility and enables the successful cultivation of MSCs [58] and osteogenic cells [59]. On the other hand, PVA is a water-soluble material and has been employed as a suitable substance for the delivery of bioactive compounds from the nanofiber core [60]. To improve the surface parameters for MSCs seeding, coaxial nanofibers were further functionalized by pulsed laser deposition of HA. Thin layers of 200, 400, or 800 nm thickness were deposited onto the nanofiber surface. HA deposition clearly modified the nanofiber surface and significantly influenced the surface properties. Scanning electron microscopy revealed the fibrous morphology of PCL nanofibers (Figure 1(a)). This is in accordance with our previous results [61]. Pulsed laser deposition of a 200 nm thick HA layer did not affect the fibrous morphology or porosity of the nanofibers (Figure 1(b)). However, the fibrous character of samples with a 400 nm thick HA layer (Figure 1(c)) was less well preserved, and the porosity of the scaffold decreased. The fibrous morphology disappeared completely in samples with a 800 nm thick HA coating (Figure 1(d)).

3.2. Biomechanical Testing. The effect of an HA layer on the biomechanical properties of the nanofibers was tested using a tensile test. Young's moduli of elasticity, the ultimate stresses, and the ultimate strains of scaffolds of PCL/PVA

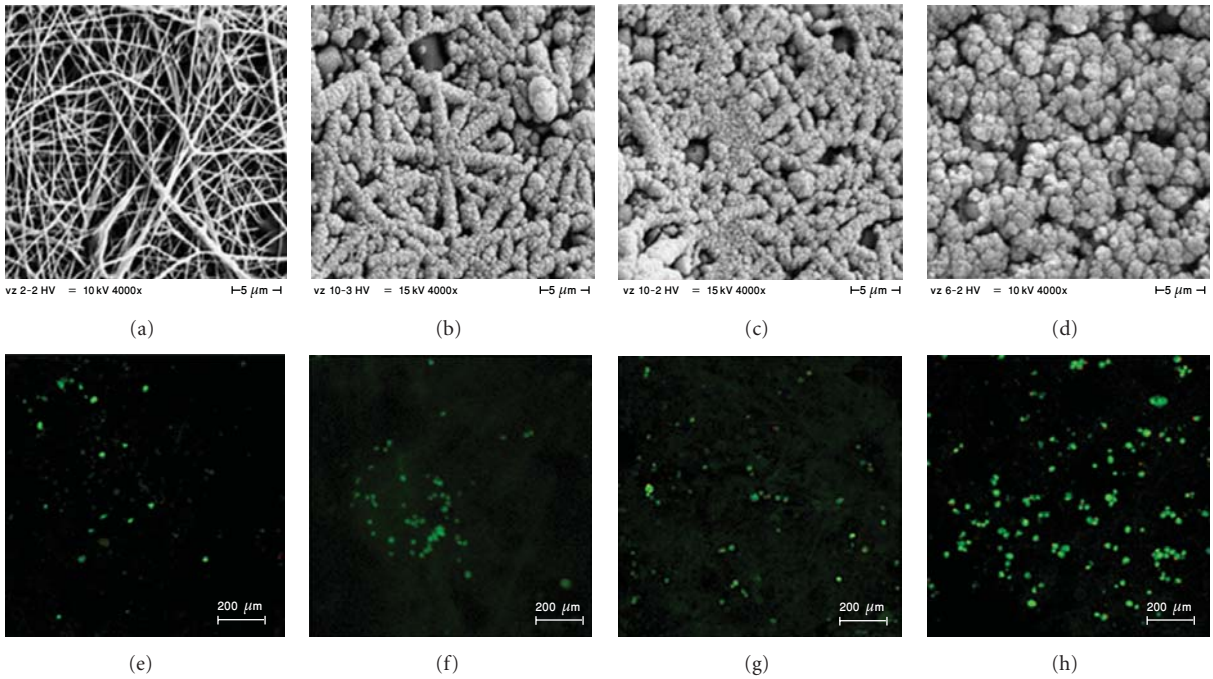

Figure 1: Visualization of scaffolds by SEM and confocal microscopy. Prepared scaffolds were visualized using SEM (a, b, c, d). On day 7, MSCs were stained using BCECF-AM and propidium iodide for live/dead staining, and samples were visualized by confocal microscopy (e, f, g, h); PCL/PVA (a, e), PCL/PVA200HA (b, f), PCL/PVA400HA (c, g), and PCL/PVA800HA (d, h).

nanofibers and various amounts of HA were determined at room temperature using a Zwick/Roell traction machine. We found significant differences in Young's moduli of elasticity between samples without an HA layer and those with an HA layer ($P = 0.04$). Young's moduli of elasticity in the case of pure PCL/PVA nanofibers was 1.76 ± 0.50 Mpa while that for the samples with an HA layer was 5.40 ± 3.09 MPa; the difference was significant (see Figure 4(a)). Significant differences between these two groups were found as well in the case of ultimate strains ($P < 0.001$). Here, the value obtained for pure PCL/PVA scaffolds was 0.23 ± 0.03, while for scaffolds with an HA layer the value was 0.09 ± 0.04, (see Figure 4(b)). No significant differences were found when analyzing ultimate stresses ($P = 0.26$), although the value for the group with an HA layer, 0.36 ± 0.27 MPa, was higher than that for the pure PCL/PVA scaffolds, 0.19 ± 0.07 MPa (see Figure 4(c)). The results showed that from the mechanical point of view, a PCL/PVA scaffolds covered by an HA layer is the relevant choice as a scaffold material for other studies and applications in which greater stiffness is required.

3.3. Proliferation and Viability of MSCs Seeded on Scaffolds. To test the scaffolds' biocompatibility and their ability to stimulate the proliferation and differentiation of MSCs into osteogenic cells, MSCs were seeded on scaffolds and cultivated for 14 days. Their proliferation and viability were determined on days 1, 7, and 14. Cell proliferation was determined by the PicoGreen assay and confocal microscopy (Figure 2). Viability was determined by the widely used MTT assay. Clearly, the deposition of a 400 nm or 800 nm thick HA layer resulted in the highest absorbance, which in turn reflected the best cell viability. However, some publications have reported that the MTT test is affected by cell number [62]. In order to correct for the possible inaccuracy of the MTT assay, we performed the PicoGreen assay as well. PicoGreen is a highly sensitive probe for dsDNA and thus can be used to determine cell numbers. Consequently, we performed both the MTT assay and the PicoGreen assay and correlated both results. This approach enabled the calibration of the absorbance measured in the MTT assay to the cell number determined by PicoGreen. By comparing the results of both assays in this manner, we were able to derive reliable data on cell viability (Figure 2). The results showed that in the control samples (PCL/PVA), cell viability was only slightly elevated. On the other hand, samples coated with HA showed a marked increase in cell viability. The highest viability was detected for samples with a 400 nm or 800 nm thick HA coating.

This conclusion was clearly supported by our confocal microscopy observations. MSC viability on the scaffolds was characterized by BCECF-AM and propidium iodide in the presence of an HA coating (Figures 1(e)–1(h)). The largest cell population was found in the samples with an 800 nm thick HA coating (Figure 1(h)), which is in agreement with the results of the PicoGreen assay.

3.4. Osteogenic Differentiation of MSCs. A positive influence of HA on osteogenesis has been demonstrated in many reports [4, 63, 64]. On the other hand, Wang et al. pointed out the significance of HA structures for proliferation and found higher cell proliferation rates on microsized HA particles than on nanosized ones [65]. Ribeiro et al. also found improved cell viability and proliferation of osteoblastic

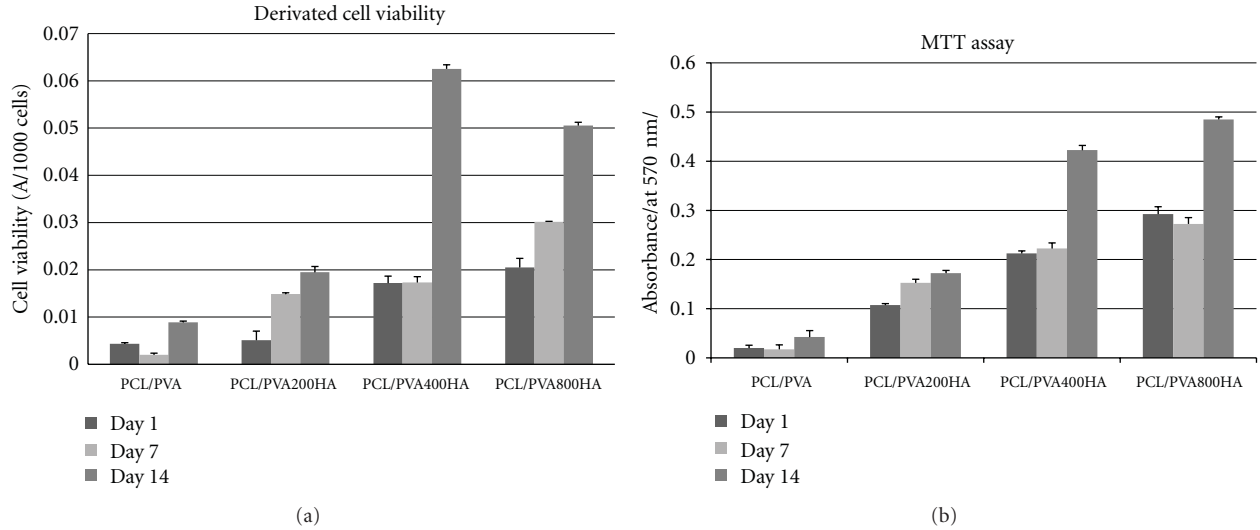

Figure 2: Cell metabolic activity and viability. Metabolic activity of viable MSCs was detected by MTT assay on day 1, 7, and 14 (mean ± standard deviation). Results of MTT assay for PCL/PVA, PCL/PVA200HA, PCL/PVA400HA, and PCL/PVA800HA samples (a). Cell viability calculated as derivation of absorbance values from MTT assay to cell counts determined by PicoGreen assay (b).

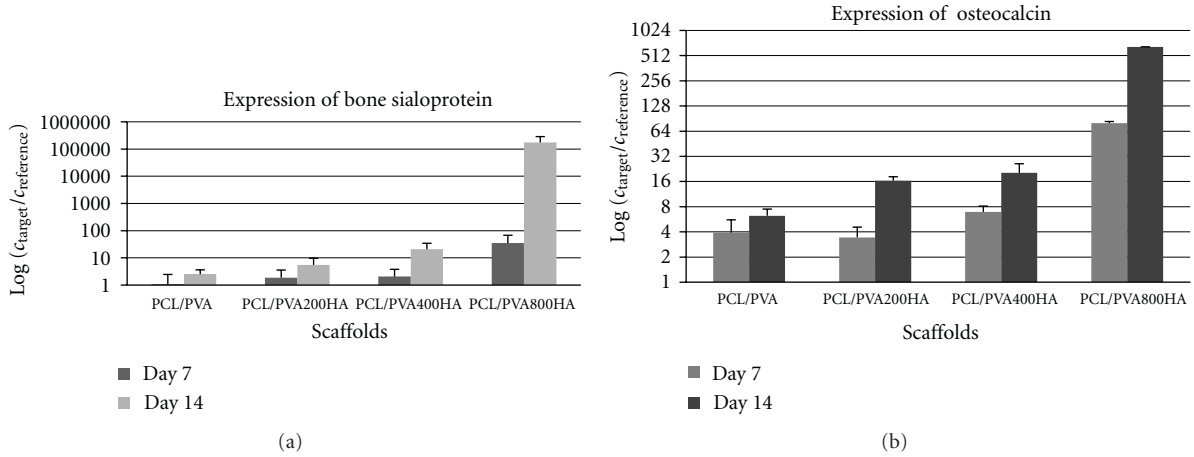

Figure 3: Expression of BS (a) and OC (b) genes. Osteogenic differentiation of MSCs was detected by PCR analysis of expresion BS and OC genes on day 7 and 14 (mean ± standard deviation).

MC3T3-E1 cells on HA particles of larger size [66]. However, there is no clear evidence so far on the effect of HA on differentiation into osteogenic cells. Therefore, the effect of HA coating of nanofibers on the osteogenic differentiation of MSCs was studied using real-time PCR analysis. The expression levels of BS and OC mRNAs, osteogenic markers, were detected on day 7 and 14 for all samples (Figures 3(a) and 3(b)). Interestingly, the samples with an 800 nm thick HA coating were characterized by the significantly higher expression of BS and OC genes than the pure PCL/PVA samples. Based on our results, we can hypothesize that HA-modified nanofibers induced cell differentiation and also improved cell viability (Figure 2).

3.5. Release Profile of FITC-Dextran. Besides surface modifications, possibilities exist for drug distribution into the nanofiber core. The encapsulation of different proliferation agents inside the nanofibers can increase their ability to stimulate proliferation and thus further improve the positive effect of nanofiber scaffolds on MSCs proliferation and differentiation. This could be especially important in combination with the already described positive effect of HA deposition on MSCs viability and differentiation. Knowledge of the release profile from HA-coated nanofibers seems to be a key point for the construction of novel drug-delivery systems suitable for bone tissue engineering.

To study the release profile from coaxially electrospun nanofibers with different concentrations of core polymer, FITC-dextran incorporated into the nanofiber core was employed as the monitoring fluorescence probe. The FITC-dextran samples were incubated at room temperature in TBS buffer, which was subsequently replaced with fresh buffer as

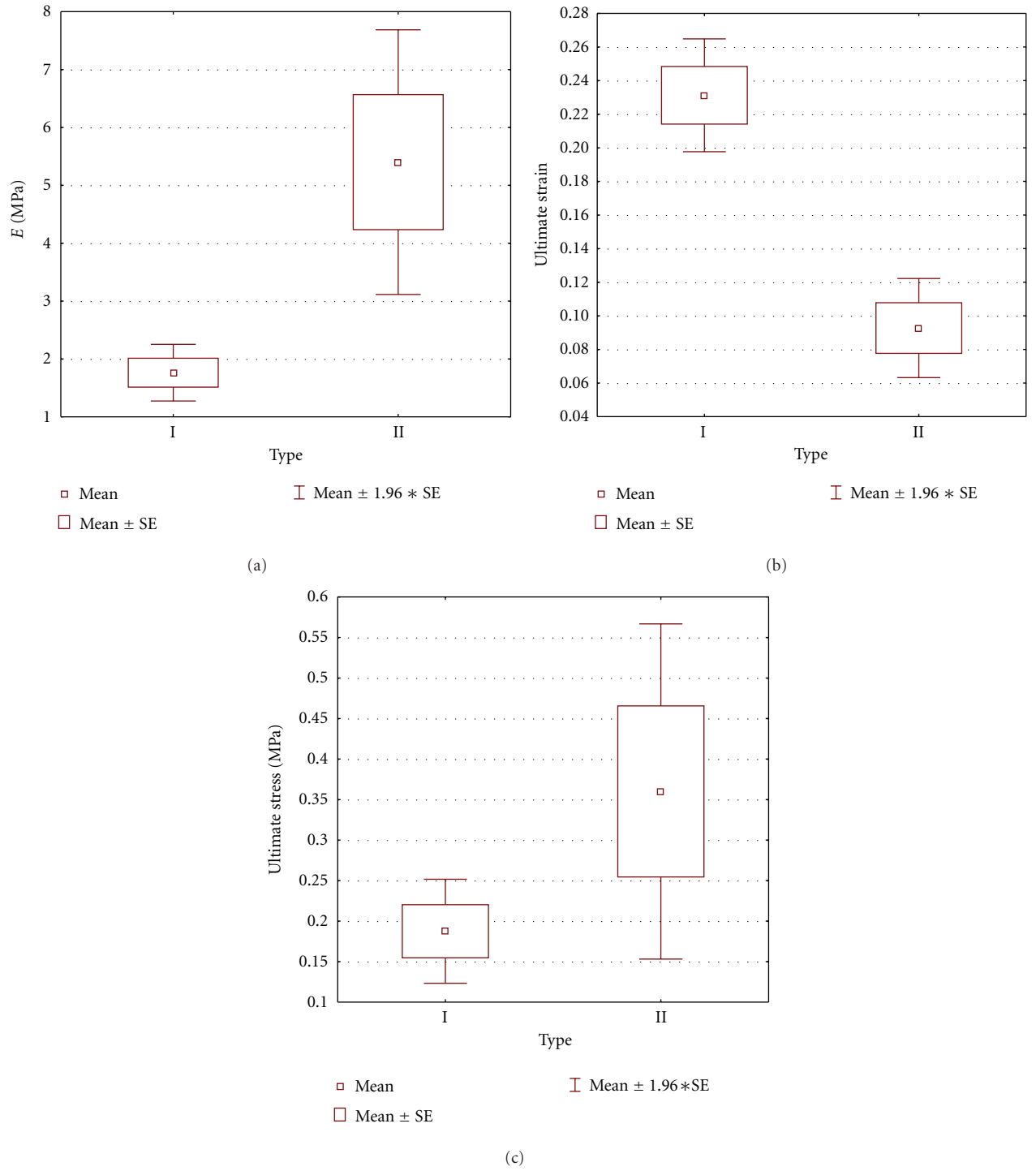

Figure 4: The moduli of elasticity, the ultimate strain, and ultimate stress of the group of pure PCL/PVA composite (type I) and the group of the PCL/PVA composite covered by HA layer (type II). There is a significant difference in the moduli of elasticity between these groups (determined by t-test; $P = 0.04$) (a) and also in the ultimate strain ($P < 0.001$) (b), but not in the ultimate stress ($P = 0.26$) (c). Mean is the mean value, SE is the standard error.

described in Section 2. The collected fractions were analyzed by fluorescence spectroscopy, and the cumulative release profile of FITC-dextran was calculated (Figure 5). The half-time of release from coaxial nanofibers was strongly dependent on the presence of a hydrophilic core polymer. Core/shell nanofibers containing FITC-dextran dissolved in 1% PVA showed the highest burst release (79% of FITC-dextran released in 24 h). The half time of release was calculated as $\tau_r = 18$ h. The release of FITC/dextran from fibers with 3% PVA showed a slower release; however, an intense burst

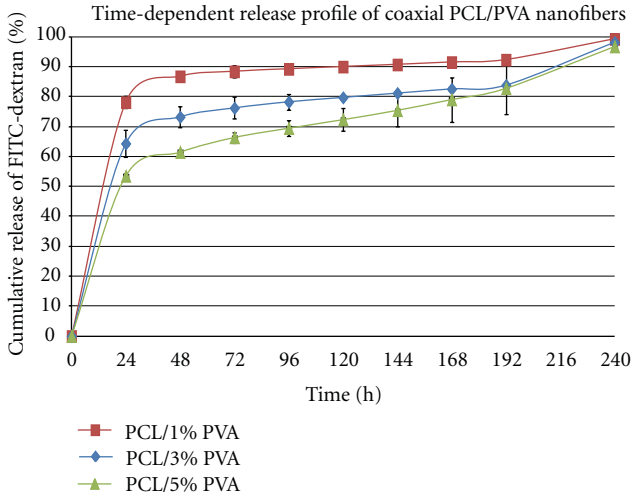

FIGURE 5: Time-dependent release profile of coaxial PCL/PVA nanofibers. Release of FITC-dextran from samples with different content of PVA core was analyzed using fluorescence spectroscopy. Samples were analyzed for 240 h, and supernatants were collected in 24 h intervals (mean ± standard deviation).

release was observed (65% of FITC-dextran released in 24 h). The half-time of release was prolonged to 24 h. Interestingly, samples with 5% PVA as the core polymer showed the most sustained release profile. The burst release was reduced to 52% of FITC-dextran release in 24 h, and the half-time of release was shifted to 54 h. The results clearly show that different concentrations of the water-soluble core significantly affect the release profiles of incorporated substances.

4. Conclusion

Pulsed laser deposition was proven to be a suitable method for HA coating of coaxial PCL/PVA nanofibers. The fibrous morphology of PCL/PVA nanofibers was preserved when the nanofiber scaffold was coated with thin layers of HA (200 nm and 400 nm). Increasing the thickness of HA, however, resulted in a gradual loss of this fibrous character. In addition, the biomechanical properties were improved after HA deposition on PCL/PVA nanofibers as the value of Young's moduli of elasticity significantly increased after HA deposition.

The proliferation and differentiation of MSCs on HA-coated scaffolds are separate processes. Our HA-coated nanofiber scaffolds clearly displayed a positive effect on the differentiation of MSCs into osteogenic cells but not on cell proliferation. The moderate effect of HA-coated nanofiber scaffolds on cell proliferation observed in our study could be improved, however, by exploiting core/shell nanofibers. Such a delivery system, based on coaxial spinning, can encapsulate proliferation stimulating factors that could be subsequently steadily released. This system seems to be a potentially promising one for the development of artificial bone tissue and bone healing. To conclude, thin-layer hydroxyapatite deposition on a nanofiber surface stimulated mesenchymal stem cell proliferation and their differentiation into osteoblasts. The 800 nm HA layer was demonstrated to be optimal for bone tissue engineering application.

Authors Contribution

Both authors contributed equally.

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Research Article

BMP9-Induced Osteogenetic Differentiation and Bone Formation of Muscle-Derived Stem Cells

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Efficient osteogenetic differentiation and bone formation from muscle-derived stem cells (MDSCs) should have potential clinical applications in treating nonunion fracture healing or bone defects. Here, we investigate osteogenetic differentiation ability of MDSCs induced by bone morphogenetic protein 9 (BMP9) *in vitro* and bone formation ability in rabbit radius defects repairing model. Rabbit's MDSCs were extracted by type I collagenase and trypsin methods, and BMP9 was introduced into MDSCs by infection with recombinant adenovirus. Effects of BMP9-induced osteogenetic differentiation of MDSCs were identified with alkaline phosphatase (ALP) activity and expression of later marker. In stem-cell implantation assay, MDSCs have also shown valuable potential bone formation ability induced by BMP9 in rabbit radius defects repairing test. Taken together, our findings suggest that MDSCs are potentiated osteogenetic stem cells which can be induced by BMP9 to treat large segmental bone defects, nonunion fracture, and/or osteoporotic fracture.

1. Introduction

Skeletal muscles are always regarded as the source of satellite stem cells or muscle precursor cells. These kinds of stem cells are at the quiescent condition under normal circumstances and activated when the repairing of muscle tissue is needed. They will be differentiated and blended together into new muscle fibers, reaching the purpose of repairing defected muscles as a result [1]. In recent years, studies have shown that there is also another kind of stem cells in skeletal muscle, called muscle-derived stem cells (MDSCs). They have mesenchymal stem cells-like differentiation potential [2], and the ability of being differentiated into several types of terminal cells is maintained. Instead of being differentiated into muscle cells, MDSCs can also be differentiated into other type of cells, such as hematopoietic cells [3], osteoblasts [4], and chondroblasts [5] under particular conditions. Therefore, the potential of MDSCs as candidate seed cells in osteogenetic tissue engineering has been paid much more attention than before.

Bone morphogenetic proteins (BMPs) belong to one of the β-superfamily members of transforming growth factors (TGFs); BMPs are a kind of multifunctional growth factors and are one of the most potent osteogenetic growth factors [6–8]. BMP9 belongs to BMPs family and is expressed in liver [9]. BMP9 possible roles include inducing and maintaining the cholinergic phenotype of embryonic basal forebrain cholinergic neurons, inhibiting hepatic glucose production and inducing the expression of key enzymes of lipid metabolism, and stimulating murine hepcidin 1 expression [10–12]. Although the functional role of BMP9 in the skeletal system remains to be fully understood, the potent osteogenic activity of BMP9 suggests that it may be used as one of the most effective bone regeneration agents compared with other BMPs, such as BMP2, BMP4, or BMP7 [13, 14].

Presently, among the overwhelming majority of many osteogenetic studies of BMPs, mouse mesenchymal stem cells (such as C3H10), mouse embryonic fibroblasts (MEFs), and bone marrow stromal cells (BMSCs) are subjected as studying objects. However, there have been no literature reports on whether BPM9 can induce osteogenetic differentiation of muscle-derived stem cells. Therefore, in this paper, we have demonstrated the potential osteogenetic ability of MDSCs induced by BMP9 *in vitro* and *in vivo* testing.

2. Materials and Methods

2.1. Materials. BMP9, BMP2, and GFP expression adenoviruses (AdBMP9, AdBMP2, and Ad-GFP) were provided by Dr. He (Molecular Oncology Laboratory, Medical Center, the University of Chicago, USA) and amplified in our laboratory. Hank's solution, DMEM culture medium, and high-quality fetal calf serum (FBS) were used in cells culture (Hyclone Company). Alkaline phosphatase (ALP) staining kit and quantitative testing kit were purchased from BD Company. Alizarin red S staining kit, type I collagenase, trypsin, polylysine, and vitamin C were purchased from Sigma Company. Anti-Sca-1 antibody (Wuhan Boster Biological Technology Co., Ltd.) and nanohydroxyapatite/polyamide bone cement were provided by Research Center of Nano-biomaterials, Sichuan University.

Healthy rabbits with an average age of 6–8 weeks, as the experimental animal, were provided by the Experimental Animal Center, Chongqing Medical University.

2.2. Methods

2.2.1. Separation and Cultivation of MDSCs. After anesthesia, muscle strips (2 cm × 0.8 cm × 0.5 cm, approximately 5 g) were cut from the rabbits and then placed into a sterilized bottle. Carry out sequential digestion by two-step method (type I collagenase and trypsin methods), then filter through no. 100, 200, and 400 stainless steel screens, and aspirate the obtained cells into a 100 mL culture bottle (PP1). 9 mL of DMEM culture solution (contained 100 mL/L fetal calf serum) was added. 1 h later, transfer the cell suspension into another culture dish (PP2) by the differential-rate wall-adhering growth method. Hereafter, repeat the procedure above to obtain PP3, PP4, PP5, and PP6 every 24 h.

2.2.2. Identification of MDSCs. MDSCs were fixed with acetone and prepared for immunohistochemical staining analysis at 48 hours after subculture. Immunohistochemical staining was subjected with mouse anti-rabbit Sca-1 monoclonal antibodies.

2.2.3. ALP Staining and ALP Activity Quantitative Measurement. ALP activity was assessed by a modified Great Escape SEAP Chemiluminescence Assay (BD Clontech, Mountain View, CA, USA) and/or histochemical staining assay (using a mixture of 0.1 mg/mL of naphthol AS-MX phosphate and 0.6 mg/mL of fast blue BB salt) [13, 14]. Cultured MDSCs were seeded in 24-well plate with subconfluent of 30% and infected with AdBPM9 (experimental group), AdBPM2 (positive control group), and Ad-GFP (negative control group), respectively. At 5, 7, and 9 days after infection, ALP activity would be measured and histochemical staining would be done as indicated. The results were repeated in at least three independent experiments. ALP activity was normalized by total cellular protein concentrations among the samples.

2.2.4. Calcium Salt Sedimentation Experiment. MDSCs were seeded into a 24-well plate and infected with AdBPM9, AdBMP2, and Ad-GFP. Alizarin red S staining was subjected at 14 days after infection. Cells were fixed with 0.05% (v/v) glutaraldehyde at room temperature for 10 minutes. After being washed with distilled water, fixed cells were incubated with 0.4% alizarin red S (Sigma-Aldrich) for 5 minutes, followed by extensive washing with distilled water. The staining of calcium mineral deposits was recorded under bright-field microscopy.

2.2.5. Preparation of Bone Defect Model and Animal Grouping. Anesthetize 36 New Zealand white rabbits, then dissection was cut (longitudinally approximately 30 mm along the inner side of the forearm) to adequately expose the middle segment of the radius, and prepare a bone defect model of approximately 12 mm by an electric saw, in accordance with the length of the implant. Randomly assign the rabbits into 3 groups and implant Adv-hBMP9 + nanohydroxyapatite/polyamide bone cement, Adv-hBMP2 + nanohydroxyapatite/polyamide bone cement, and Adv-GFP + hydroxyapatite/polyamide bone cement at the left and right bone defect sites, respectively. Suture the wound layer by layer and wrap with sterile dressings. After the surgery, each rabbit in each group is injected with gentamicin sulfate 10,000 U, for 3 d; the rabbits of each group are fed in different cages.

2.2.6. Detection by X-Ray Radiography. Carry out X-ray radiography in weeks 2, 4, 8, 12, and 16 after the surgery, respectively.

2.2.7. Collection and Detection of Histological Specimens. In Weeks 2, 4, 6, 8, 12, and 16 after the surgery, sacrifice 2 rabbits of each group, respectively, macroscopically observe the healing condition of the implant and surrounding tissues, and carry out a histological examination of the tissue in the stent.

3. Results

3.1. Separation and Cultivation of MDSCs. The cells of early wall-adhering growth (PP1) are mostly fibroblasts, with large body and with rapid growth, without significant directional growth. The cells of late-stage wall-adhering growth (PP6) are mostly short, fusiform, polygonal cells (Figure 1(a)), with obviously directional growth that may form into a long chain; however, their growth is slower than that of fibroblasts. In 7–10 d later, intercellular lamellar fusion occurs; hereafter, cell growth rate is slower than before.

3.2. Identification of MDSCs. Sca-1 is one of the most characteristic phenotypes of MDSCs; when the *in vitro* culture density of cells reached 70%–80%, Sca-1 immunohistochemical staining was carried. The results showed that 80%–90% of cells were positive in Sca-1 staining (Figure 1(b)).

3.3. ALP Staining and ALP Activity of BMP9 Induced in Muscle-Derived Stem Cells. ALP is the early marker for osteogenous differentiation of cells. ALP staining of MDSCs transfected with BMP9, BMP2, and GFP at day 9 was shown (Figure 2(a)). It suggested that ALP expression induced by

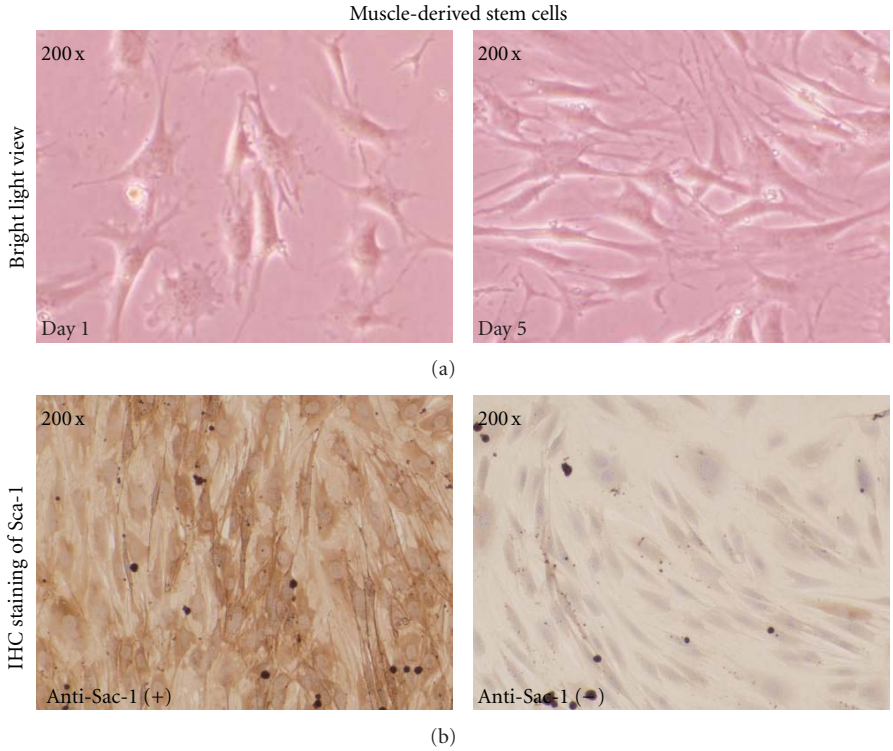

Figure 1: (a) Culture of muscle-derived stem cells. MDSCs were cultured and subcultured at confluent of 80%. Bright view images were taken at day 1 and day 5 after subconfluent. (b) Immunohistochemical staining of Sca-1. MDSCs were subcultured. Expression of Sca-1 was assessed by immunohistochemical staining analysis at day 2 using an anti-Sca-1 antibody (Wuhan Boster Biological Technology Co., Ltd.).

BMP9 in MDSCs was higher than other groups. And also, ALP activities of MDSCs after infected by BMP9, BMP2, and GFP expression adenovirus were assessed at days 5, 7, and 9 (Figure 2(b)). ALP activities induced by BMP9 in MDSCs were significantly higher in indicated time points.

3.4. Expression of BMP9-Induced Calcium Salt Sedimentation in Muscle-Derived Stem Cells. Alizarin red S staining was carried out at day 14 after infection; it was found that, under the induction of BMP9, obvious calcium salt sedimentation occurred in MDSCs, the density of calcification was higher than other groups, and at the same time, the size of calcification in BMP9 group was larger (Figure 3).

3.5. Results of X-Ray Radiography. Week 2 after the surgery: no obvious callus formation was seen around the implant in each group.

Week 4 after the surgery: the boundary between the broken ends of bone and the implant was veiled in the BMP9 group, and the transmittancy of the implant was decreased. No obvious changes occurred in the remaining two groups.

Week 8 after the surgery: callus grew into the implant from the two broken ends of bone and from the side of the ulna (Figure 4). The amount of formed callus in the BMP2 group was less than that in the phase in the BMP9 group (Figure 4). A small amount of callus was seen in the side of ulna in the GFP group (Figure 4).

Week 12 after the surgery: in the BMP9 group, the two broken ends of bone were completely connected, and the majority of bone marrow cavities were recanalized. However, the two broken ends of bone were not completely connected in the BMP2 group.

Week 16 after the surgery: in the BMP9 group, the two broken ends of bone were completely connected, and the majority of bone marrow cavities were recanalized (Figure 4). However, the ends of bone broken were not completely connected in the BMP2 group (Figure 4). In the GFP group, there was only a certain amount of formed callus in the side of ulna and in the proximal end of bone broken (Figure 4).

3.6. Morphologic Macroscopic Observation. Week 2 after the surgery: in the BMP9 group, the implant was wrapped by noncohesive fibrous tissues, the two ends of the implant were connected with the recipient bone fibers, a small amount of soft tissues with blood vessels grew into the lateral aperture of the implant, no obvious new bone formation was seen, and the adhesion between the implant and the recipient bone was not firm and the implant could be shaken. In the BMP2 group, it was seen that the implant was wrapped by noncohesive fibrous tissues, no obvious new bone formation was seen, and the implant could be shaken. In the GFP group, it was seen that the implant was wrapped by a small amount of fibrous tissues.

Week 4 after the surgery: in the BMP9 group, the implant was wrapped by fibrous tissues, the proximal and distal ends of the implant were connected with the recipient bone and could not be shaken, a large amount of soft tissues with blood

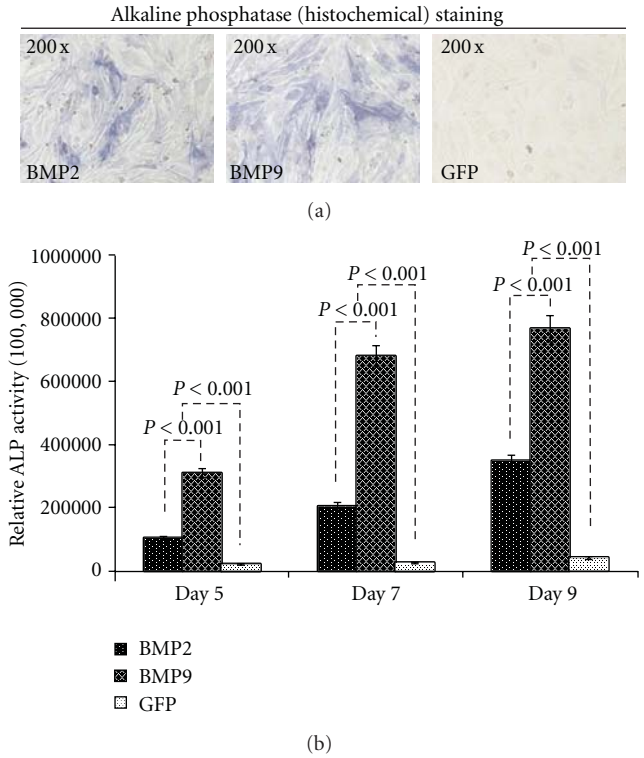

Figure 2: Potentiation of BMP-induced early osteogenetic marker ALP activity in muscle-derived stem cells. (a) ALP staining in MDSCs. MDSCs were infected with AdBMP9, AdBMP2, and Ad-GFP. ALP staining was done at the indicated time points. ALP expression induced by BMP9 in MDSCs was shown higher than other groups. (b) BMP-induced ALP activity in MDSCs. MDSCs were infected with AdBMP9, AdBMP2, and Ad-GFP. ALP activity was measured at the indicated time points. Each assay condition was dose in triplicate. ALP activities induced by BMP9 in MDSCs were significantly higher in indicated time points ($P < 0.001$).

vessels grew into the lateral aperture of the implant, and there was a small amount of formed new callus. In the BMP2 group, a small amount of soft tissues with blood vessels grew into the lateral aperture of the implant, and no new bone formation was seen. In the GFP group, a small amount of soft tissues with blood vessels grew into the lateral aperture of the implant, and no new bone formation was seen.

Week 6 after the surgery: in the BMP9 group, the implant was wrapped completely by fibrous membranous tissues, and there were hard new bone and callus formation. In the BMP2 group, there was a small amount of formed new callus. In the GFP group, new bone callus formation occurs initially.

Week 8 after the surgery: in the BMP9 group, there was a large amount of formed new callus. It was seen that hard calluses grew into the implant, and a bone-wedge-like structure was formed in the lateral aperture of the implant. In the BMP2 group, the lumen was full of connective tissues, and there was new callus formation. In the GFP group, a small amount of new callus was formed.

Weeks 12 and 16 after the surgery: in the BMP9 group, callus was increased. In the BMP2 group, it was seen that hard calluses grew into the implant. In the GFP group, a large amount of new callus was formed.

3.7. Results of Histological Examination. In the BMP9 group: in Week 2 after the surgery, it was seen that connective tissues grew into the lumen of the implant, and there were much inflammatory cell infiltration and immature capillary formation. In week 4 after the surgery, the lumen was full of connective tissues, inflammatory cells were reduced, a mature vascular net was formed, and it was seen that a small amount of cartilage grew. In week 6 after the surgery, a mature vascular net was formed, and there was fibrous callus formation in the stent. In week 8 after the surgery, the lumen was full of mature vascular net and cartilages, and there were much fibrous callus and osteoid formations, and a bone wedge-like structure was formed in the lateral aperture of the implant. Some cartilages were directly adhered to the surface of the implant (Figure 5). In week 12 after the surgery, the lumen was full of cartilages; after bone formation and moulding, mature bone trabecular formation was seen, similar to peripheral bones, observed under microscope, and was connected with the recipient bone mutually. In week 16 after the surgery, the bone cavity was filled with mature trabecular bone, and immature trabecular bone between meshes were connected into mesh-like and plate-like shapes (Figure 5).

In the BPM2 group: vasoformation and bone formation were similar to those in the BMP9 group; however, the times for vasoformation and bone formation were approximately 2 weeks later than those in the BMP9 group (Figure 5).

In the GFP group: the times for vasoformation and bone formation were much later than those in the BMP2 group (Figure 5).

4. Discussion

There are several classes of cell populations in the skeletal muscle, of which the muscle-derived stem cells (MDSCs) have multidirectional differentiation ability [4, 15–17]. In the experiment, MDSCs were extracted by the preplate technique; the principle of this technique was based on different times for wall-adhering growth of different cells: the majority of cells of initial wall-adhering growth are fibroblasts (PP1), the main portion of cells of wall-adhering growth in 24–48 h are satellite cells (PP2-PP4), and cells of last wall-adhering growth were Sca-1 staining-positive MDSCs. Prior to wall-adhering growth, MDSCs were of small and round spherical shape, with strong refractivity, and were also of round shape at the time of initial wall-adhering growth; after spontaneous growth, they are fused into mature polynuclear myotubes. It is different from fibroblasts in this point. After MDSCs transfected with BMP9 and BMP2, it is found by ALP staining and quantitative analysis that BMP9 induced ALP activities of muscle-derived stem cells, and the activities were increased with time, reaching peak in day 9, suggesting that MDSCs had the osteogenous differentiation ability *in vitro*. Meanwhile, the ALP activities induced by BMP9 were obviously higher than those in the BMP2 group, suggesting that BMP9 had higher potential ability of osteogenesis stimulating than BMP2. The results

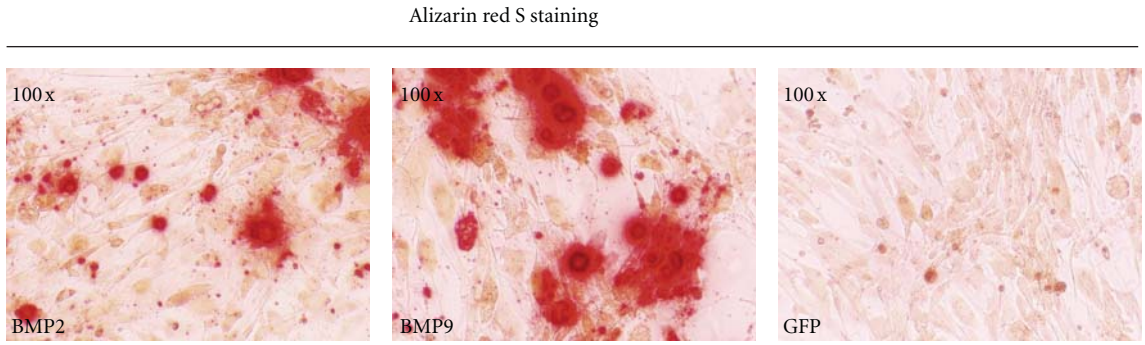

Figure 3: Alizarin red S staining. MDSCs were infected with adenoviruses as indicated. Alizarin red S staining was conducted at 14 days. The density of calcification induced by BMP9 was higher than other groups, and at the same time, the size of calcification in BMP9 group was larger.

Figure 4: Bone formation of MDSCs induced by BMP2 and BMP9 in rabbit radius defects repairing test. BMP9 or BMP2 expression MDSCs were implanted in rabbit radius defects model. X-ray images of bone formation by MDSCs implantation assay had been taken at week 8 and week 16. Representative X-ray images are shown as indicated.

of the later-stage marker, that is, calcium salt sedimentation, for osteogenous differentiation of cells showed that there was obvious calcium salt sedimentation in the BMP9 group; moreover, compared with that in the BMP2 group, BMP9 has stronger ability of stimulating calcium salt sedimentation in MDSCs. These demonstrated that BMP9 was probably a more efficient osteoinductive growth factor compared with BMP2. The result was consistent with that from the studies conducted by Wada et al. [18].

Previously, the studies by D. S. Musgrave found that, under the action of BMP2, MDSCs resulted in ectopic osteogenesis in upper limbs [19]. In the studies by V. Wright,

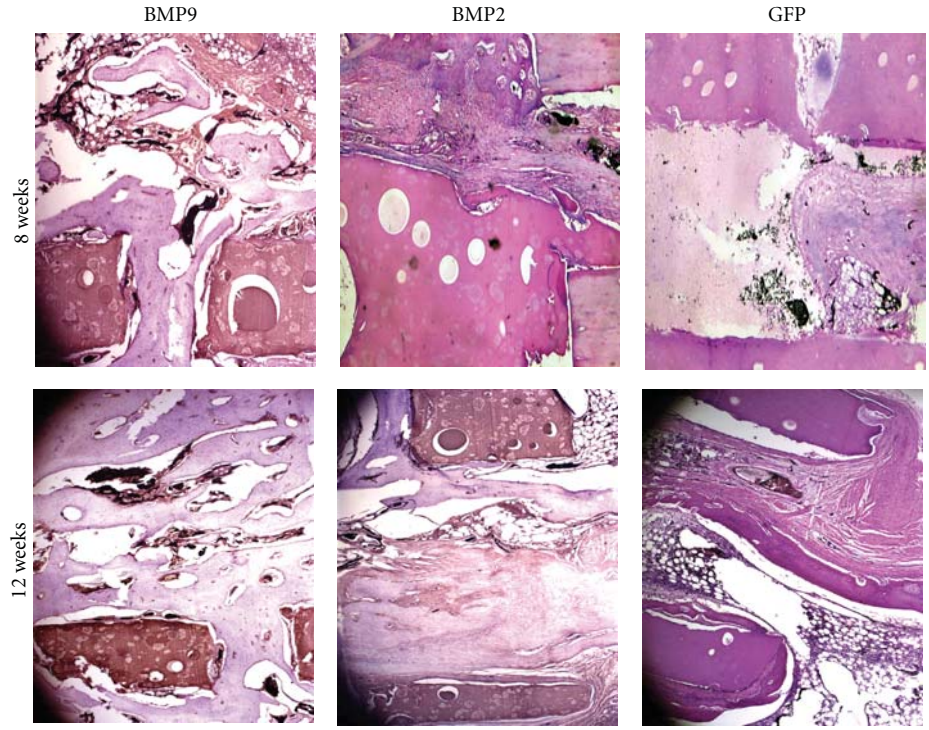

Figure 5: Histologic evaluation of the retrieved bone samples. Retrieved bone sample from BMP9- or BMP2-treated groups were fixed and decalcified. The paraffin-embedded sections was subjected to hematoxylin and eosine (H&E) staining. Representative images are shown. Magnification, 200.

MDSCs transfected with BMP4-induced ectopic osteogenesis and promoted bone healing [20]. Therefore, during our study, the effects of BMP9/2/GFP in repairing radius defect were observed by using the method in which, after the middle segment defect model of radius was prepared, Adv-hBMP9/2/GFP + nanohydroxyapatite/polyamide bone cement was implanted. As a result, new bone was initially emerged in week 4 in the BMP9 group; in the BMP9 group, by week 8, large mounts of blood vessels, soft tissues, and bone calluses were formed, and hard calluses grew into the implant. In week 16 after the surgery, the two broken ends of bone were completely connected, and the majority of bone marrow cavities were recanalized; by histological examination, mature bone trabecular was seen, and new bone trabecular between meshes was connected into mesh-like and plate-like shapes. The repairing duration of bone defects in the BMP2 group was approximately 2 weeks longer than that in the BMP9 Group. Bone defects repairing in GFP group is much more slow than that in the BMP2 group. This result is consistent with the prior reports submitted by Cheng et al. [13] and Kang et al. [14]. They demonstrated that BMP9 (in addition to BMP-2, BMP4, or BMP-7) is identified as the most potent BMPs to induce orthotopic bone formation *in vivo* [14].

From what have been discussed above, BMP9 has strong effects of inducing directional osteogenous differentiation of muscle-derived stem cells; compared with other stem cells in tissue engineering, the *in vitro* cultivation of MDSCs has the advantages of good material availability, as well as strong proliferation and differentiation abilities. Meanwhile, it has also demonstrated that it is a research direction of vast potential to carry out research on artificial bone in tissue engineering, by using highly efficient osteogenous induction factor and readily available, adequate tissue stem cells in combination.

Acknowledgments

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Review Article

Adult Bone Marrow: Which Stem Cells for Cellular Therapy Protocols in Neurodegenerative Disorders?

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The generation of neuronal cells from stem cells obtained from adult bone marrow is of significant clinical interest in order to design new cell therapy protocols for several neurological disorders. The recent identification in adult bone marrow of stem cells derived from the neural crests (NCSCs) might explain the neuronal phenotypic plasticity shown by bone marrow cells. However, little information is available about the nature of these cells compared to mesenchymal stem cells (MSCs). In this paper, we will review all information available concerning NCSC from adult tissues and their possible use in regenerative medicine. Moreover, as multiple recent studies showed the beneficial effect of bone marrow stromal cells in neurodegenerative diseases, we will discuss which stem cells isolated from adult bone marrow should be more suitable for cell replacement therapy.

1. Introduction

Neurodegenerative disease is a generic term used for a wide range of acute and chronic conditions whose etiology is unknown such as Parkinson's disease, Huntington's disease, amyotrophic lateral sclerosis (ALS), Alzheimer's disease, but also now for other neurological diseases whose etiology is better known but which are also concerned by a chronic lost of neurons and glial cells such as multiple sclerosis (MS), stroke, and spinal cord injury. Although the adult brain contains small numbers of stem cells in restricted areas, the central nervous system exhibits limited capacity of regenerating lost tissue. Therefore, cell replacement therapies of lesioned brain have provided the basis for the development of potentially powerful new therapeutic strategies for a broad spectrum of human neurological diseases. However, the paucity of suitable cell types for cell replacement therapy in patients suffering from neurological disorders has hampered the development of this promising therapeutic approach.

Stem cells are classically defined as cells that have the ability to renew themselves continuously and possess pluripotent or multipotent ability to differentiate into many cell types. Beside the germ stem cells devoted to give rise to ovocytes or spermatozoids, those cells can be classified in three subgroups: embryonic stem cells (ES), induced pluripotent stem cells (iPS), and somatic stem cells (Figure 1). ES cells are derived from the inner mass of blastocyst and are considered as pluripotent stem cells as these cells can give rise to various mature cells from the three germ layers. iPS cells are also pluripotent stem cells; however, those cells derived from adult somatic cells such as skin fibroblasts are genetically modified by introduction of four embryogenesis-related genes [1, 2]. Finally, tissue-specific stem cells known as somatic or adult stem cells are more restricted stem cells (multipotent stem cells) and are isolated from various fetal or adult tissues (i.e., hematopoietic stem cells, bone marrow mesenchymal stem cells, adipose tissue-derived stem cells, amniotic fluid stem cells, neural stem cells, and so forth) [3].

In recent years, neurons and glial cells have been successfully generated from stem cells such as embryonic stem cells [4], iPS [5], mesenchymal stem cells (MSCs) [6, 7], and adult neural stem cells [8], and extensive efforts by investigators to develop stem cell-based brain transplantation therapies have been carried out. Over the last decade, convincing

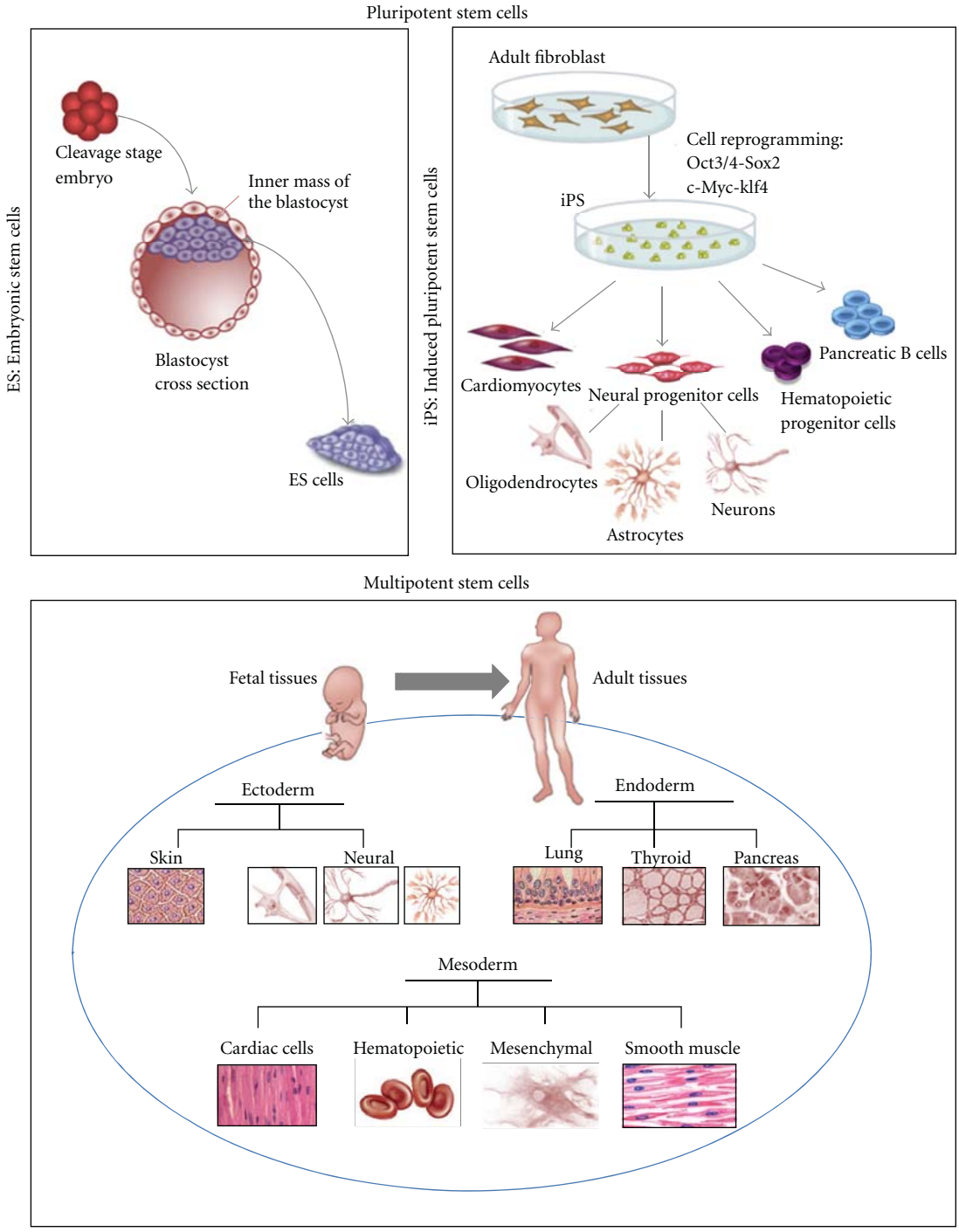

Figure 1: Stem cell type and origin. Beside germ stem cells, three group of stem cells can be defined according to their differentiating abilities: (a) pluripotent embryonic stem cells (ES), (b) induced pluripotent stem cells (iPS), and (c) multipotent fetal or adult somatic stem cells.

evidence has emerged of the capability of various stem cell populations to induce regeneration in animal models of Parkinson's disease (PD), Huntington's disease, Alzheimer's disease (AD), multiple sclerosis, or cerebral ischemia [9]. Some of the studies have already been carried out to clinical trials. In example, in the case of Parkinson's disease, transplantation of fetal ventral mesencephalon tissue directly into the brains of PD patients has been done in a few centers with varying results [10–12], and it appeared that using fetal ventral mesencephalon tissue raised numerous problems from ethical issues to heterogeneity and relative scarcity of tissue [13] suggesting that other stem cells (like adult somatic stem cells) may be more suitable for such a therapy. Likewise, ES cells have also been grafted in patients with injured spinal cord, as USA Federal Regulators have cleared the way for the first human trials of human ES cell

Table 1: Maturation steps of bone marrow derived neuron-like cells.

Maturation of BMDN	5 Days *in vitro*	8 Days *in vitro*	12 Days *in vitro*
Neurotransmitter sensitivities	GABA, glycine, glutamate	GABA, glycine, glutamate	GABA, glycine, glutamate
Potassic voltage-gated channels	+++	+++	+++
Sodic voltage-gated channels	−	+++	+++
Action potentials	−	+++	+++
Trains of action potentials	−	−	−
Synaptic activities	−	−	−
Membrane potential (mV)	-37 ± 3	$-50,3 \pm 2$	$-57,7 \pm 2,3$

research, authorizing researchers to test whether those cells are safe or not [14]. It is still too early to know the effect of ES cells on patient recovery; however, several concerns have been previously raised on animal models as ES cells induced teratocarcinomas and some exploratory clinical trials are confirming the animal studies [15].

In thispaper , we will review our results concerning identification and characterization of neural crest stem cells (NCSCs) in adult bone marrow as a potential source for cellular therapy in neurological disorders. We will also discuss what are the main questions that remain pending concerning the use of those cells in cellular therapy protocols for neurological disorders.

2. Somatic Stem Cells Isolated from Adult Bone Marrow

The postnatal bone marrow has traditionally been seen as an organ composed of two main systems rooted in distinct lineages—the hematopoietic tissue and the associated supporting stroma. The evidence pointing to a putative stem cell upstream of the diverse lineages and cell phenotypes comprising the bone marrow stromal system has made marrow the only known organ in which two (or more) separate and distinct stem cells and dependent tissue systems not only coexist but functionally cooperate, defining hematopoietic stem cells (HSCs) and mesenchymal stem cells (MSCs) [16].

MSCs were first isolated from the bone marrow (BM-MSC) stem cell niche. More recently, extensive research has revealed that cells with morphological and functional characteristics similar to BM-MSC can be identified in a large number of organs or tissues including adipose tissue and peripheral blood. Despite having different origins, these MSC populations maintain cell biological properties typically associated with stem cells. These include continuous cell cycle progression for self-renewal and the potential to differentiate into highly specialized cell types of the mesodermal phenotype including chondroblast, osteoblast, and adipocyte lineages. Interestingly, BM-MSCs have also been reported to be inducible via the ectodermal or endodermal germline, demonstrating the expression of neuron-like factors,insulin production , or hepatic lineage-associated genes, respectively. In addition to these general stem cell properties, the International Society for Cellular Therapy proposed a more specific panel of markers for the characterization of MSC. Due to the failure to identify a certain unique MSC cell-surface molecule, a set of minimal criteria for MSC was recommended, which includes the capability of adherence to plastic surfaces and the expression of the cell surface markers CD44, CD73, CD90, and CD105 with a concomitant absence of CD14, CD19, CD34, CD45, and HLA-DR expression [17].

Originally analyzed because of their critical role in the formation of the hematopoietic microenvironment (HME), bone marrow stromal cells became interesting because of their surprising ability to differentiate into mature neural cell types. More recently, a third stem cell group has been identified as originating from the neural crest, which could explain the capacity of stromal stem cells to differentiate into functional neurons.

3. Neural Phenotypic Plasticity of Adult Bone Marrow Stromal Cells

Several years ago, we demonstrated that a fraction of bone marrow stromal cells were able to differentiate into functional neurons. Those specific cells were characterized as nestin-positive mesenchymal stem cells [6, 7, 18]. Electrophysiological analyses using the whole-cell patch-clamp technique revealed that adult rat bone marrow stromal cells [6, 7] were able to differentiate into excitable neuron-like cells when they were cocultivated with mouse cerebellar granule neurons. First, we demonstrated that those cells express several neuronal markers (NeuN and Beta-III tubulin; Figure 2), an axonal marker (neurofilament H and M protein recognized by the monoclonal antibody, SMI31), and a dendritic marker (MAP2ab). Electrophysiological recordings of these nestin-positive bone-marrow-derived neuron-like cells (BMDN) were performed, and three maturation stages were observed (Table 1).

At 4–6 days of coculture, BMDN showed some neurotransmitter responsiveness (GABA, glycine, serotonin, and glutamate) and voltage-gated K^+ currents inhibited by TEA (tetraethylammonium). However, those cells did not express functional sodium voltage-gated channels and have a high membrane potential (Vrest) ($-37.6^\circ \pm 3$ mV, $n = 61$). During the second week of coculture, BMDN started to display Na^+ currents reversely inhibited by TTX (tetrodotoxin) and became able to fire single spike of action potential. In those older cocultures, the Vrest reaches a more negative value, which was closer to the value usually measured in neurons (7–9 days, -50.3 ± 2 mV, $n = 76$ and 10–15 days, -56.7 ± 2.3 mV, $n = 97$).

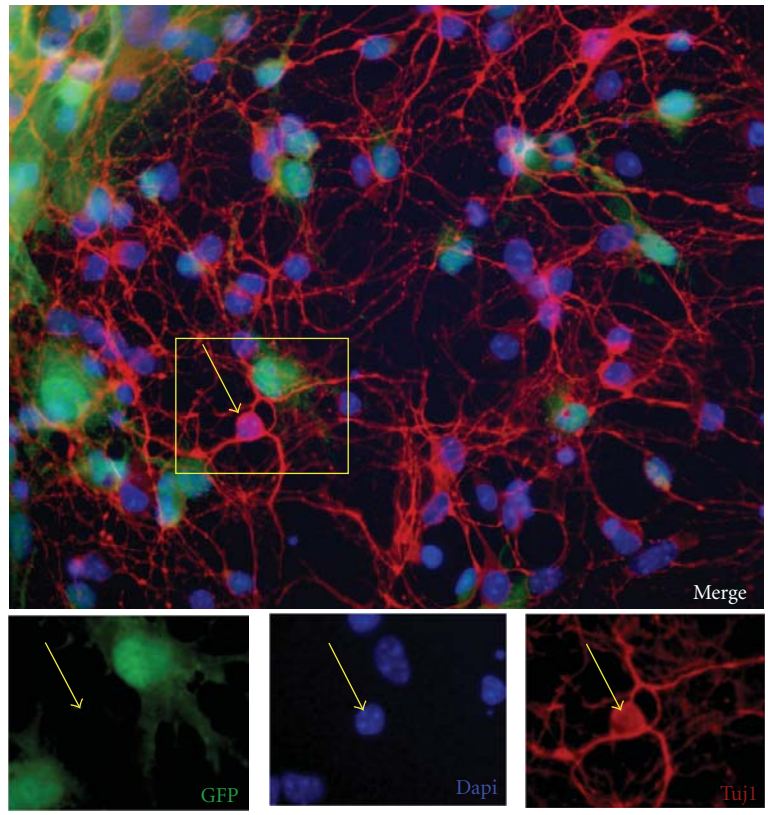

Figure 2: Neuronal marker expressed by bone marrow stromal cells. Bone marrow stromal cells were cocultivated for 5 days with GFP-positive cerebellar granule neurons (green). Immunofluorescence labeling showed that beta-III tubulin recognize by Tuj1 antibodies (red) was expressed by about 20% of bone marrow stromal cells (GFP-negative or nongreen cells) [6, 7].

As only nestin-positive bone marrow stromal cells were able to differentiate into functional neurons, we performed several proteomic and transcriptomic comparisons that pointed out several characteristics like ErbB3 and Sox10 overexpression in nestin-positive MSCs, suggesting that these cells could actually be neural-crest-derived cells [19]. Few months later, Nogoshi et al. [20] confirmed the presence of neural-crest-derived cells in adult bone marrow.

4. Characterization of Neural Crest Stem Cells from Adult Bone Marrow

4.1. Neural Crest Stem Cell Origin. In early vertebrate development, the neural crest is specified in the embryonic ectoderm at the boundary of the neural plate and the ectoderm. Once specified, the neural crest cells undergo a process of epithelium to mesenchyme transition (EMT) that will confer them the ability to migrate. The EMT involves different molecular and cellular machineries and implies deep changes in cell morphology and in the type of cell surface adhesion and recognition molecules. When the EMT is complete, they delaminate from the neural folds/neural tube and migrate along characteristic pathways to differentiate into a wide variety of derivates (Figure 3) [21].

Takahashi et al. [1] was the first to address the biological origin of MSCs and showed that they are generated in waves, with the neuroepithelium unexpectedly providing the first wave and a second wave of nonneural-derived MSCs taking precedence in the adult [22]. Indeed, using protocols that differentiate ES cells to mesodermal versus neural/neural crest lineages, they demonstrated that both lineages generated PDGFRa-positive cells (a marker for MSC) that could make adipocytes. However, the surprise came when they found that the neural, but not mesodermal, differentiations contained MSCs that could proliferate extensively as multipotent clones. Moreover, these MSCs were generated from cells expressing Sox1, a definitive marker for neuroepithelium, demonstrating their neural origin. Thus, for ES cells, differentiation along a mesodermal pathway did not generate MSCs, but differentiation toward a neural/neural crest fate did.

In order to address the *in vivo* relevance of these findings,Takahashi et al. [1] used a transgenic mice expressing GFP under Sox1 promoter. They then isolated the trunk of these embryos at E9.5 (thereby excluding the cranial neural crest, which is known to generate mesenchymal cells) and demonstrated that Sox1-GFP-positive cells gave rise to PDGFRa-positive MSC. In contrast, GFP-negative, PDGFRa-positive cells (which expressed mesodermal markers) did not generate MSCs, although they did make adipocytes. Thus,

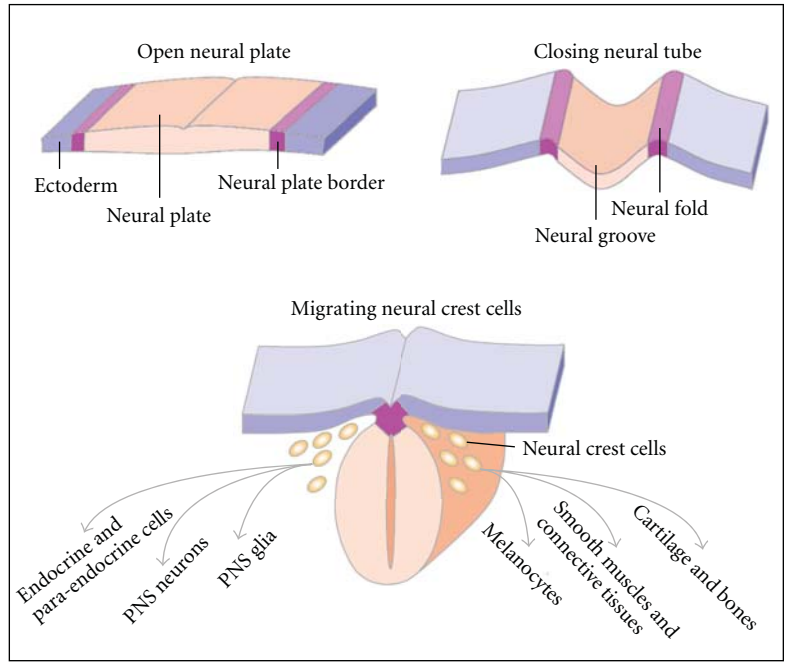

Figure 3: Neurulation and neural crest migration. As neurulation proceeds, the neural plate rolls up and the neural plate border becomes the neural folds. Near the time of neural tube closure (depending on the species), the neural crest cells go through an epithelial to mesenchymal transition (EMT), delaminate from the neural folds or dorsal neural tube, and migrate along defined pathways.

Table 2: Presence of neural-crest-derived cells in adult tissues.

Place	Marker	Animal	Genotype	Reference
Gut	P75NTR	Rat	Wild type	[23]
DRG		Rat	Wild type	[24]
DRG, Whisker pad, bone, marrow	EGFP	Mouse	P0 Wnt1-CRE/CAG-EGFP	[20]
Skin		Mouse	Wild type	[22]
Skin	Lacz	Mouse	Wnt1-CRE/ROSA-Lacz	[25]
Skin	EYFP	Mouse	Dct-Cre/ROSA-EYFP	[26]
Cornea	EYFP	Mouse	P0 Wnt1-CRE/CAG-EGFP	[27]
Carotid body	EYFP	Mouse	GFAP promoter-EGFP	[28]

just as seen with ES cells, MSC could be generated from trunk neuroepithelial cells but not from mesodermal cells in mid-gestation embryos. These experiments demonstrated that trunk neuroepithelium could make MSC. To demonstrate that it actually did so, the authors made Sox1-Cre/YFP mice in which the progeny of Sox1-positive neuroepithelial cells were persistently labeled and confirmed the presence of YFP cells in adult bone marrow.

In parallel, using a two-component genetic system based on Cre/lox recombination to label indelibly the entire mouse neural crest population at the time of its formation [29], several groups used *Wnt1-Cre/R26R* double transgenic mice, in which virtually all neural crest stem cells express β-galactosidase, to identified NCSC in various tissues. Indeed, using this transgenic model, Sieber-Blum et al. [25] demonstrated the presence of pluripotent neural crest stem cells in adult follicle hairs, Wong et al. [26] demonstrated the presence of neural crest cells in the mouse adult skin, and Nagoshi et al. [20] confirmed the presence of NCSC in adult bone marrow (Table 2).

4.2. Self-Renewal Ability and Multipotency of Adult Bone Marrow NCSC. To consider NCSC from adult bone marrow as a potential source for cellular therapy protocol, a better characterization of those cells was mandatory. In our study, we first address the self-renewal ability, as first characteristic of stemness. Indeed, we demonstrated that NCSCs were able to grow as spheres, which is one of the main hallmarks of immature neural cells and proliferate from a single cell culture (clonal culture). We then addressed

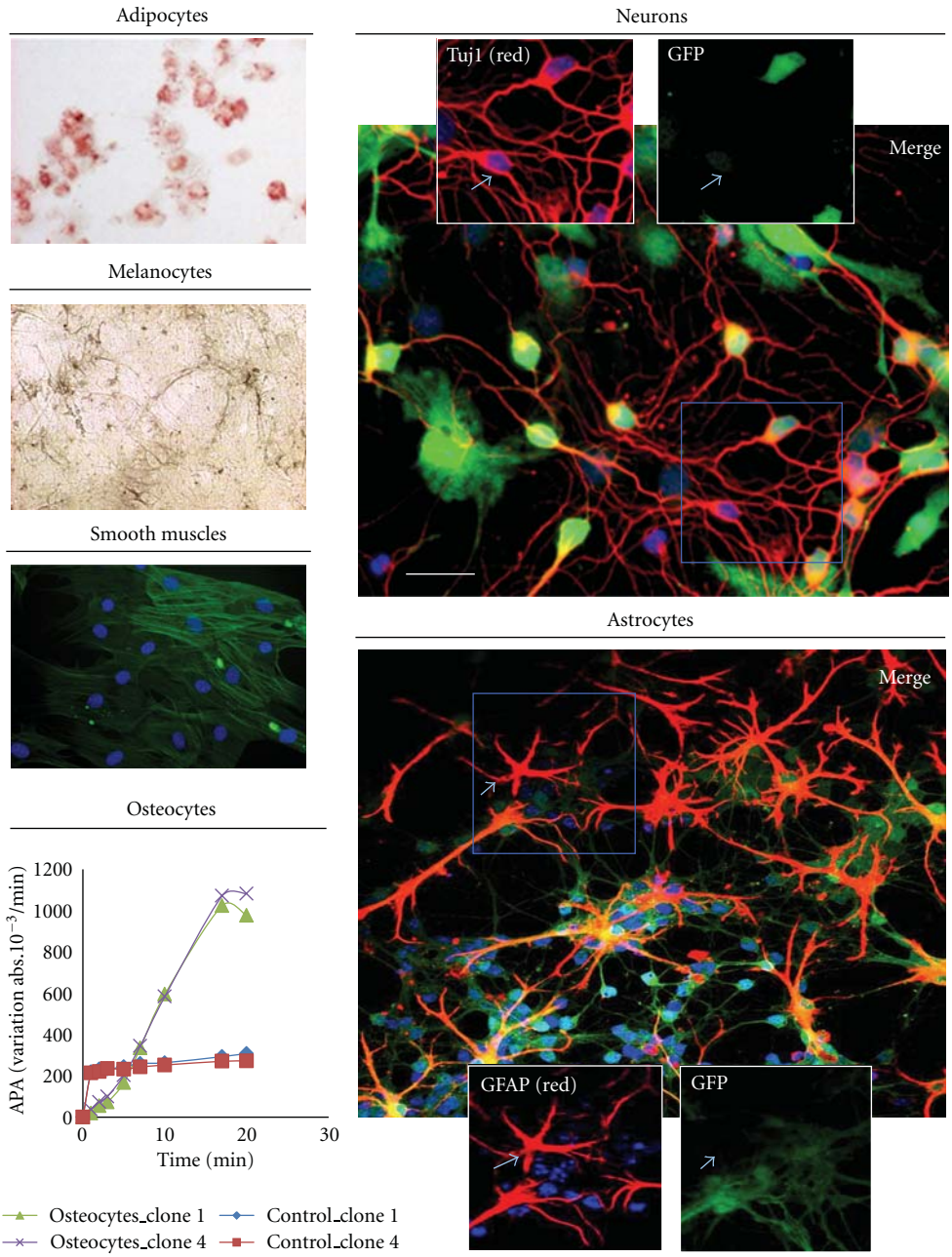

Figure 4: Multipotency of adult bone marrow NCSC. NCSC clones were subjected to differentiating protocols and were shown to be able to differentiate into adipocytes (Oil Red O labeling), melanocytes (L-DOPA labeling), smooth muscles (SMA-labeling), and osteocytes (alkaline phosphatase activity). Moreover, when co-cultured with cerebellar granule neurons, we were able to differentiate NCSC clones into neurons (beta-III tubulin labeling by Tuj1 monoclonal antibody) or glial cells (GFAP labeling).

the multipotency and verified if those NCSC clones were able to differentiate into multiple mature cell types. Indeed, we observed that NCSC were able to differentiate into adipocytes, melanocytes, smooth muscles, osteocytes, neurons, and astrocytes (Figure 4) [30].

4.3. Maintenance and Proliferation of Adult Bone Marrow NCSC. Before using NCSC from adult bone marrow, we have to face some limiting factors like the fact that NCSCs are a minority population (less than 1%) in adult bone marrow. As Wnt1 and BMP2 factors were described to help for maintenance and proliferation of NCSC isolated from embryo [31], we tested those two factors, on adult NCSC isolated from adult bone marrow. Interestingly, we demonstrated that Wnt1 and BMP2 were able to increase the number of NCSCs present in bone marrow stromal cell culture, up to four times within 2 passages [30] reaching 20% of NCSC.

5. *In Vivo* Characterization of Neural Crest Stem Cells and/or Bone Marrow Stromal Cells in Neurological Disorder Mice Models

5.1. Spinal Stroke. Among others, the spinal cord is the collection of fibers that runs from or to the brain through the spine, carrying signals from or to the brain to or from the rest of the body. Those signals control a person's muscles and enable the person to feel various sensations. The main consequence of injuries to the spinal cord is the interference with those signals. Those injuries are characterized as "complete" or "incomplete": if the injured person loses all sensation and all ability to control the muscles below the point of the injury, the injury is said "complete"; in the case of an "incomplete" injury, the victim retains some ability to feel sensations or control movement below the injured area.

Main goals in spinal cord repair include reconnecting brain and lower spinal cord, building new circuits, remyelination of demyelinated axons, providing trophic support, and bridging the gap of the lesion [32]. Overcoming myelin-associated and/or glial-scar-associated growth inhibition are experimental approaches that have been most successfully studied in *in vivo* experiments. Further issues concern gray matter reconstitution and protecting neurons and glia from secondary death [32].

In this purpose, neural crest stem cells isolated from the bulge of hair follicle have been grafted in rat model of spinal cord lesion [33]. Those cells survived, integrated, and intermingled with host neurites in the lesioned spinal cord. NCSC were nonmigratory and did not proliferate or form tumors. Significant subsets of grafted cells expressed the neuron-specific beta-III tubulin, the GABAergic marker glutamate decarboxylase 67 (GAD67), the oligodendrocyte markers RIP, or myelin basic protein (MBP) [25]. More interestingly, functional improvement was shown by two independent approaches, spinal somatosensory-evoked potentials (SpSEP) and the Semmes-Weinstein touch test [34]. The strength of NSCS was fully characterized as they can exert a combination of pertinent functions in the contused spinal cord, including cell replacement, neuroprotection, angiogenesis, and modulation of scar formation. However, those results have never been confirmed with human NCSC, which should be the next promising step.

Similar studies were previously performed with bone marrow stromal cells. Indeed, several researches reported the antiproliferative, anti-inflammatory, and anti-apoptotic features of bone marrow stromal cells [35]. Indeed, Zeng et al. [36] demonstrated that BMSC seeded in a three dimensions gelatin sponge scaffold and transplanted in a transected rat spinal cord resulted in attenuation of inflammation, promotion of angiogenesis, and reduction of cavity formation. Those BMSCs were isolated from 10 weeks old rats and passaged 3 to 6 times. Likewise, Xu et al. [37] demonstrated that a co-culture of Schwann cell with BMSC had greater effects on injured spinal cord recovery than untreated BMSC. Indeed, analyses of chemokine and cytokine expression revealed that BMSC/Schwann cell co-cultures produced far less MCP-1 and IL-6 than BMSCs or Schwann cells cultured alone. Transplanted BMSC may thus improve recovery in spinal cord injured mice through immunosuppressive effects that can be enhanced by a Schwann cell coculturing step. These results indicate that the temporary presence of BMSC in the injured cord is sufficient to alter the cascade of pathological events that normally occur after spinal cord injury and therefore generating a microenvironment which favours an improved recovery. In this study, BMSCs were isolated from adult mice and used after 4 passages.

5.2. Krabbe's Disease. Krabbe's disease, a demyelinating disorder caused by mutations in the lysosomal enzyme galactocerebrosidase (GALC), is a disorder of the nervous system where cell transplantation is the only available therapy [38]. In this leukodystrophy, apoptosis of myelin-forming oligodendrocytes and Schwann cells is caused by accumulation of a GALC substrate, galactosylsphingosine (psychosine), which causes a severe demyelination of both the peripheral (PNS) and central (CNS) nervous systems. Effective treatment of Krabbe's disease is challenging given the rapid decline of patients and the need to correct both the PNS and CNS.

So far, the most effective treatment for Krabbe's patients is hematopoietic stem cell (HSC) transplantation, which supplies the missing enzyme to the nervous system; however, this option showed only a mild and temporary beneficial effect on peripheral nerves. As a consequence of a lack of appropriate treatment, a recent study analyzed the therapeutical properties of MSCs in such a disease [38]. The authors demonstrated that MSCs had a multilevel mechanism of action targeting neurons, Schwann cells, and macrophages that coordinately promoted recovery of nerve pathology following intravenous transplantation, demonstrating that MSC could also be used in peripheral nervous system pathology.

5.3. Multiple Sclerosis. Multiple sclerosis (MS) is a common neurological disease and a major cause of disability, particularly affecting young adults. It is characterized by patches of damage occurring throughout the brain and spinal cord with loss of myelin sheaths accompanied by loss of cells that make myelin (oligodendrocytes) [39]. In addition, we now know that there is damage to neurons and their axons too, and that this occurs both within these discrete patches and in tissue between them. The cause of MS remains unknown, but an autoimmune reaction against oligodendrocytes and myelin is generally assumed to play a major role, and early acute MS lesions almost invariably show prominent inflammation. Efforts to develop cell therapy of nervous system lesion in MS have long been directed towards directly implanting cells capable of replacing lost oligodendrocytes and regenerating myelin sheaths.

To our knowledge, no experiment has been performed to characterize the effect of neural crest stem cells on the improvement of multiple sclerosis disease; however, several data can be collected concerning the positive effect of Schwann cells (derived from NCSCs) and of bone marrow stromal cells.

As previously described in injured spinal cord, bone marrow stromal cells have been characterized on their antiproliferative, anti-inflammatory, and antiapoptotic features. These properties have been exploited in the effective treatment of experimental autoimmune encephalomyelitis (EAE), an animal model of multiple sclerosis where the inhibition of the autoimmune response resulted in a significant neuroprotection [35]. Based on recent experimental data, a number of clinical trials have been designed for the intravenous (IV) and/or intrathecal (ITH) administration of BMSCs in MS patients [40].

5.4. Parkinson's Disease. Parkinson's disease (PD) is a chronic, progressive neurodegenerative disorder characterized by a continuous and selective loss of dopaminergic neurons in the *substantia nigra pars compacta* with a subsequent reduction of dopamine release mainly in the striatum. This ongoing loss of nigral dopaminergic neurons leads to clinical diagnosis mainly due to occurrence of motor symptoms such as rigidity, tremor, and bradykinesia, which result from a reduction of about 70% of striatal dopamine [41].

Levy et al. [42] analyzed the effect of differentiated human BMSC onto dopaminergic precursor on hemi-Parkinsonian rats, after transplantation into striatum. This graft resulted in improvement of rat behavioral deficits quantified by apomorphine-induced rotational behavior. The transplanted induced neuronal cells proved to be of superior benefit compared with the transplantation of naive BMSC. Immunohistochemical analysis of grafted brains revealed that abundant induced cells survived the grafting procedure and some of these cells displayed dopaminergic traits.

Similarly, authors in [43] isolated and characterized MSCs from Parkinson's disease (PD) patients and compared them with MSCs derived from normal adult bone marrow. These authors show that PD-derived MSCs are similar to normal MSCs in phenotype, morphology, and differentiation capacity. Moreover, PD-derived MSCs are able of differentiating into neurons in a specific medium with up to 30% having the characteristics of dopamine cells. At last, PD-derived MSCs could inhibit T-lymphocyte proliferation induced by mitogens. These findings indicate that MSCs derived from PD patients' bone marrow could be a promising cell type for cellular therapy and somatic gene therapy applications.

5.5. Huntington's Disease. Huntington's disease (HD) is an autosomal dominant genetic disorder caused by the expansion of polyglutamine encoded by CAG repeats in Exon 1 of the *IT15* gene encoding for Huntingtin (Htt). The polyglutamine repeat length determines the age of onset and the overall level of function but not the severity of the disease [44]. Although the exact mechanism underlying HD disease progression remains uncertain, the hallmark of this disease is a gross atrophy of the striatum and cortex and a decrease of GABAergic neurons [45].

One strategy for HD therapy is to enhance neurogenesis, which has been studied by the administration of stem/progenitor cells, including BMSCs. Several studies [46] showed that BMSCs promote repair of the CNS by creating a more favorable environment for neuroprotection and regeneration through the secretion of various cytokines and chemokines. Moreover, Snyder et al. [46] demonstrated that BMSC injected into the dentate gyrus of HD mice model increased neurogenesis and decreased atrophy of the striatum.

5.6. Alzheimer's Disease. Alzheimer's disease (AD) is the most common form of dementia, affecting more than 18 million people worldwide. With increased life expectancy, this number is expected to rise in the future. AD is characterized by progressive memory deficits, cognitive impairment, and personality changes associated with the degeneration of multiple neuronal types and pathologically by the presence of neuritic or amyloid plaques and neurofibrillary tangles [47]. Amyloid β-peptide (Aβ) appears to play a key pathogenic role in AD, and studies have connected Aβ plaques with the formation of intercellular tau tangles, another neurotoxic feature of AD [48]. Currently, no treatment is available to cure or prevent the neuronal cell death that results in inevitable decline in AD patients.

The innate immune system is the vital first line of defense against a wide range of pathogens and tissue injuries, triggering inflammation through activation of microglia and macrophages. Many studies have shown that microglia are attracted to and surround senile plaques both in human AD samples and in rodent transgenic models that develop AD-related disease [49]. In this context, Lee et al. [50] demonstrated that treated APP/PS1 mice (mouse model of AD) with BM-MSCs promoted microglial activation, rescued cognitive impairment, and reduced Aβ and tau pathology in the mouse brain.

6. Conclusions

The NCSC is one of the most intriguing cells in the field of regenerative medicine, because it is easily harvested from various accessible peripheral tissues, which could make autologous transplantation possible. Autologous transplantation would avoid immunological complications as well as the ethical concerns associated with the use of embryonic stem cells. Of the various NCSCs, research on skin-derived NCSC is the most advanced mainly due to their easy isolation process. One of the critical questions for the application of NCSC to regenerative medicine is whether cells that are differentiated from NCSCs are functional. Some evidence supports this [51]; however, lots of questions remained pending. By example, a very important question is the differentiation abilities of NCSC isolated from various tissues: are they similar or different?

On the other hand, even if bone marrow stromal cells did not show a strong ability to replace lost neurons in neurodegenerative disorders such as Parkinson's or Huntington's disease, their impact on inflammation modulation or stimulation of endogenous cells were quite remarkable. This impact is also illustrated by a high number of ongoing

clinical trials with these cells [52]. However, the main challenges remain the standardization of cell culture and isolation, to meet the international rules. Indeed, more than ever, it has been demonstrated that bone marrow stromal cells are constituted of an heterogenous population containing multiple stem/progenitor cell types including mesenchymal stem cells and neural crest stem cells, among others. Most of the studies describing the effects of BMSCs on inflammation modulation or stimulation of endogenous cells were performed on low passages (<4), which mainly contain MSC and less than 10% of NCSCs. So we could stipulate that most of these effects were probably due to MSCs. However, in a perspective of cell therapy, a strong characterization of the role of each cell type in neuronal recovery seemed mandatory to establish strong and safe protocols.

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Research Article

Amniotic Mesenchymal Stem Cells: A New Source for Hepatocyte-Like Cells and Induction of CFTR Expression by Coculture with Cystic Fibrosis Airway Epithelial Cells

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Cystic fibrosis (CF) is a monogenic disease caused by mutations in the CF transmembrane conductance regulator (CFTR) gene, with lung and liver manifestations. Because of pitfalls of gene therapy, novel approaches for reconstitution of the airway epithelium and CFTR expression should be explored. In the present study, human amniotic mesenchymal stem cells (hAMSCs) were isolated from term placentas and characterized for expression of phenotypic and pluripotency markers, and for differentiation potential towards mesoderm (osteogenic and adipogenic) lineages. Moreover, hAMSCs were induced to differentiate into hepatocyte-like cells, as demonstrated by mixed function oxidase activity and expression of albumin, alpha1-antitrypsin, and CK19. We also investigated the CFTR expression in hAMSCs upon isolation and in coculture with CF airway epithelial cells. Freshly isolated hAMSCs displayed low levels of CFTR mRNA, which even decreased with culture passages. Following staining with the vital dye CM-DiI, hAMSCs were mixed with CFBE41o- respiratory epithelial cells and seeded onto permeable filters. Flow cytometry demonstrated that 33–50% of hAMSCs acquired a detectable CFTR expression on the apical membrane, a result confirmed by confocal microscopy. Our data show that amniotic MSCs have the potential to differentiate into epithelial cells of organs relevant in CF pathogenesis and may contribute to partial correction of the CF phenotype.

1. Introduction

Human placenta may represent a fruitful reserve of stem cells for regenerative medicine. Amniotic epithelial cells (hAECs) and amniotic mesenchymal stromal cells (hAMSCs) are known to have unique characteristics, such as derivation from early embryological development, low level expression of major histocompatibility complex antigens, and a less-restricted differentiation potential [1]. In culture, hAECs and hAMSCs can differentiate toward "classic" mesodermal lineages (osteogenic, chondrogenic, and adipogenic), as well as toward cell types of all three germ layers-ectoderm, mesoderm, and endoderm (reviewed in [2, 3]). Because the amniotic membrane is discarded after delivery, it is easy to obtain without harming mothers or babies and would thereby overcome the ethical issues associated with the use of embryonic stem cells. Based on these considerations, human amniotic membrane/amnion-derived cells are considered to be a useful biological material and also a novel cell source for cell transplantation. The availability of hAECs and hAMSCs and

the lack of ethical concerns for this source of stem cells are considered advantageous for their widespread use and acceptance.

Cystic fibrosis (CF) is a lethal autosomal recessive disorder due to mutations in the CF transmembrane conductance regulator (CFTR) gene, a cAMP-dependent chloride channel expressed on the apical side of epithelial cells [4]. Although CF involves many organs with secretory/absorptive properties, including the liver, the main cause of morbidity and mortality is a chronic inflammatory lung disease. Because of its monogenic nature, and since the lung is easily accessible, CF has been a target disease for gene-based therapeutic intervention; however, this approach has given unsatisfied results in terms of efficiency of gene delivery to the lung and of efficacy outcomes [5]. This partial success was due to the inefficiency of passing the mucus barrier overlying the epithelial cells and to the immune response against the gene therapy vectors [6]. Cell therapy could be a more effective treatment because allogenic normal cells and autologous engineered cells express CFTR gene. Bone marrow-derived stem cells have been the first source evaluated for homing to the lung and curative potential, but the in vivo efficiency of bone marrow stem cells to differentiate in airways epithelium is very low (0.01–0.025%) [7], as also demonstrated by different studies in CF mice [8, 9].

Recently, new cell sources for CF treatment have been characterized; MSCs from cord blood [10] and amniotic fluid stem cells [11] can differentiate in vitro and in vivo in airway epithelium. Stemming from these results on MSCs, and based on the demonstrated high plasticity of amniotic-derived stem cells, after an extensive characterization of the expression of phenotypic and pluripotency markers by hAMSCs and their differentiative potential, we preliminarily evaluated their usefulness in CF by in vitro experiments using cocultures of hAMSCs and CF-respiratory epithelial cells.

2. Materials and Methods

2.1. Isolation and Culture of Human Amniotic Mesenchymal Stromal Cells. Human amniotic mesenchymal stromal cells (hAMSCs) were isolated from term placentas ($n = 3$) which would normally be discarded after delivery. Tissues were obtained under appropriate Ethical Committee approval and signed informed consent. All infectious pathogen-positive deliveries including those involving HBV, HCV, and HIV, as well as cases of prediagnosed genetic abnormalities, were excluded. Placenta samples were procured immediately after delivery and processed under sterile conditions. After peeling from the placenta and washing with calcium- and magnesium-free HBSS (CMF-HBSS, Lonza, Treviglio, Italy) supplemented with 0.5 mM EGTA (Sigma, Milan, Italy), amnion membranes were processed to remove epithelial cells as previously reported [12]. Once epithelial cells were removed, the amniotic membranes were digested in order to collect hAMSCs [13]. Briefly, amniotic membranes were washed three times with cold HBSS, cut into pieces, and transferred into 50-mL centrifuge tubes, containing about 30–40 mL of digestion solution composed by EMEM (Lonza) supplemented with 25 mM HEPES buffer without L-glutamine (Lonza), 1 mg/mL collagenase type IV, and 25 μg/mL DNase I (both from Sigma, Milan, Italy). Membranes were incubated on a rotator between 45 min to 1.5 h, depending on tissue thickness, at 37°C. After blocking the enzymatic reaction with cold HBSS, cell suspensions were centrifuged 2 times for 5 min at 200 ×g, 4°C and counted using a Bürker chamber.

After isolation, DNA was obtained from hAMSCs and hAECs by phenol/chlorophorm extraction. Purified DNA was investigated for most frequent mutations in CFTR gene using a commercial kit (Inno-Lipa CFTR19, Inno-Lipa CFTR17+TnUpdate, Inno-Lipa CFTR-Italian Regional-Innogenetics, Ghent, Belgium).

hAMSCs were plated at a density of 1×10^5 cells per cm^2 in standard culture medium composed by DMEM (Lonza) supplemented with 1% sodium pyruvate, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 1% nonessential amino acid, 55 μM β-mercaptoethanol (all by Invitrogen, Milan, Italy), 1% L-glutamine, 1% antibiotics solution (both by Cellgro, Manassas, VA, USA), and 10 ng/mL epidermal growth factor (EGF, Sigma), according to the previously reported protocol [13]. Medium was replaced 2 h after plating in order to remove unattached contaminating epithelial cells and then every 2 days.

Every time cells reached 80% of confluence, cells were detached with trypsin-EDTA (Invitrogen), washed, counted with a Bürker chamber, and replated in a new plastic flask at a density of 1×10^5 cells per cm^2 in order to calculate their growth curve. Doubling time was calculated inserting times and cell counts on the website http://www.doubling-time.com/compute.php.

2.2. Characterization of hAMSCs

2.2.1. Flow Cytometry. Flow cytometry analyses of hAMSCs were performed immediately after dissociation and at second culture passage as previously described [14]. Briefly, cells were detached from culture flask using trypsin and, after washing, were incubated with 4% normal mouse serum/PBS/NaN_3 for 20 minutes at 4°C in order to block nonspecific sites on cell membrane. Cells were then stained in the dark at 4°C for 20 minutes with 7-amino actinomycin-D (7AAD) to discriminate viable cells from fragments and dead cells and with the following monoclonal antibodies (moabs): against CD13, CD29, CD31, CD34, CD44, CD45, CD49f, CD73, CD90, CD105, CD146, CD166, EpCAM, SSEA4 (all from Becton Dickinson Biosciences, BD, Franklin Lakes, NJ, USA), and CD133-1 (Miltenyi Biotech, Bergisch Gladbach, Germany). Moabs were conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE) or PE-Cyanin 7 (PE-Cy7) or allophycocyanin (APC) or APC-Cyanin 7 (APC-Cy7).

For internal labelling, cells were fixed at room temperature with 4% paraformaldehyde (PFA) for 10 min and permeated with 100% ethanol for 2 min after washing with PBS. Cells were incubated with 10% FBS to block nonspecific binding, followed by primary antibodies against Oct-4 and Nanog (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for 1 h; secondary PE-conjugated antibody (Invitrogen) was applied for 30 min. The fluorescence threshold between negative and positive cells was set on the basis of the reactivity of

appropriate nonspecific fluorochrome-conjugated isotypic controls. At least, 10^6 cells were finally analysed using a FACSCanto II equipped with FACSDiva software (BD).

2.2.2. Immunofluorescence Microscopy of Cultured Cells. Plated cells were stained as reported elsewhere [14]. Fixed (4% PFA or 70% ethanol for 30 min) and permeabilized (HEPES-Triton X-100 buffer 0.25% in PBS for 20 min) cells were incubated with a blocking buffer containing 0.5 M NaCl, 20 mM $NaHPO_4$, 0.1% Triton X-100, and 30% horse and goat serum for 30 min (all reagents were from Sigma) and then immunostained with the following primary moabs: anti-EpCAM, cytokeratin (CK)18, alpha-fetoprotein (Sigma), CK19 (Novocastra, Newcastle, UK), albumin (DakoCytomation, Milan, Italy), CK7, CD49f, CD29, S100A4, CD90, CD31, CD146, zonula occludens-1 (ZO-1), fibronectin, alpha1-antitrypsin, E-cadherin, and beta-catenin (BD) for 2 h. After washing, cells were incubated with the appropriate secondary FITC or Texas Red-conjugated antibodies (BD) for 1 h in the dark. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, Sigma) for 5 min in the dark.

Images were taken using a Leica Microsystems DM IRE 2 microscope and analysed with the FW4000I software (Leica Microsystems, Milan, Italy).

2.2.3. Reverse-Transcriptase (RT)-Polymerase Chain Reaction (PCR). CFTR mRNA expression was investigated by semiquantitative RT-PCR. Total RNA was isolated from freshly isolated and cultured cells with TRIzol® Reagent (Invitrogen), according to the manufacturer's protocol. One μg of RNA was reverse transcribed into first strand cDNA with the High-Capacity cDNA Reverse Transcription kit (Applied Biosystems) using random primers following manufacturer's instructions. In order to analyze the expression of CFTR gene, 100 ng of cDNA was used in a final volume of 25 μL with 200 nM dNTP, 10 pM of each outer primer (Table 1), 0.3 U Taq-DNA-polymerase, reaction buffer, and $MgCl_2$ (Invitrogen). A second nested PCR was performed using inner primers (Table 1). Cycling conditions consisted of 95°C for 30 seconds, annealing at 60°C for 1 min and elongation at 72°C for 2 min. Cycle numbers consisted of 35 cycles. cDNA from nasal brushing from healthy control was used as positive control for CFTR analysis; no reverse-transcribed sample was used as negative control.

In parallel, β-actin was used as house-keeping gene (Table 1). PCR products were evaluated on 1.5% agarose gel electrophoresis.

2.3. Differentiation of hAMSCs Towards Different Lineages

2.3.1. Adipogenic and Osteogenic Differentiation. To induce adipogenic and osteogenic differentiation, cells at passages 1–3 were harvested and plated on tissue culture dishes (BD) at a density of 4×10^3 cells per cm^2. Cells were then treated with either adipogenic or osteogenic differentiation media (Lonza) for three weeks. The adipogenic protocol consisted of 4 rounds of adipogenic induction medium for 2 days followed by adipogenic maintenance medium for 3 days. The presence of adipose elements in induced cultures was determined by Oil-Red-O (Sigma) staining as follow: cells were washed in PBS, then fixed in 10% formalin for 1 h, washed in isopropanol 60%, and air dried. Cells were then incubated with Oil-Red-O staining solution for 10 min, then washed several times in PBS, and observed with an inverted microscope Eclipse TS100 (Nikon, Tokyo, Japan) equipped with a DS-FI1 CCD camera (Nikon).

In order to induce osteogenesis, cells were treated with osteogenic medium for 3 weeks with medium changes 3 times a week. The presence of calcium deposits in induced cultures was determined by Alizarin Red (Sigma) staining as follow: cells were fixed in 10% formalin for 1 h, then washed in deionized water, and incubated for 30 min at room temperature with Alizarin Red 2% in water at pH 4.2. The cells were finally washed several times to remove the excess of staining and analyzed as described above.

2.3.2. Hepatocyte Differentiation. A simple protocol [15] was used for hepatic differentiation of hAMSCs; cells were plated on type 1 collagen-coated culture dishes in DMEM supplemented with 10% FBS, 1% nonessential aminoacids, 1% L-glutamine, beta-mercaptoethanol, and 10 ng/mL of EGF for 8 days and then with IMDM with the same compounds plus 10^{-7} M dexamethasone (Sigma) for 6 days. One of the functions in cultured hepatocytes is that of the cytochrome P450-dependent mixed function oxidases (MFOs). Diethoxy (5,6) chloromethylfluorescein (Invitrogen) is a probe suitable for use as an in situ stain for MFO activity since this colorless molecule is metabolized in a fluorescent green compound retained in the cells [16]. Five mg of probe was eluted in 1143 μL DMSO (stock solution 10 mmol). Test medium was prepared as follow: 987 μL of RPMI, 12 μL HEPES 1 M (12 mmol final), and 1 μL probe 10 mmol (10 μmol final). Control medium was prepared as follow: 987 μL RPMI, 12 μL HEPES 1 M (12 mmol final), and 1 μL DMSO. The cells were washed in PBS and incubated with the test (or control) medium for 2 h at 37°C in a CO_2 incubator. Cells were analyzed using a Leica Microsystems DM IRE 2 microscope.

After differentiation for 21 days (8 days in DMEM supplemented as above +13 days in IMDM supplemented with dexamethasone), cells were stained by means of immunofluorescence as reported above in order to verify the expression of epithelial markers.

2.4. hAMSC Labelling. Passage two hAMSCs were labeled with chloromethylbenzamido (CellTracker CM-DiI) [17]. Stock solutions of CM-DiI were prepared in dimethylsulfoxide (DMSO) at 1 ng/μL. Immediately before labelling, the stock solution was diluted up to a final concentration of 0.005 ng/μL in DMEM without phenol red. Cells grown at confluence in a T25 flask were washed with phosphate-buffered saline (PBS) and then incubated with the dye working solution for 30 min at 37°C. After labelling, cells are washed twice with PBS, then incubated at 37°C 5% CO_2 for at least 24 h in the presence of fresh medium.

Table 1: Primer sequences for CFTR RT-PCR analysis.

Gene	Forward primer	Reverse primer	Product length (bp)
CFTR			
Outer primers	CGAGAGACCATGCAGAGGTC	GCTCCAAGAGAGTCATACCA	1108
Inner primers	CGAGAGACCATGCAGAGGTC	TGTACTGCTTTGGTGACTTCCCC	301
β-actin	CAACTGGGACGACATGGA	ACGTCACACTTCATGATGGA	610

2.5. Cultures of Airway Epithelial Cells. 16HBE14o- and CFBE41o- are human epithelial bronchial cell lines, wild type and homozygous for the F508del allele (F508del/F508del), respectively, a generous gift of Professor D. Gruenert (University of California at San Francisco, USA). Epithelial cells were grown in MEM, 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin, alone or in Coculture with hAMSCs.

2.6. Coculture of hAMSCs with CFBE41o- Cells. Labelled hAMSCs were mixed with CFBE41o- cells at different ratios (1 : 5, 1 : 10, 1 : 15, and 1 : 20) and, in order to obtain polarized cocultures, cells were seeded on 6.5-mm diameter Snapwell, 0.4-μm pore size (Corning, Acton, MA, USA) at 1×10^5 per filter coated with a solution of 10 μg/mL fibronectin (BD Biosciences, CA, USA), 100 μg/mL albumin from bovine serum (Sigma-Aldrich, Milan, Italy), and 30 μg/mL bovine collagen type I (BD) dissolved in MEM. As controls, hAMSCs and CFBE41o- were seeded at 2.5×10^4 and 1×10^5 per filter, respectively. Cocultures were maintained at 37°C 5% CO_2 for at least 6–8 days.

Separate cocultures were obtained by seeding hAMSCs onto the filter and CFBE41o- cells onto the bottom of the lower chamber. To obtain 1 : 5 and 1 : 10 ratios, hAMSCs were seeded at 2×10^4 and 1×10^4 and CFBE41o- cells at 8×10^4 and 9×10^4, respectively. As controls, hAMSCs were seeded at 1×10^5 per filter. Medium was changed daily in each chamber for 5 days, and cultures were analyzed at day 6.

2.7. CFTR Cytofluorimetric Assay. Cells were detached with trypsin-EDTA treatment and fixed in PBS containing 2% PFA for 5 min. After centrifugation at 250 ×g, the resulting pellets were washed and resuspended in PBS. The cells were then incubated with CFTR antibody MAB25031 mouse IgG2a (R&D Systems, Minneapolis, MN, USA) used at 1 : 20 dilution for 1 h at 4°C. After washing in PBS, the cells were incubated with the FITC-conjugated secondary antibody (anti-mouse used at 1 : 100; Sigma) for 1 h at 4°C, followed by two washes in PBS, and analyzed. As a background control, cocultures were incubated with secondary antibody only, and the resulting fluorescence was subtracted from the analyzed samples incubated both with primary and secondary antibodies. Data were collected using a Coulter Epix XL flow cytometer (Beckman Coulter, Fullerton, CA, USA) and analyzed with WinMDI 2.9 (http://www.cyto.purdue.edu/flowcyt/software/Winmdi.htm). Ten thousand cells were examined in each experiment. Since physical parameters (forward scatter and side scatter) did not allow us to distinguish hAMSCs from CFBE41o- cells, specific expression of CFTR on hAMSCs was detected in the CM-DiI-labelled cells. Analyses were performed by plotting the FLH-1 channel (525 nm) against the FLH-2 channel (575 nm), identifying the CFTR-specific green signal and the red-labelled hAMSCs, respectively. The vitality was evaluated by trypan blue exclusion assay and resulted to be >98%.

2.8. Confocal Analysis of CFTR Protein. Polarized cells were washed three times with PBS and incubated in PBS, 2% BSA for 30 min on ice. Cells were incubated with CFTR antibody MAB25031 diluted 1 : 20 in PBS containing 0.2% BSA for 1 h on ice. Cells were rinsed three times with PBS and incubated with the FITC-conjugated secondary antibody diluted 1 : 100 in PBS added with 0.2% BSA for 30 min on ice. After two washes in PBS, cells were fixed in 3% PFA and 2% sucrose for 10 min. After three washes in PBS, filters were excised and placed side up on a glass slide and overlaid with a drop of Mowiol (Calbiochem, San Diego, CA, USA) followed by a coverslip. Cells were analyzed using a Nikon TE2000 microscope coupled to a Radiance 2100 confocal dual-laser scanning microscopy system (Bio-Rad, Segrate, Italy). Specimens were viewed through a 60x oil immersion objective. Digital images were processed using the program Laser Sharp 2000 (Bio-Rad).

2.9. Statistical Analysis. Statistical significance of differences was evaluated by a two-tailed unpaired Student's t-test. Data were analyzed using Prism 4 (GraphPad Software, Inc., La Jolla, CA, USA). P values of less than 0.05 were considered significant.

3. Results

3.1. Isolation and Characterization of hAMSCs from Human Amnion. At least 33×10^6 hAMSCs (range 26–160 $\times 10^6$) were recovered in each isolation ($n = 3$) with a viability of 85–90%. Inno-lipa screening revealed the absence of most frequent mutation of CFTR (86% of detection rate) in hAMSCs used in this study. After plastic adhesion, hAMSCs were characterized by a fibroblastic morphology very similar to that described for mesenchymal cells isolated from bone marrow (Figures 1(a) and 1(b)) and could be kept in culture until passages 5–10. Proliferation slowed beyond passage two. In the exponential growth phase, approximately two cell doublings were observed over 15 days, giving these cells an average doubling time of 18.03 days calculated over 28 days of culture. An example of a growth curve for hAMSCs is presented in Figure 1(c).

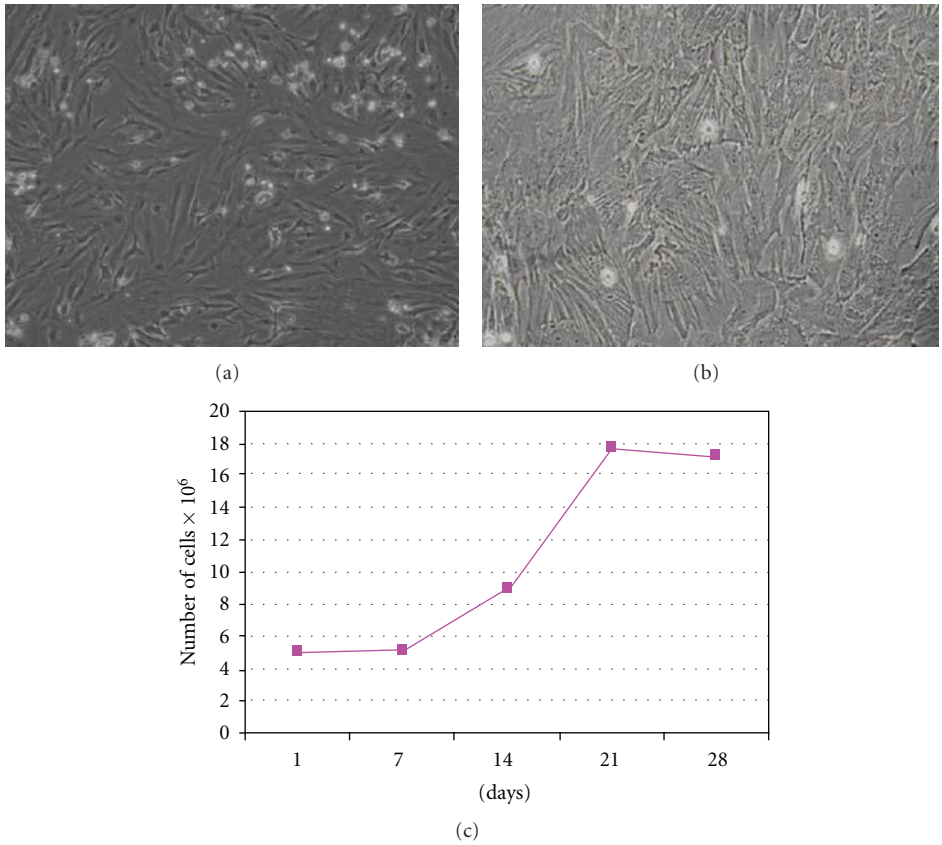

Figure 1: hAMSCs morphology and growth. Cell morphology at passage one (a) and passage three (b), original magnification 10x. Growth kinetics of hAMSCs in culture (c).

3.2. Flow Cytometry and Immunofluorescence Analysis. hAMSCs showed an immunophenotypic profile very similar to that of mesenchymal stem cells derived from bone marrow; that is, they are positive for CD29, CD44, CD73, CD90, and CD105 and negative for the hematopoietic markers CD34, CD133, and CD45. Freshly isolated hAMSCs showed a low expression of epithelial markers (EpCAM and CD49f), which decreased up to null expression after the first passage (Table 2).

hAMSCs showed the embryonic stem cell associated surface marker SSEA4 (Figure 2(f)), while very low expression of molecular markers associated with pluripotent stem cells (Nanog and Oct-4) by flow cytometry was observed (Figures 2(d) and 2(e)).

Fluorescence microscopy confirmed the positivity for CD29 and CD90 and revealed the expression of other mesenchymal markers such as fibronectin and vimentin (Figure 3). hAMSCs were almost negative for ZO-1, a marker of tight junctions and cytokeratin (CK) 7, while stained positive for CK18.

3.3. Cell Differentiation Ability. To determine whether hAMSCs could differentiate into adipocytes, cells were allowed to grow to 70% confluence prior to induction. Morphological changes as well as formation of lipid droplets within the cells were noticeable starting from one week after induction and were visualized by Oil-Red-O staining (Figure 4(b)). Cells maintained in control medium did not show any sign of adipogenic differentiation (Figure 4(a)).

To investigate the osteogenic potential of hAMSCs, cells were cultured under appropriate condition for differentiation. The presence of calcium deposits in induced cultures was determined by Alizarin Red (Figure 4(d)). Cells maintained in control media did not show any change in their morphology and no calcium deposit (Figure 4(c)).

Hepatocyte differentiation of hAMSCs was evaluated after 14 days of induction. Cells were incubated for 2 h with diethoxy (5,6) chloromethylfluorescein. The generation of fluorescent products was evaluated by fluorescence microscopy. Although hAMSCs were of mesenchymal origin, they showed signs of hepatocyte differentiation (Figure 4(f)). Cells maintained in control medium did not show any sign of hepatocyte differentiation (Figure 4(e)).

Moreover, we performed cell immunophenotyping after hepatocyte induction (Figure 5). After hepatocyte differentiation, the number of cells expressing CK7 increased, while some cells expressed albumin and, weakly, alpha1-antitrypsin. Finally, we observed also the presence of CK19- positive cells. No alpha-fetoprotein expression was detected (not shown).

3.4. CFTR mRNA Expression. In order to see whether hAMSCs express CFTR mRNA, a semiquantitative RT-PCR

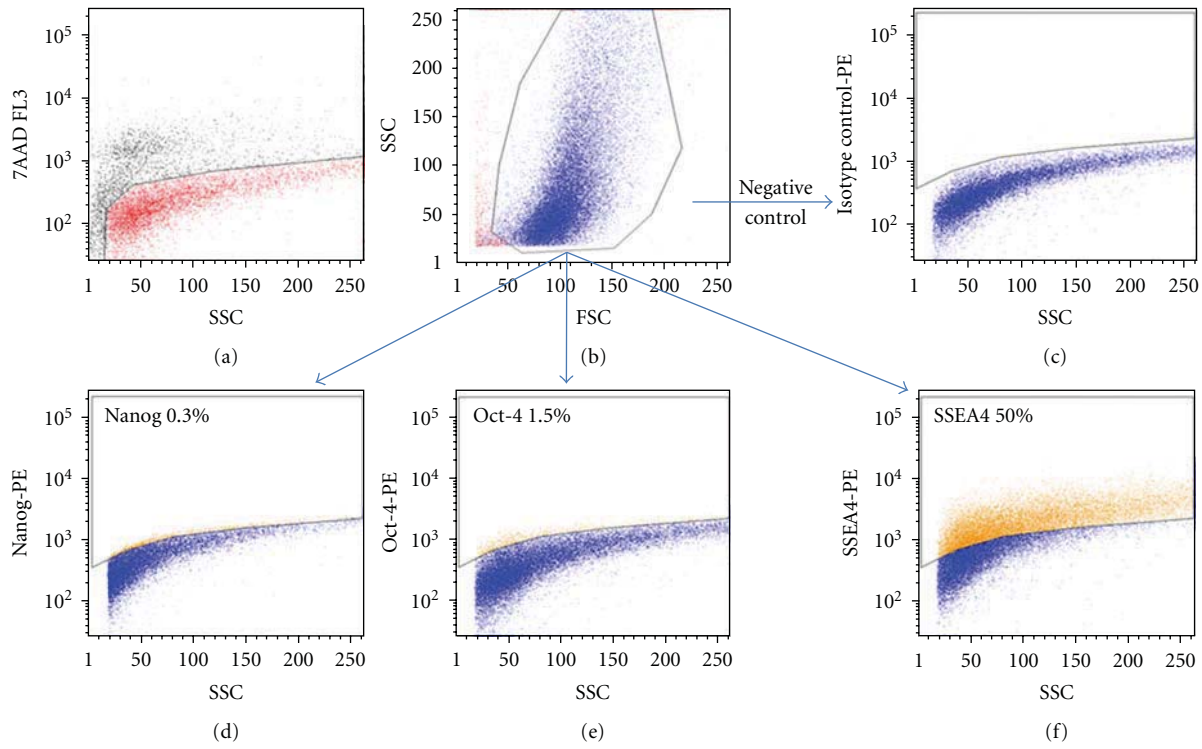

Figure 2: hAMSCs pluripotent stem cells and ESC marker expression. Flow cytometry representative expression of the pluripotent and embryonic stem cells markers in freshly isolated hAMSCs. (a) For each staining, a gate on viable cells (red) was drawn; (b) hAMSCs were gated on the basis of morphological features; (c) cells incubated with isotypic control were used as negative controls; (d) Nanog, (e) Oct-4, and (f) SSEA4 expression in hAMSCs.

Table 2: hAMSCs membrane marker expression.

Surface antigens	Freshly isolated		Passage two	
	Median %	Range	Median %	Range
CD45	2	0–4	4	3-4
CD34	0	0-1	0	0-1
CD133	0	0-1	0	0-1
CD13	80	70–89	95	89–99
CD44	81	71–90	90	89–92
CD73	90	88–91	94	88–99
CD90	79	69–89	94	89–99
CD29	76	66–86	98	95–99
CD105	49	30–66	58	40–76
CD166	83	71–95	85	71–98
CD49f	16	13–31	3	2–5
EpCAM	16	12–20	0	0-1
CD31	0	0	ND	ND
CD146	0	0	ND	ND

Data were expressed as median percentage and ranges of three different experiments.

assay was carried out. CFTR was detected in hAMSCs by RT-PCR only after nested PCR (Figure 6). The expression of CFTR in hAMSC appeared to decrease dramatically during culture. hAECs showed a similar expression of CFTR mRNA when studied upon isolation (Figure 6).

3.5. CFTR Protein Expression by Flow Cytometry. hAMSCs stained with CM-DiI (as described in Materials and Methods section) were mixed with CFBE41o- cells at different increasing ratios (1 : 20, 1 : 15, 1 : 10, and 1 : 5) and seeded onto semipermeable filters. In order to analyze the CFTR protein

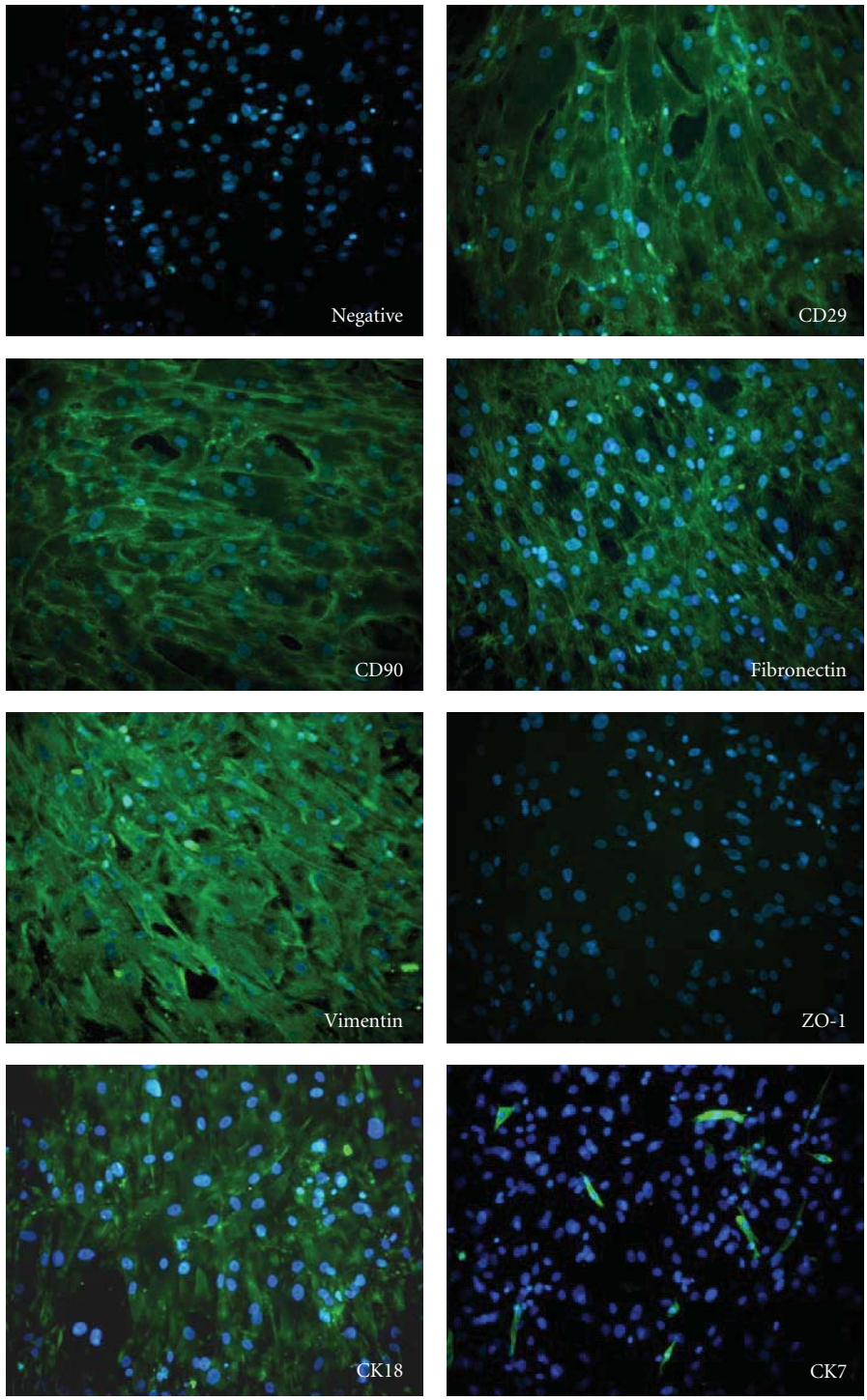

Figure 3: Immunofluorescence characterization of hAMSCs. Representative images of fluorescence microscopy staining. The upper left panel (denoted as "negative") shows cells incubated with the secondary antibody only. hAMSCs were positive for CD29, CD90, fibronectin, vimentin, and CK 18 and negative for ZO-1 and CK 7. Nuclei were counterstained with DAPI. Original magnification 20x.

expression in hAMSC–CFBE41o- cocultures at different ratios, a flow cytometric assay was performed. This mixed population was analyzed after labelling with the CFTR antibody MAB25031 in the absence of permeabilization followed by an incubation with FITC-conjugated secondary antibody. As a positive control, CFTR labelling was assessed in normal human airway 16HBE14o- cells, resulting in 50 ± 5.0% of positive cells, as previously shown [18]. CFBE41o- cells showed less CFTR-specific labelling on the membrane (11% of positive cells), consistent with the lack of CFTR transport on the plasma membrane which is a characteristic of these cells. Plasma membrane CFTR expression was detected in

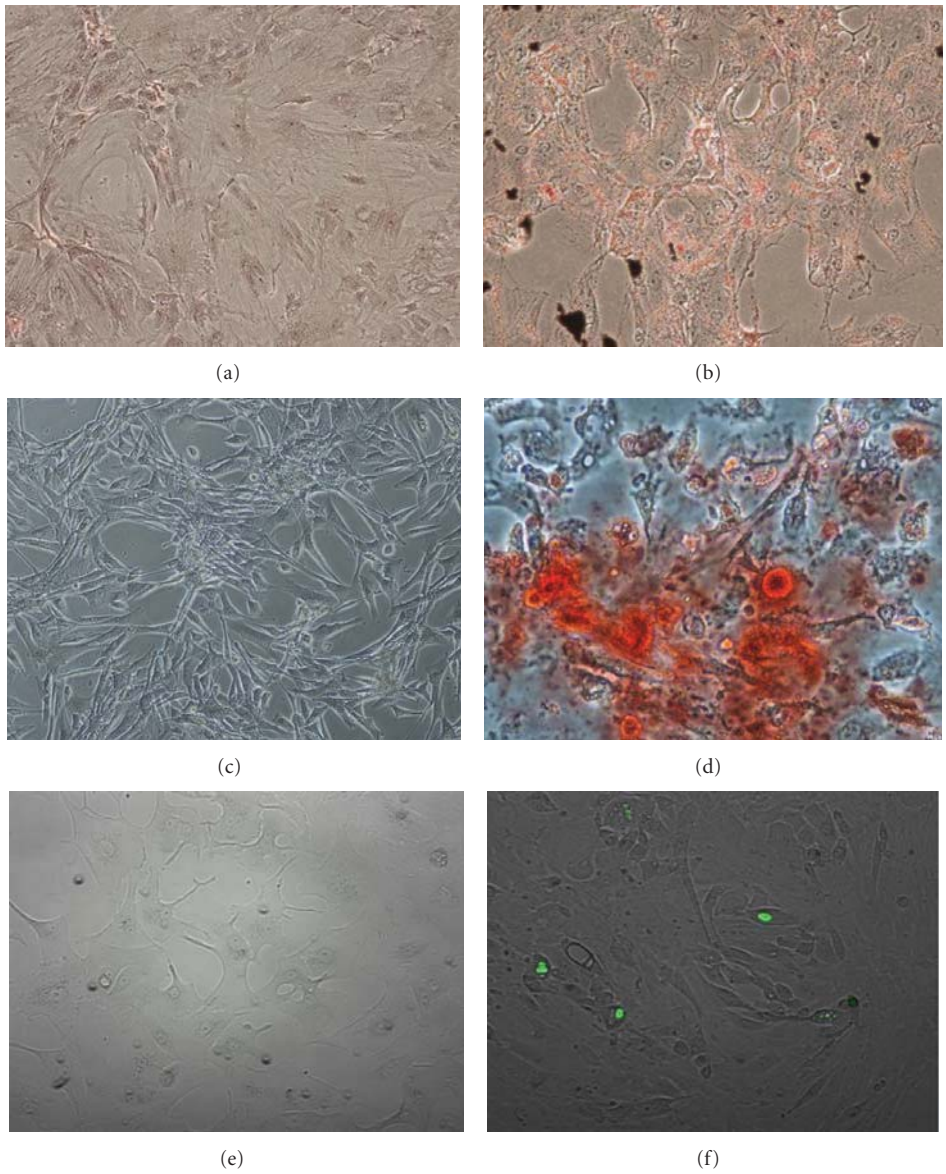

Figure 4: In vitro differentiation capability. Representative images of in vitro osteocyte (b), adipocyte (d), and hepatocyte (f) differentiation of hAMSCs. (a), (c), and (e) represent respective negative controls (i.e., uninduced cells). Original magnification 20x.

only 6.2% of hAMSCs (Table 3). It was possible to detect an increase of CFTR-specific signal in CM-DiI-labeled cells at all hAMSC–CFBE41o- ratios as compared with hAMSCs cells alone. The lower the ratio of hAMSCs : CFBE41o- the lower the increase in CFTR-specific signal in CM-DiI-labeled cells, these data indicating that a critical number of hAMSCs is important in order to obtain a meaningful effect on CFTR expression. Overall, these data show that a population of hAMSCs with low CFTR expression have increased this expression upon cocultures with CF epithelial cells.

To investigate the mechanism underlying the expression of CFTR in hAMSCs after cocultures with CFBE41o- cells, we performed separate cocultures of hAMSCs and CFBE41o- cells. Thus, hAMSCs were grown onto the filter whereas CFBE41o- cells were seeded onto the bottom well. After 6 days of culture, hAMSCs were analyzed for CFTR expression by cytofluorimetry. Results showed that, at the hAMSCs : CFBE41o- ratios of 1 : 5 and 1 : 10, the percentages of $CFTR^+$ hAMSCs were 10.5 ± 3.8 and 11.6 ± 5.0, respectively ($n = 3$). These data, compared with those obtained in direct Coculture conditions (column "% of $CFTR^+$ in whole CM-DiI^+ population" of Table 3), indicate that a direct contact between hAMSCs and CFBE41o- is necessary to obtain a significant increase of CFTR-specific signal in hAMSCs.

3.6. CFTR Expression and Localization by Confocal Microscopy. To confirm cytofluorimetric data and to analyze CFTR expression in cell compartments, hAMSC–CFBE41o- cocultures were assayed by means of confocal microscopy. In previously published work [19], we showed that CFTR protein is expressed on the apical side of 16HBE14o-cells, while

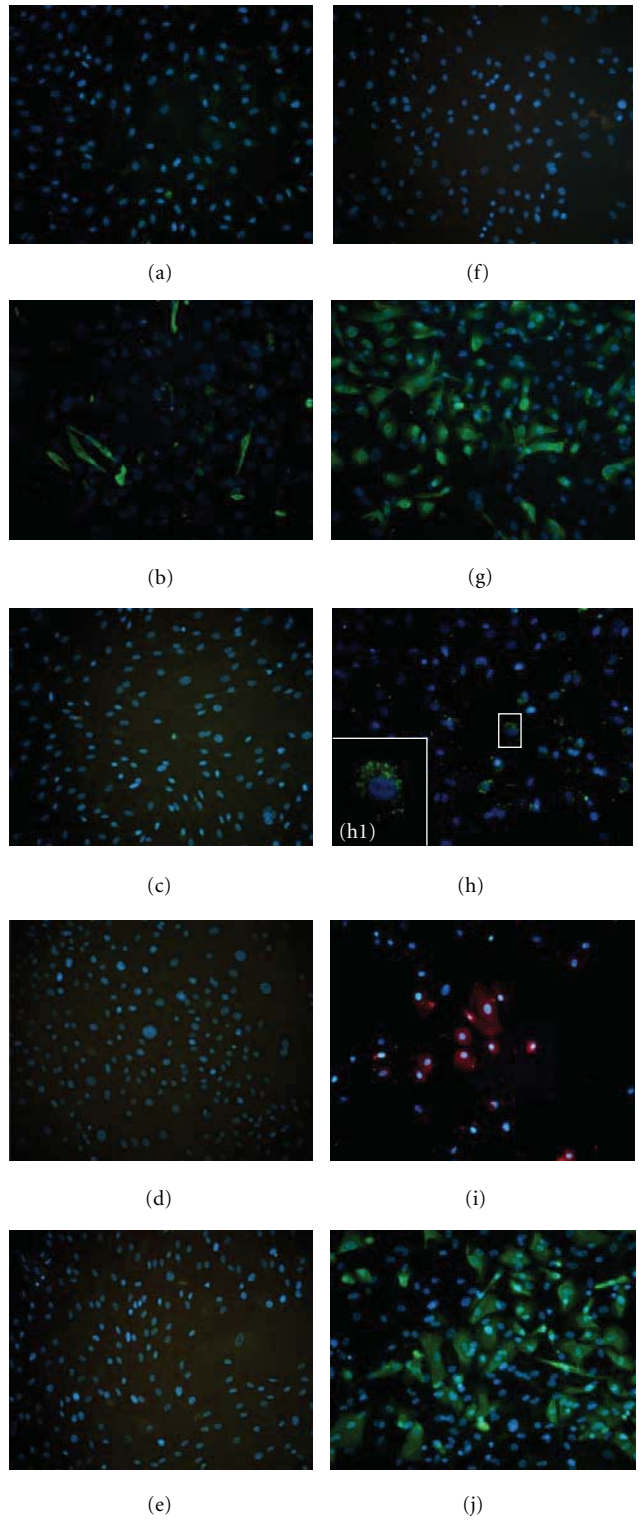

Figure 5: Immunophenotype of mesenchymal stem cells prior (left) and after (right) hepatocyte differentiation for 21 days. (a–f) negative controls, (b–g) CK7, (c–h) albumin, (d–i) alpha1-antitrypsin, and (e–j) CK19. Original magnification 20x. The insert h1 represents an enlargement of the cell highlighted by the white square.

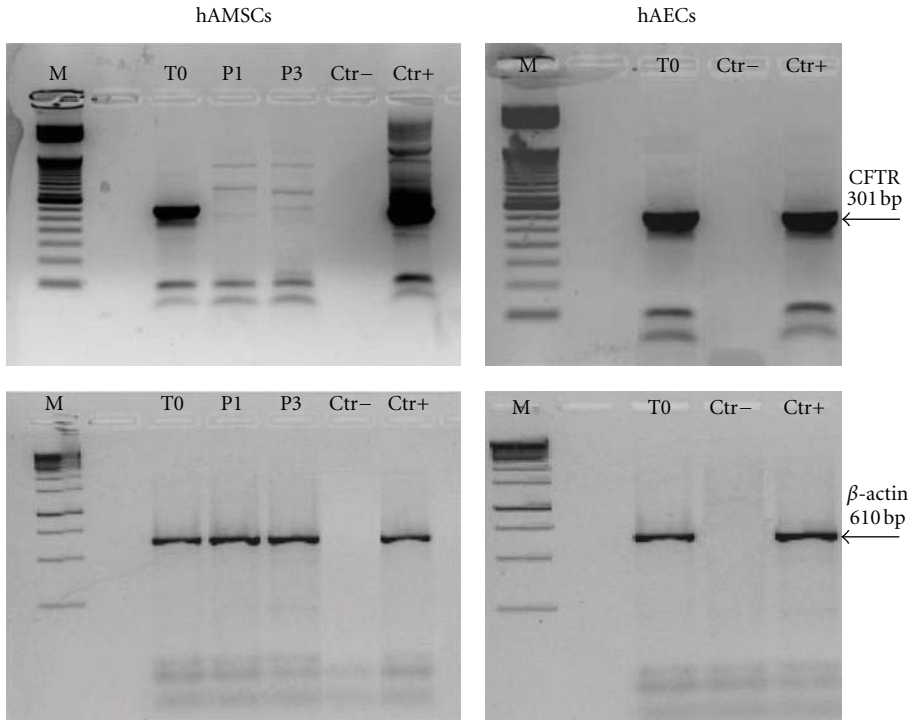

Figure 6: CFTR mRNA expression in hAMSCs and hAECs. CFTR on hAMSCs and hAECs upon isolation (T0) and on hAMSCs at passages one (P1) and three (P3). Upper panels: CFTR; lower panels: β-actin. M: molecular weight markers; Ctr+: positive control (nasal brushing); Ctr−: negative control (no RT). On the right, arrows indicate the specific band along with PCR-product length.

Table 3: Percentages of CFTR$^+$ hMSCs labelled with CM-DiI in cocultures with CFBE41o- cells.

	% of CM-DiI$^+$ CFTR$^+$ cells	% of CFTR$^+$ in whole CM-DiI$^+$ population	P
hAMSCs	—	6.2 ± 2.0	—
CFBE	—	11.2 ± 1.3	0.0006
hAMSC-CFBE 1 : 5	12.1 ± 2.5	50.0 ± 6.1	<0.0001
hAMSC-CFBE 1 : 10	7.5 ± 2.1	46.7 ± 9.3	<0.0001
hAMSC-CFBE 1 : 15	3.0 ± 0.4	33.2 ± 6.5	<0.0001
hAMSC-CFBE 1 : 20	2.2 ± 0.9	34.6 ± 8.7	<0.0001

Percentages of CM-DiI$^+$CFTR$^+$ cells were obtained by plotting the FLH-1 channel, identifying CFTR-specific green signal, against FLH-2 channel, identifying red-labelled hAMSCs. Percentages of CFTR-expressing hAMSCs in whole CM-DiI$^+$ population were obtained by dividing the double positive hAMSCs for all CM-DiI$^+$ cells (with and without green signal). Data are shown as the mean ± SD of five experiments. Significance is referred to CFTR$^+$ cells in the whole CM-DiI$^+$ population in all conditions as compared with hMSCs alone.

CFBE41o- cells display only intracellular staining. CFTR expression and localization was evaluated by epifluorescence with a protocol which allows to detect only surface and not intracellular CFTR (see Materials and Methods section), followed by confocal microscopy analysis. As can be seen in Figure 7, CFTR was highly expressed on the apical membrane of some hAMSCs since red labelled cells showed a green staining at membrane level (Figures 7(b)–7(d)), whereas CFBE41o- monolayers in absence of hAMSCs showed essentially no specific signal for CFTR expression on the membrane (Figure 7(a)), consistent with the lack of CFTR transport to the apical membrane in CF cells. hAMSCs showed a very faint signal related to CFTR (Figure 7(e)). These data confirm cytofluorimetric analysis as to the plasma membrane expression of CFTR in labelled hMSCs which increases when Cocultured with CF cells.

4. Discussion

Human MSCs are pluripotent stem cells initially identified in postnatal bone marrow (BM) [20], which is the most common source used in clinical settings [21]. However, the use of BM has some limitations, including the low frequency of MSCs and the invasive procedure for obtaining them. Moreover, the age and disease state may affect the collection of sufficient healthy autologous BM for transplantation [22–24]. Finally, expansion of autologous BM cells could represent a cumbersome and low-yield approach. In the present study, we directed our attention on a source, the amniotic membrane, which is rich in MSCs [25], is easily accessible and ethically acceptable, since the term placenta is discarded after delivery. hAMSCs have been shown to be superior in proliferation and differentiation potential to BM cells [26] and to

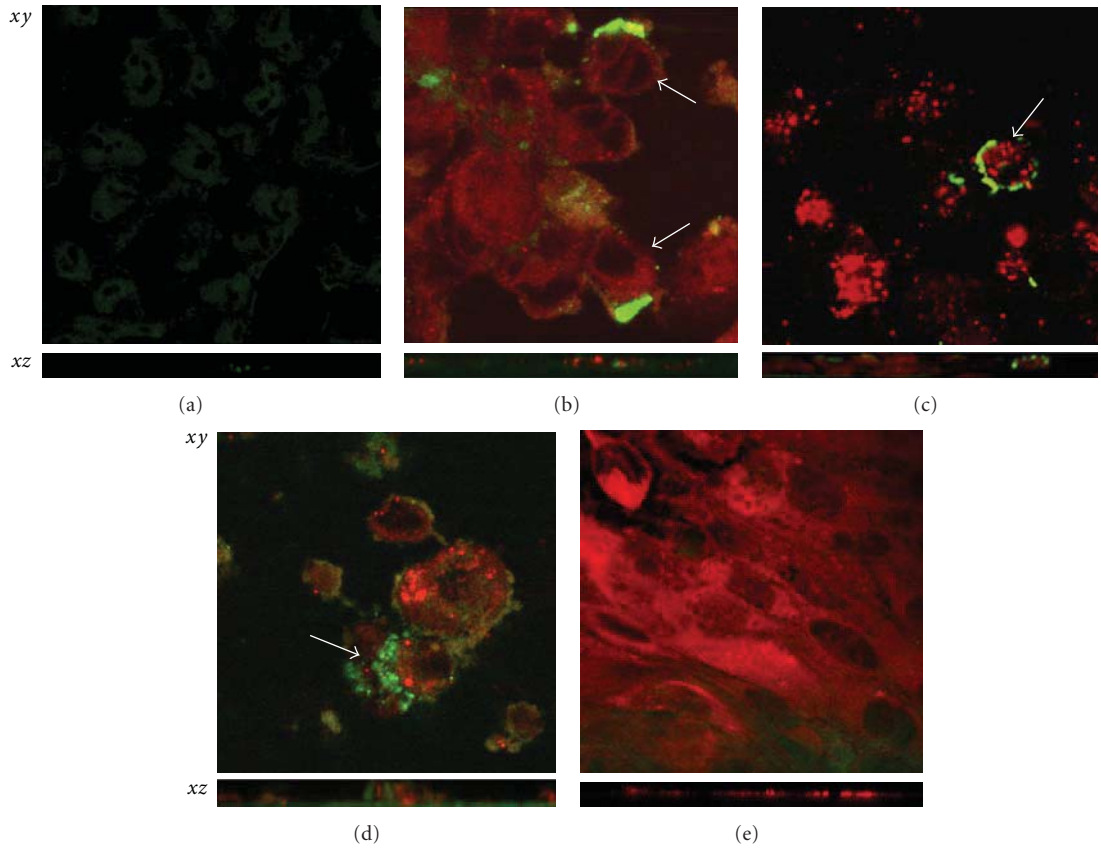

Figure 7: CFTR immunodetection by confocal analysis. Confocal scans are shown in the horizontal cross-section (xy) plane and vertical cross-section (xz) plane. (a) CFBE41o- cells; (b) hAMSCs- CFBE 1 : 5 ratio; (c) hAMSCs- CFBE 1 : 10 ratio; (d) hAMSCs- CFBE 1 : 15 ratio; (e) hAMSCs alone. The white arrows point to CM-DiI-labelled hAMSCs expressing CFTR on their membrane (green signal). Note in (d) that hAMSCs harbour some CFTR-specific signal in discrete regions under the apical plasma membrane.

display differentiation potential towards mesoderm lineages (osteogenic, chondrogenic, and adipogenic) similar to BM cells [25–27]. Importantly, various studies have reported differentiation of hAMSCs to ectoderm (neural) [27, 28], mesoderm (skeletal muscle, cardiomyocytic, and endothelial) [26, 29–31], and endoderm (pancreatic) [32] lineages.

In the present study, we isolated and characterized hAMSCs according to previously published protocols, that is, by removing the epithelial cells by enzymatic digestion and obtaining the hAMSC suspension by collagenase and DNase treatment [26, 27, 30, 33, 34]. hAMSCs show a higher proliferative potential than BM MSCs [26, 35], and, in our culture conditions, they reached a plateau after 21 days in culture, similarly to what has been seen in a previous study [26], while others have observed a plateau already at day 11 [25]. hAMSCs displayed a fibroblastic morphology and presented surface markers expressed also by BM-MSCs and cells isolated from both the amnion and other regions of the full-term placenta such as CD29, CD44, CD105, CD73, CD90, and vimentin and were negative for the hematopoietic markers CD45 and CD34 [26, 27, 35–39]. They also displayed positivity for the epithelial markers CD49f and CK18; since these markers are lost upon culture, they could represent a small contamination by epithelial cells, which has been described also previously [33, 40]. This hypothesis is corroborated by CFTR mRNA expression in hAECs upon their isolation from the placenta (as shown in Figure 6). Interestingly, it has been observed that adherent cells obtained from human amniotic membranes were comprised of both round-shaped epithelial cells and spindle-shaped fibroblast-like cells prior to the first passage, whereas the epithelial cells were rarely detected after the third passage [37]. In alternative, this hybrid phenotype of hAMSCs [41] is interpreted as a sign of pluripotency and suggests that the amnion-derived cells had not completely differentiated into epithelial or mesenchymal cells [3]. Nevertheless, as shown here, the amniotic cells derived from term placenta seem to remain somewhat "plastic" and maintain the capability to differentiate and contribute to cells from different germ layers. Mesodermal differentiation of MSC from various sources was widely reported in the literature and is considered one of the principal assay to prove "stemness" of mesenchymal cells [20, 22, 29]. Not differently from previous papers, hAMSCs isolated in our experiments were able to differentiate into both adipocytes and osteocytes. In recent years, the exploitation of adipose tissue or bone marrow-derived MSC for hepatocyte differentiation and liver repair was explored by many researchers [42–44] but, to the best of our knowledge, only one previous study has demonstrated that hAMSCs can differentiate into hepatocyte-like cells, although only at gene-expression level [37].

Because amniotic cells can differentiate into different cell types, we examined them with antibodies directed against well-known surface markers characteristic of embryonic stem cells. Amniotic cells express the stage-specific embryonic antigen SSEA-4 [45] although the relative proportion of SSEA-4-positive cells in initial isolates is lower than that observed with embryonic stem cells [46]. In addition to characteristic stem cell surface markers, amniotic cells show very low expression of Oct-4 and Nanog, transcription factors involved in regulating ES cells' self-renewal and differentiation, as it has been previously shown for freshly isolated MSCs obtained from bone marrow, adipose tissue, and heart [47]. Further studies are needed to understand whether these genes are regulated during the in vitro culture conditions, so to identify regulatory pathways that mimic in vivo activation.

CF is a potential model disease for stem cell therapy because of the persistent lung inflammation that leads to damage and remodeling and can promote engraftment of stem cells [7]. A developing potential therapeutic approach for CF and other lung diseases has been stimulated by recent reports demonstrating that several cell populations derived from adult bone marrow, from amniotic fluid or from umbilical cord blood, including MSCs, endothelial progenitor cells, and circulating fibrocytes, can localize to the lung and acquire phenotypic and functional markers of mature lung-specific cells [10, 11, 48]. The results published by Wang et al. [49] and Loi et al. [8] strongly suggest that the population of BM cells relevant for repopulating the lung epithelium may be found in the plastic adherent stromal cell compartment. Besides the drawbacks presented by BM-MSCs discussed above, amniotic fluid contains a heterogeneous population of cells from fetal origin [25], whereas MSCs could not be reliably isolated from all term umbilical cordon blood samples [10, 50–52].

In the present study, we propose human placenta as an ethical source of MSCs for CF therapy. The first goal was to investigate the CFTR expression in these cells. At the earliest stages of human development, CFTR protein and function have been detected in early blastocysts in the apical membrane of trophectoderm cells, while its expression at mRNA level has been shown in first trimester placenta (8-week gestation) [53]. However, no data are available concerning its mRNA and protein levels in specific cell types of term placenta. In this study, we show that a nested RT-PCR was necessary for obtaining a detectable signal from freshly isolated hAMSCs, indicating very low levels of CFTR mRNA in these cells. We have recently shown that also hematopoietic stem/progenitor cells display such low levels upon purification from the bone marrow [18]. The reason why CFTR must be kept at low expression levels in stem/progenitor cell compartments is not known at the moment. Although at this moment we do not know whether freshly isolated hAMSCs show CFTR expression or the specific band by RT-PCR is given by an epithelial contamination, CFTR mRNA was barely visible at passages one and three. Confocal microscopy confirmed these results at the protein level. Notably, CFTR was reexpressed by hAMSCs upon Coculture with epithelial cells, as demonstrated unequivocally by flow cytometry and confocal microscope analysis. At this stage, we do not know why the lower the hAMSCs : CFBE41o- ratios the lower the CFTR expression in hAMSCs. It can be speculated that this effect might be due to cross-talk between amniotic and epithelial cells, for which a critical number of hAMSCs are needed. Indeed, in other Coculture systems, developed with MSCs and chondrocytes, it has been shown universally that the more chondrocytes the lower the expression of extracellular matrix genes and functional properties of engineered cartilage [54, 55].

Also, the mechanism underlying this effect is to be discovered yet. However, indirect cocultures data give us an indication that this effect is primarily due to the contact between amnion MSCs and epithelial cells, and not to factors acting by a paracrine manner. Lung morphogenesis is an orchestrated molecular and cellular process controlled by cellular interactions with growth factors and morphogenic factors [56]. Since the cellular interactions between epithelial and mesenchymal cells in monolayer Coculture are likely to be bidirectional, a possible mode of action could be cross-talk between cells via gap junctions, which has been observed in vivo in the lung between transplanted MSCs and resident epithelial cells [57]. Recently, it has been found that MSCs could be induced to differentiate into corneal epithelium [58] or endothelium [59] in Coculture condition, but not in the indirect Coculture system where MSCs and endothelial cells were cultured in separate inserts [59]. More importantly, BM-MSCs acquired an airway epithelium phenotype when Cocultured with respiratory epithelial cells and determined a partial resumption of the chloride secretion defect in CF epithelia [49]. Although we have not analyzed the correction of the chloride transport defect in CFBE14o- monolayers by hAMSCs, based on the work by Wang et al. [49], it can be anticipated that we should see the same effect on the basic electrophysiological defect. Furthermore, since only 6–20% of corrected cells are needed to revert the basic defect in chloride secretion [60], our data showing that 33–50% of hAMSCs acquired CFTR expression shed a positive light on the use of amnion MSCs in the CF treatment.

5. Conclusions

Our data indicate hAMSCs as a novel, promising, readily accessible, and ethically compatible source of pluripotent cells that could be used in regenerative medicine. In this respect, hAMSCs present promising features as indicated by their expression of embryonic stem cell markers such as SSEA4 and by their differentiation potential towards mesodermal and endodermal lineages.

Although CF is a clinically heterogeneous disease caused by a defect in the CFTR gene affecting multiple organ systems, major morbidity and mortality are given by the lung disease; however, hepatobiliary complications of CF are increasingly common and clinically relevant as the age of patients increases [61, 62]. This study shows the differentiative potential of hAMSCs towards hepatocyte-like cells, which might be useful in CF, and highlights the need for further investigations to elucidate the mechanism mediating CFTR expression in hAMSCs upon cell to-cell interactions.

Conflict of Interests

The authors declare that they have no conflict of interests.

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Review Article

Therapeutic Implications of Mesenchymal Stem Cells in Liver Injury

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Mesenchymal stem cells (MSCs), represent an attractive tool for the establishment of a successful stem-cell-based therapy of liver diseases. A number of different mechanisms contribute to the therapeutic effects exerted by MSCs, since these cells can differentiate into functional hepatic cells and can also produce a series of growth factors and cytokines able to suppress inflammatory responses, reduce hepatocyte apoptosis, regress liver fibrosis, and enhance hepatocyte functionality. To date, the infusion of MSCs or MSC-conditioned medium has shown encouraging results in the treatment of fulminant hepatic failure and in end-stage liver disease in experimental settings. However, some issues under debate hamper the use of MSCs in clinical trials. This paper summarizes the biological relevance of MSCs and the potential benefits and risks that can result from translating the MSC research to the treatment of liver diseases.

1. Introduction

The liver has a remarkable regenerative capacity in response to acute injury. Mature hepatocytes can reenter the cell cycle and undergo several cell divisions to restore the hepatic mass. However, following chronic liver damage, the regenerative ability of hepatocytes is lost. In such conditions, the liver is unable to maintain its functional mass; this is clinically mirrored by the so-called "liver failure." Currently, orthotopic liver transplantation (OLT) represents the most suitable therapeutic option for patients with advanced liver diseases and hepatic failure. Nevertheless, only a minority of candidates undergo OLT, given the organ shortage. Hence, alternative strategies for the treatment of decompensated liver diseases are needed to be developed [1].

Cell-based therapy has been proposed as a potential alternative to OLT. Indeed, it has been known for more than 30 years that hepatocytes isolated from a donor liver and infused intraportally in animal models of liver damage can be engrafted into the recipient hepatic parenchyma and express metabolic activity. These results have encouraged clinical trials using hepatocytes transplantation to treat a variety of liver diseases [2]. The best outcome of allogeneic hepatocytes transplantation was reported for the treatment of acute liver failure, in which hepatocytes infusion provides the rapid metabolism of liver toxins and the stabilization of hemodynamic parameters. However, transplantation of liver cells provides serious practical problems: donor scarcity, risk of rejection, low hepatocyte viability (only 30% of hepatocytes survive transplantation) and inability maintain and amplify cell cultures [3, 4].

Given this background, a growing enthusiasm has greeted the development of stem-cell-based therapies for liver diseases. In particular, transplantation of hematopoietic bone marrow (BM) stem cells and mesenchymal stem cells (MSCs) has been extensively investigated as potential sources for liver regeneration.

In 1999, Petersen et al. first showed that liver stem cells might be derived from BM, in a rat model of liver injury [5], and it was suggested that BM could contribute to the mature

hepatocyte population. Subsequent studies have shown that BM-derived hepatocytes might arise from cell fusion and not only by direct differentiation [6] and that BM cells give a limited contribution to the hepatocyte population, under physiological conditions or in response to mild injury [7].

MSCs represent another promising candidate for liver stem cell therapy. Several studies have demonstrated that MSCs can differentiate *in vitro* along the hepatogenic lineage [8, 9]. To date, studies on animal models reported the beneficial effect of MSCs in promoting hepatic tissue regeneration. Kuo et al. have shown that both MSC-derived hepatocytes and MSCs, transplanted by either intrasplenic or intravenous route, can be engrafted into the recipient liver and differentiate into functional hepatocytes. Intravenous transplantation was more effective in rescuing liver failure than intrasplenic transplantation. Moreover, MSCs were more resistant to reactive oxygen species *in vitro*, reduced oxidative stress in recipient mice, and accelerated repopulation of hepatocytes after liver damage, suggesting a possible role for paracrine effects [10]. These results have been confirmed also by Banas et al., who evaluated the therapeutic potential of MSCs for the treatment of liver failure and postulated that the beneficial effects of human MSC transplantation were due at least in part to the cells' ability to produce a large number and volume of bioactive factors [11]. To date, only a few clinical trials have been performed in patients with end-stage liver disease caused by hepatitis B, hepatitis C, and alcoholic fibrosis. The results of these studies have shown that MSC injection can be used for the treatment of end-stage liver diseases, with satisfactory tolerability and clinically relevant effects [12]. Nonetheless, these studies have not provided definitive evidence that MSCs have a capability to differentiate into functional hepatocytes *in vivo* [13], because the observed improvements could be attributed to the secretion of soluble growth factors by MSCs, rather than to their transdifferentiation into hepatocytes [7]. MSC cells have also emerged as promising candidate cells for immunomodulation therapy, especially in the setting of liver transplantation, given their ability to interact at various levels with the immune system [14, 15].

Overall, a number of different mechanisms contribute to the therapeutic effects exerted by MSCs, which can differentiate into functional hepatic cells and also produce a series of growth factors and cytokines that can suppress inflammatory responses, reduce hepatocytes apoptosis, regress liver fibrosis, and enhance hepatocytes functionality [16].

2. MSC Properties

MSCs were first described by Friedenstein in the early 1990s, as an adherent, fibroblastoid cell population that showed inherent osteogenic properties [17]. Numerous studies have demonstrated that MSCs have a high degree of plasticity, as they differentiate into cells of the mesenchymal lineage, but they can also transdifferentiate into neurons, splenocytes, and various epithelial cells, including lung, liver, intestine, and kidney cells. BM was originally considered the reference source for MSC isolation, although they have been isolated from a multitude of adult tissues, including muscle, adipose tissue, connective tissue, trabecular bone, synovial fluid, along with perinatal tissues, such as umbilical cord, amniotic fluid, and placenta [18]. In particular, adipose tissue (AT) has several advantages compared to other adult tissues as a source of MSCs. Indeed, AT is abundant and can be easily removed by simple lipoaspirate. Moreover, adipose-tissue-derived MSCs (AT-MSCs) can be maintained longer in culture and possess a higher proliferation capacity than BM-derived MSCs. Thus, AT may be an ideal source of large numbers of autologous stem cells [19].

MSCs do not express the hematopoietic surface markers CD34 and CD45, but stain positive for CD44, CD29, CD105, CD73, and CD166 [20]. Moreover, MSCs express human leukocyte antigen (HLA) class I, but not HLA class II, and secrete several extracellular matrix (ECM) molecules, such as collagen, fibronectin, laminin, and proteoglycans. For this reason it has been postulated that MSCs might play a central role in ECM organization. We performed a high-throughput molecular analysis of BM- and AT-MSCs. The gene expression profile analysis has revealed that they share 190 coherently modulated transcripts, which might represent the molecular "MSC stemness signature." Among them, we found several genes involved in basic biologic mechanisms, such as embryogenesis, organogenesis, signal transduction, cell adhesion, stress response, and transcription regulation. In particular, a key role in determining the outcome of MSC fate determination is played by KLF4, highlighting the specific binding of KLF4 to regulatory sequences of genes involved in adult stem cell maintenance [19].

BM-derived MSCs are known to naturally support hematopoiesis by secreting a number of trophic molecules, including soluble extracellular matrix glycoproteins, cytokines, and growth factors [21, 22]. Recent studies have demonstrated that MSCs can produce some antiapoptotic cytokines such as stromal-cell-derived factor-1 and vascular endothelial growth factor, which efficiently reduce the apoptosis of recipient cells via the stromal cell-derived factor-1/CX chemokine receptor-4 axis. The antiapoptotic effects of MSCs have been observed in liver injury models [23–26]. Furthermore, MSCs can secrete several cytokines such as hepatocyte growth factor (HGF), epidermal growth factor, IL-6, and TNF-α; in turn, these cytokines stimulate hepatocyte proliferation and maintain hepatocyte function, as indicated by the high levels of albumin and urea secretion granted upon MSC transplantation [27]. Finally, MSCs can produce a series of cytokines and signal molecules that can potentially suppress inflammatory responses such as IL-1 receptor antagonists and can upregulate anti-inflammatory cytokines such as IL-10 [25].

3. MSC Plasticity

Given their wide differentiation potential and their self-renewal capacity, MSCs have been considered a promising candidate for cell-based therapy and tissue engineering. Moreover, these cells have the ability to proliferate to an extensive but finite degree, an important characteristic that

should reduce concerns about potential tumorigenicity upon *in vivo* transplantation.

The high degree of plasticity of MSCs has been widely demonstrated during the last decade [28–31]. In particular, *in vitro* models, using culture medium supplemented with a cocktail of growth factors, were used to successfully induce the transdifferentiation of MSCs into hepatic cells with functional properties, such as the production of albumin and urea, along with glycogen storage [32]. Moreover, the *in vivo* transdifferentiation of MSCs into hepatic cells has been described in rats [33], mice [34], and humans [35].

Seo et al. first reported that human AT-MSCs injected into SCID mice, following toxic liver damage, were able to differentiate into hepatocyte-like cells [36]. Several reports have confirmed the possibility of generating hepatocyte-like cells from AT-MSCs [37, 38]. In particular, in a xenogeneic transplantation model of liver regeneration, the engraftment of AT-MSCs predifferentiated *in vitro* to hepatocyte-like cells was significantly more efficient *versus* undifferentiated AT-MSCs, and AT-MSCs were better candidates than BM-MSCs for cell therapies [39].

We confirmed that AT-MSCs can transdifferentiate *in vitro* into hepatocyte-like cells, using a two-step protocol with sequential addition of growth factors. Under this regimen, spindle-shaped fibroblastoid cells differentiated to a layer of compact polygonal epithelial cells. These cells acquired specific liver functions, as shown by their ability to store glycogen and to express hepatic-associated genes and proteins. Moreover, the comparative high-throughput molecular analysis of AT-MSCs, before and after hepatogenic conversion, allowed the identification of a complex interplay between cell receptors, signaling pathways, and transcription factors, responsible for tissue cross-lineage conversion through the mesenchymal-epithelial transition (MET). Our study showed that the AT-MSC plasticity is dependent on MET and suggested that subtle regulations of the canonical pathways of BMP, WNT, and TGF-β may be important to allow MSCs to transdifferentiate into other lineages [40].

The pivotal role that MET plays in determining AT-MSCs transdifferentiation in hepatocytes was also confirmed in an interesting article by Yamamoto and colleagues [41]. The authors compared the transcriptomes of three cell populations, undifferentiated AT-MSCs, AT-MSC-derived hepatocytes (AT-MSC-Hepa) and human primary hepatocytes, and human liver tissue, using microarray analysis. The results indicated that AT-MSC-Hepa and hepatocytes displayed a similar gene expression profile, while undifferentiated AT-MSCs showed a different pattern. The list of genes upregulated in AT-MSC-Hepa, liver cells, and tissue comprised, in particular, genes encoding hepatocyte-specific metabolic enzymes and markers [41]. Interestingly, the microarray data indicated the downregulation of two regulators of the epithelial-mesenchymal transition (EMT), Twist and Snail, along with the upregulation of epithelial markers, such as E-cadherin and a-catenin, in AT-MSC-Hepa. In contrast, the expression of mesenchymal markers, such as N-cadherin and vimentin, was downregulated. These findings support the notion that MET is activated during the hepatic differentiation of AT-MSCs, representing a pivotal step for stem cell transdifferentiation [41].

4. MSCs and Immune System

MSCs express few HLA class I and no HLA class II molecules, allowing them to evade allogeneic immune response. This is the so-called "immunoprivilege," an interesting feature in MSC biology, which makes these cells extremely suitable for both autologous and allogeneic transplantation [42]. Moreover, several studies have established that MSCs exert a generally suppressive effect on a wide variety of cells belonging to both adaptive and innate immunity, including T and B lymphocytes and natural killer cells (NKs). This immunomodulatory effect provides a rational basis for the application of MSCs in the treatment of immune-mediated diseases, such as graft-versus-host disease (GVHD). To date, the mechanisms underlying this immunoregulation remain unclear: some investigators suggested a cell-to-cell contact-mediated suppression, while others hypothesized a soluble-factor-mediated mechanism [43].

MSCs can suppress the activity of CD8+ cytotoxic T lymphocytes both directly by inhibiting their proliferation following antigen stimulation and indirectly by increasing the relative proportion of CD4+ T helper-2 (TH2) lymphocytes and CD4+ regulatory T lymphocytes [44]. Since B-lymphocyte activation is largely T cell dependent, the influence of MSCs on T lymphocytes may also indirectly suppress B-cell functions [45]. Additionally, MSCs exert a direct influence on B-lymphocytes via cell-cell contact and through secretion of paracrine molecules [46].

MSCs exert significant effects on the innate immune system cells, including monocytes, dendritic cells (DCs), macrophages, NKs, and neutrophils. The mechanisms by which MSCs exert their inhibitory effect on DC maturation is still poorly defined. Spaggiari et al. have shown *in vitro* that MSCs inhibit the early stages of the progression from monocytes to immature DCs, induced by interleukin-4 (IL-4) and granulocyte-macrophage colony-stimulating factor (GM-CSF). The authors have shown that different soluble factors mediate the inhibitory effect exerted by MSCs, and they provided a convincing evidence of the pivotal role of prostaglandin E2 (PGE2) [47]. MSCs have a profound inhibitory effect on NK function, suppressing the IL-2-induced cell proliferation, their cytolytic activity, and the production of cytokines. MSCs can inhibit NK-cell function *via* the production of soluble factors, including indoleamine 2,3-dioxygenase (IDO) and PGE2 [48]. Lastly, an *in vitro* study demonstrated that MSCs inhibit apoptosis, expression of adhesion molecules, and migration capability of neutrophils. These results are consistent with the hypothesis that, within the BM niche, MSCs protect neutrophils of the storage pool from apoptosis, preserving their effector functions. Moreover, MSCs reduce intensity of the respiratory burst preventing the excessive or inappropriate activation of the oxidative metabolism. This may be a critical mechanism through which MSCs can limit the severity of tissue damage following ischemic and ischemia/reperfusion (I/R) injury [49].

5. Therapeutic Implications of MSC-Based Treatments of Liver Diseases

The therapeutic potentialities of MSCs are also based on their inherent ability to home in sites of inflammation following tissue injury when injected intravenously. This involves their capability of migrating across endothelial cell layers and being attracted to and retained in the ischemic tissue but not in the remote or intact tissue. Although the mechanisms driving this property are not fully understood, it is likely that injured tissues express specific receptors or ligands that facilitate trafficking, adhesion, and infiltration of MSCs to the damaged site, similarly to leukocytes [50, 51]. It is well known that chemokines are released after tissue damage and that migratory direction follows the chemokine density gradient. In this regard, it has been recently demonstrated that MSCs express chemokine receptors and ligands that are involved in leukocyte migration during inflammation, including the stromal-derived factor-1 (SDF-1) chemokine receptor (chemokine (C-X-C motif) receptor 4, CXCR4) that stimulates the recruitment of progenitor cells to the site of tissue injury [52–55]. MSCs also express several adhesion molecules that respond to SDF-1, as well as chemokines, such as CX3CL1, CXCL16, CCL3, CCL19, and CCL21 [56–58]. Hence, the increase of inflammatory chemokine concentration at the site of inflammation is a key mediator of MSC trafficking to the site of injury [52]. In addition, many integrins, selectins, and chemokine receptors involved in the tethering, rolling, adhesion, and transmigration of leukocytes have also been reported to be expressed on MSCs. In particular, E- and P-selectin, CD44, and VCAM-1, which function in leukocyte adhesion, have been shown to be functionally important in the adhesion of MSCs to the endothelium [59–61].

The therapeutic role of MSCs has been investigated using either autologous or allogeneic transplantation of cells, which were previously expanded in culture and then introduced intravenously or directly into the tissue of interest. To date, infusion of MSCs has shown encouraging results in the treatment of several immune- and inflammatory-mediated conditions including GVHD, diabetes, and ulcerative colitis and in the protection of solid organ grafts from rejection [62]. Recent experimental studies have shown the successful application of MSC transplantation in the treatment of fulminant hepatic failure (FHF), end-stage liver disease (ESLD), and inherited metabolic disorders (IMDs). These studies have shown that MSC transplantation can partially restore the liver function, ameliorate the symptoms, and enhance the survival rates [8, 62].

Different studies have shown that administration of MSC-conditioned medium (MSC-CM), or MSC-derived molecules, might function as alternative or adjuvant tool *versus* MSC direct transplantation alone, for the treatment of FHF [43, 62]. Indeed, Parekkadan et al. showed that the administration of MSC-derived molecules, either by a bolus of MSC-CM or by extracorporeal support using a bioreactor, significantly improved short-term survival in a D-galactosamine-induced rat model of FHF [62]. In another study, van Poll et al. confirmed the effectiveness of MSC-CM in a rat model of FHF. These authors demonstrated that systemic infusion of MSC-CM provides significant survival benefit and prevents the release of liver injury biomarkers [62]. Furthermore, MSC-CM therapy had profound inhibitory effects on hepatocellular death, resulting in a 90% reduction of hepatocyte apoptosis, and enhanced the liver regeneration programs, incrementing the number of proliferating hepatocytes. Taken together, these data support the theory that MSC-CM induces an integrated beneficial response to liver damage [62]. Compared to MSC-CM, transplanted MSCs have the capability to home in the site of injury and ensure continued delivery of trophic signal molecules. However, long-term engraftment rates are low, and invasive methods for the local delivery of MSCs are necessary [11, 63].

A study by Kanazawa and colleagues showed an interesting application for MSCs in the treatment of the hepatic I/R injury that occurs after liver transplantation [64]. These authors reported that transplanted BM-MSCs were able to ameliorate hepatic I/R injury and improve liver regeneration, in a rat model of Hepatectomy plus I/R; the cellular treatment constrained the increase of serum transaminase levels, the most sensitive marker for hepatic I/R injury evaluation. In addition, a significantly lower percentage of apoptotic hepatocytes were observed in the MSCs group compared with the controls. These findings suggested that MSCs might have the potential to protect the liver against I/R injury-induced hepatocyte apoptosis and to enhance liver regeneration [64].

MSCs have been proposed for the treatment of liver cirrhosis, characterized by distortion of the hepatic architecture and formation of regenerative nodules. Liver cirrhosis is generally considered an irreversible process and represents a frequent cause of death worldwide [65]. The autologous MSC injection could be a valid alternative to OLT in the treatment of liver cirrhosis. Indeed, several animal studies and clinical trials have demonstrated that MSCs have the potential to reverse the fibrotic process by inhibiting collagen deposition and transforming growth factor-β1 production [11, 66, 67]. The molecular mechanism underlying the antifibrotic properties of MSCs can mainly reside in the high expression levels of matrix metalloproteinase (MMPs), especially MMP-9, which may directly degrade the extracellular matrix and lead to hepatic stellate cell apoptosis [68, 69].

Recently, Pan et al. have shown that BM-MSCs were able to attenuate liver fibrosis by a direct suppression of hepatic stellate cell activation through the inhibition of delta-like 1 (Dlk1) protein, a member of the EGF-like family of homeotic proteins, in a carbon-tetrachloride- (CCl4-) induced liver fibrosis animal model [70]. In addition, Mohamadnejad and colleagues have conducted a phase 1 clinical trial to determine the safety and feasibility of MSC peripheral vein infusion in patients with decompensated liver cirrhosis: liver function and MELD scores were improved in half of the patients after six months [71].

Despite these encouraging results, the use of MSCs in the hepatologic clinical practice is hampered by the inability to monitor the transplanted cells within the patients and by the lack of standardized clinical protocols. Moreover, the

antifibrotic effect of MSCs is still debated, as MSCs could also potentially differentiate into fibrogenic cells [13, 72].

6. MSCs in Liver Transplantation: Risks and Benefits

Transplantation tolerance is an important goal in the effort to reduce long-term morbidity and mortality in organ-transplant recipients. MSCs can be induced toward hepatic differentiation *ex vivo* and used as a potential valid alternative or a bridging to OLT [10–12], as they could prevent allograft rejection. Such potentiality is based on MSC immunomodulatory properties along with their healing and trophic functions, which could help to minimize ischemia, I/R, and inflammation [15, 73]. The immunomodulatory effect exerted by MSCs on T-lymphocyte response appears to be of primary importance in their ability to prevent allograft rejection. As previously discussed, MSCs suppress the proliferation and function of cytotoxic T lymphocytes while promoting the activities of helper and regulatory T lymphocytes. The precise mechanisms responsible for this effect and whether or not it persists long-term remain to be determined, and further studies are needed to address this issue.

An additional benefit to the use of MSCs for the prevention of solid organ allograft immunorejection is that infusion of these cells at the time of organ transplantation may have the potential to promote a state of immunologic chimerism and long-term tolerance of the transplanted organ by the host immune system [74]. This was achieved in distinct animal models and, in a few notable cases, was associated with long-term graft survival in the absence of immunosuppression [74–76].

Despite the important benefits arising from the use of MSC-based therapy, there are still safety issues to debate about, in particular regarding the long-term effects on immune function and the tumorigenic risk.

Several evidences suggest that MSCs might promote tumor growth *via* transformation, suppression of the antitumor immune response, and direct trophic action on tumor cells [77–86]. The transplantation into nude mice of colon cancer cells mixed with MSCs resulted in larger tumors than did transplantation of cancer cells alone [85]. This effect was associated with a higher degree of neoangiogenesis and lower apoptotic indexes in the tumor mass. MSCs were recruited by colon cancer cells, and in turn they stimulated the migration and invasion of tumor cells through the release of soluble factors [85]. The proangiogenic properties of MSCs can be due to their potential to differentiate into pericytes [86] and, perhaps, endothelial cells, along with the secretion of angiogenic growth factors, including vascular endothelial growth factor, fibroblast-derived growth factor, platelet-derived growth factor, and stromal-derived factor-1 [87]. Moreover, MSCs can provide a stromal scaffold for growing tumors, being a source of carcinoma-associated fibroblasts (CAFs), implicated in important aspects of epithelial solid tumor biology such as neoplastic progression, tumor growth, angiogenesis, and metastasis [88].

However, MSCs immunomodulatory properties may play a potent antitumor effect [89–98]. The exact mechanisms behind the tumor suppressive effects of MSCs are not yet entirely clear, but appear to be related to the modulation of the inflammatory environment that characterizes many tumors [97]. Moreover, MSCs may exert non-immune-related effects, since they are able to interact with cancer cells and inhibit intracellular signaling pathways associated with cell growth and division [97, 98]. In a study by Abdel Aziz and colleagues, the infusion of MSCs, in a rat model of hepatocellular carcinoma, resulted in tumor suppressive effects by downregulation of Wnt signaling target genes related to antiapoptosis, mitogenesis, cell proliferation, and cell cycle regulation. This resulted in the amelioration of both liver histopathological features and function [99].

7. Concluding Remarks

MSCs are considered a potentially relevant therapeutic tool for the treatment of liver diseases, given their high degree of plasticity and immunomodulatory properties. MSCs could represent an alternative to OLT and/or an adjuvant therapy in the prevention of allograft liver rejection. However further studies *in vitro* as well *in vivo* are needed to achieve a better understanding of the potential benefits and risks of MSCs therapeutic use in clinical settings.

Authors Contributions

M. A. Puglisi and V. Tesori contributed equally to this work.

Conflict of Interests

The authors declare no conflict of interests.

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Research Article

Neurotrophic Features of Human Adipose Tissue-Derived Stromal Cells: *In Vitro* and *In Vivo* Studies

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Due to its abundance, easy retrieval, and plasticity characteristics, adipose-tissue-derived stromal cells (ATSCs) present unquestionable advantages over other adult-tissue-derived stem cells. Based on the *in silico* analysis of our previous data reporting the ATSC-specific expression profiles, the present study attempted to clarify and validate at the functional level the expression of the neurospecific genes expressed by ATSC both *in vitro* and *in vivo*. This allowed evidencing that ATSCs express neuro-specific trophins, metabolic genes, and neuroprotective molecules. They were in fact able to induce neurite outgrowth *in vitro*, along with tissue-specific commitment along the neural lineage and the expression of the TRKA neurotrophin receptor *in vivo*. Our observation adds useful information to recent evidence proposing these cells as a suitable tool for cell-based applications in neuroregenerative medicine.

1. Introduction

Adipose-tissue-derived adult pluripotent cells, commonly known as adipose tissue stromal cells (ATSCs) are mesenchymal stem cells (MSCs) residing in the connective stroma of adipose tissue. They represent a valuable source of adult stem cells, being easily isolated from an abundant and accessible tissue [1–3]. Their plasticity along with the ease of *in vitro* culturing and propagation makes them the most used cell type in a wide range of tissue regeneration applications [4–7].

We have previously shown the ATSC-specific molecular properties, by comparatively analyzing the geno-mewide expression profiles of MSCs from different adult tissues [3]. The study allowed indicating the main molecular features which regulate the stemness maintenance of MSCs and a more extensive plasticity of ATSC *in vitro*. The complete result dataset of this previous study (available at the Gene Expression Omnibus (GEO) database, http://www.ncbi.nlm.nih.gov/gds, accession number GSE8954) also indicated that ATSCs specifically express neurospecific genes.

The purpose of this study is to extract the biologically significant genes from this dataset and validate the functional relevance of the neurotrophic genes expressed by ATSC both *in vitro* and *in vivo*.

2. Materials and Methods

2.1. In Silico Biological Analysis of the Microarray Dataset. In order to identify the candidate genes involved in the neurotrophic properties of ATSCs, the gene list of ATSC-specific genes obtained through the microarray-based gene profiling of ATSC compared to bone-marrow-derived mesenchymal cells (BMSCs) and fibroblasts (http://www.ncbi.nlm.nih.gov/gds, accession number GSE8954) [3] underwent an *ad hoc* biological analysis, aimed at finding neurologically relevant genes. For this purpose, the list of 441 genes specifically upregulated in ATSC (P value <0.01), resulting from the statistical analysis (see [3] for statistical methods used in data analysis), were categorized according to the "biological function" annotations implemented from the Gene Ontology

Annotation (GOA) database (http://www.ebi.ac .uk/GOA/). Specific neuroprotective, neurodevelopmental, and/or neurotrophic functions were further studied using the "Gene Reference Into Function" tool in GenBank (http://www.ncbi .nlm.nih.gov/gene/about-generif).

2.2. Patients and Specimens. Adipose tissue (AT) specimens were obtained by lipoaspiration from healthy volunteers (mean age 40.2 ± 14.2 years) upon obtaining a written consent. A skin biopsy was obtained from the retroauricular region of an healthy male donor (aged 45) and served for the isolation of human dermal fibroblasts (HDF). Individuals data were handled confidentially and anonymously. All the procedures employed in this study were approved by the ethical committee of the Catholic University of Rome (Rome, Italy; number P552 (A.779)/CE2007).

2.3. Chemicals and Reagents. Cell culture media and supplements were purchased from Lonza (Basel, Switzerland). Enzymes, growth factors, and all other chemicals used in this study were purchased from Sigma (Sigma-Aldrich, St Louis, Mo,USA), unless otherwise specified.

2.4. ATSC Isolation and Culture. Mesenchymal stromal cells were isolated in primary culture from the lipoaspirates, as already described elsewhere [3]. Briefly, AT was extensively washed, mechanically fractionated, and digested using 0.1% collagenase type VIII. The lysed tissue was then filtered through a 100 μm mesh, and the cell suspension was centrifuged. The cell pellet was then plated in T75 tissue culture flasks using Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% FBS, 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.2 ng/mL fibroblast growth factor beta (bFGF). Cells were subcultured as previously described [3] and then used for *in vitro* and *in vivo* experiments, as detailed in the following paragraphs. ATSCs growth kinetics up to fifteen culture passages and their immunophenotype were assessed as already described elsewhere [6].

2.5. HDF Isolation and Culture. Dermal fibroblast were isolated in primary culture from the skin biopsy and cultured as previously described [8]. These cells served as a mesoderm-derived differentiated controls to produce the conditioned medium (HDF-CM) used in the *in vitro* experiments (see following paragraphs).

3. *In Vitro* Experimental Procedures: Neural Cell Line Cultures and Treatments

In order to assess the functional significance of the neurotrophic genes specifically expressed by ATSCs, LAN5 and PC12 cells were used as neural undifferentiated cell lines for the *in vitro* experiments. These cell lines are commonly employed as valuable models to study the neuronal differentiation and degeneration processes *in vitro* [9–13].

3.1. Cell Lines and Treatments. The human LAN-5 dopaminergic cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM glutamine, 40 μg/mL gentamicin, and 10% heat-inactivated fetal calf serum (FCS), according to standard protocols [13]. Cells were plated at a $10^4/cm^2$ seeding density in 24-well plates. The day after plating, ATSCs were seeded in the same wells using a $10^4/cm^2$ seeding density. In addition, separate wells of LAN5 cells were cultured in presence of ATSC-conditioned medium (ATSC-CM), which was obtained by filtering through a 0.2 μm cellulose acetate filter the supernatant medium of subconfluent ATSC cultures. Thereafter, both LAN5-ATSC cocultures and ATSC-CM-treated cells were grown for three days without changing the culture medium.

The rat PC12 noradrenergic cell line was seeded at a density of 5000 cells/cm^2 in RPMI 1640 medium containing 5% fetal calf serum and 10% horse serum and grown till 80% confluence, according to standard protocols [12]. Between the third and the fourth culture passage, cells were plated in 24-well plates, using a $10^4/cm^2$ seeding density. The day after plating, the PC12 culture medium was replaced by either ATSC-CM or HDF-CM. LAN5 and PC12 cells in standard culture medium served as controls in the experiments. As PC12 cells are known to differentiate along a functional neuronal phenotype upon NGF treatment, cells primed with 50 ng/mL of NGFβ were used as positive control of differentiation [9]. Cellular morphology was evaluated by an invertoscope up to four days of culture.

4. *In Vivo* Experimental Procedures: Neonatal Rat Brain ATSC Inoculation

4.1. Adenoviral-Mediated Cell Transduction. In order to make ATSC recognizable in living tissues, cells were transfected using a defective adenoviral vector carrying the enhanced green fluorescent protein (AdEGFP) as a reporter gene. AdEGFP stocks were kindly provided by the Vector Core Facility of the University of Pittsburgh (Pa, USA). Cells were plated at a $10^4/cm^2$ seeding density and treated with AdEGFP using a multiplicity of infection (MOI) of 100 plaque-forming units (pfu)/cell. The efficiency of cell transduction was assessed observing fluorescent cells 48 hours later using an invertoscope equipped with a fluorescent lamp. EGFP-expressing cells were then inoculated in neonatal rats, as further described.

4.2. Cell Transplantation. Human ATSCs were transduced with Ad.eGFP 48 hours prior to *in vivo* transplantation. The surgery was performed on neonatal rats at postnatal day 1 (P1), after the induction of deep anesthesia by hypothermia. A small parietal hole was made into the skull above the frontal cortex, and cells were slowly injected into the lateral ventricle (1 mm posterior to the bregma, 1 mm lateral to the midline, and 2–2.5 mm ventral to the pial surface) using a glass micropipette coupled to a Hamilton microsyringe. For each animal treated, 5×10^4 ATSCs suspended in 1 μL of Puck's saline A (Invitrogen, Carlsbad, Ca) were used. Sham-operated animals were injected with the same volume of

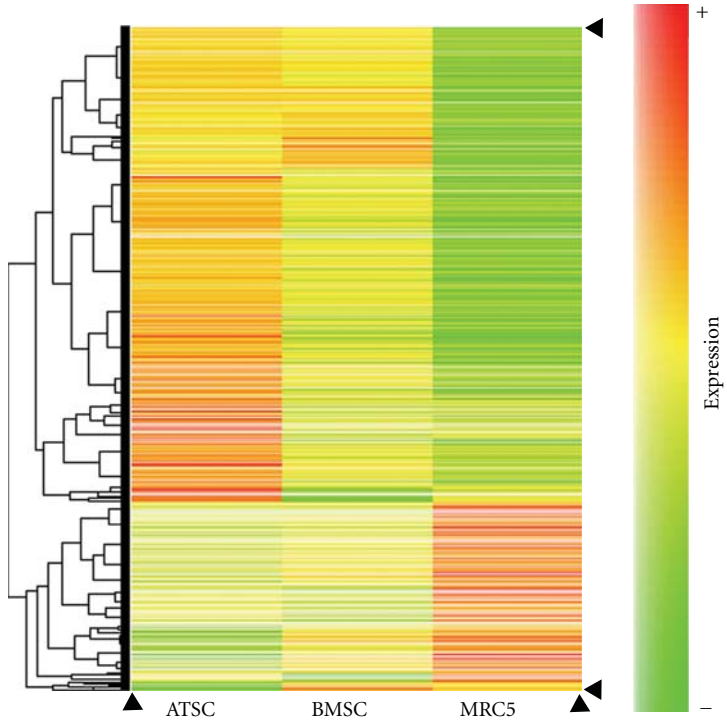

Figure 1: Hierarchical clustering of microarray data. The dendrogram shows all the 441 genes differentially expressed in ATSC (selected by *t*-test, *P* value 0.01) resulting from the statistical analysis [3]. Each row represents a single gene, while cell types are grouped in columns. The colored representation of gene expression is shown according to the scale on the right side of the figure. BMSC: bone-marrow-derived stromal cells; MRC5: human lung fibroblast cell line. See [3] for details.

saline solution. Following treatment, the skin was rapidly sutured, the pups were warmed under a lamp and returned to the dame. All animal protocols used have been approved by the Animal Experimentation Committee of the Catholic University of Rome.

4.3. Tissue Processing. The animals were sacrificed 7 and 15 days after injection ($n = 6$ for each group of ATSC treated rats, and $n = 3$ for each group of sham-treated animals). Under deep anaesthesia (ketamine/diazepam 1 : 1 i.p.), they were perfused through the aorta with 100 mL of saline solution, followed by 100 mL of 0.01 M, pH 7.4 PBS, and 4% paraformaldehyde. Thirty minutes after perfusion, the brains were removed from the skull, postfixed in 4% PBS paraformaldehyde for 2 h and immersed in 30% sucrose. Serial 40 μm thick coronal sections were cut on a freezing microtome. The first series of sections was mounted in Vectashield (Vector, UK) for fluorescent evaluation of eGFP-expressing cells. Other series of adjacent sections were processed for immunohistochemistry.

4.4. Immunohistochemistry. Anti-GFAP (polyclonal, Dako, Glostrup, Denmark, 1 : 1000 overnight at 4°C), -Doublecortin (policlonal, Chemicon, Temecula, Ca, 1 : 3000, overnight at 4°C), -NeuN (monoclonal, Chemicon, Temecula, Ca, 1 : 500, 48 h at 4°C), -O4 (monoclonal, Chemicon, Temecula, CA, 1 : 500, overnight at 4°C), and -TrKA (Santa Cruz Biotechnology, Heidelberg, Germany, 1 : 1000 overnight at 4°C) were revealed using cyanine fluorochromes-labeled secondary antibodies (donkey anti-mouse Cy3 or donkey anti-rabbit Cy3, Jackson Immunoresearch Laboratories, West Grove, Pa, 1: 400) following incubation for 1 hour at RT. Sections were mounted in Vectashield for fluorescent visualisation of labeled cells. Controls were prepared by omitting the primary antibodies.

The colocalization of eGFP with the above-mentioned markers was examined with a Zeiss LSM 510 confocal laser scanning microscopy system.

5. Results

5.1. ATSCs Express Neurospecific Genes. Data extracted from previously published microarray data showed the selective upregulation of 441 genes ($P < 0.01$) in ATSC compared to BMSC and human fibroblast MRC5 cells (Figure 1). The *in silico* biological analysis of the microarray data (GEO dataset number GSE8954) allowed to identify a short list of biologically relevant genes, involved in neuroprotection, neural developmental processes, and neurotrophic functions (see Table 1). In particular, this 12-transcript list included genes, namely, nerve growth factor beta (NGFB), neuropilin 1

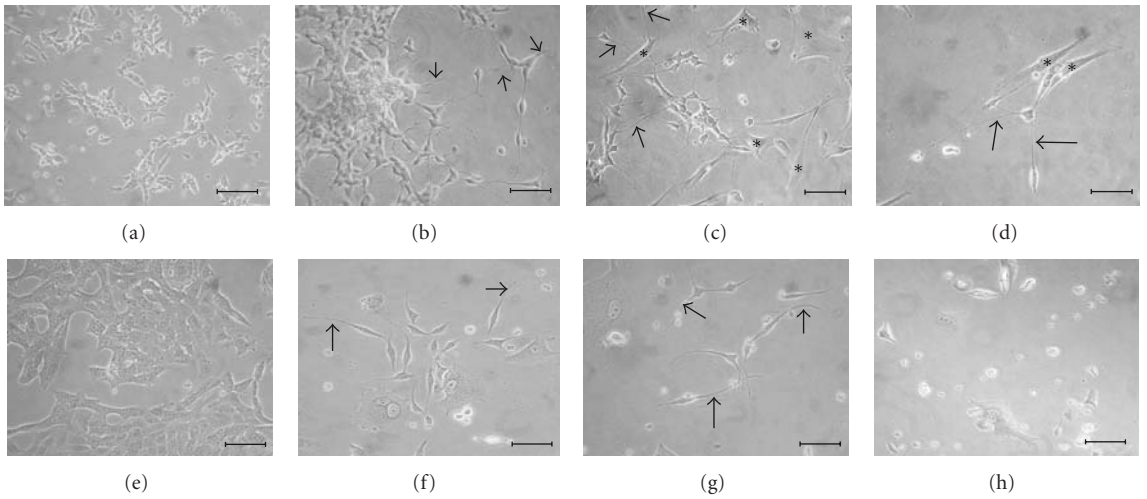

Figure 2: *In vitro* neurotrophic effects of ATSC. LAN-5 human neuroblasts and PC12 rat cells were cultured either ATSC-conditioned medium or co-cultured with ATSC and morphological modifications were monitored over time: (a) LAN5 in standard culture medium; (b) LAN5 cultured in ATSC-CM for 72 hours; (c) and (d) LAN-5 co-cultured with ATSC using a cell density of 10^4 cell/cm^2 for both cell populations; (e) PC12 in standard culture medium; (f) PC12 cultured in ATSC-CM for 4 days; (g) PC12 cultured in βNGF 100 ng/mL for 4 days; (h) PC12 cultured in HDF-conditioned medium for 4 days. Arrows show evidence of neurite outgrowth; asterisk ($*$) indicate ATSC in culture. Scale bar 100 μm in all panels except for panel $d = 10\,\mu$m.

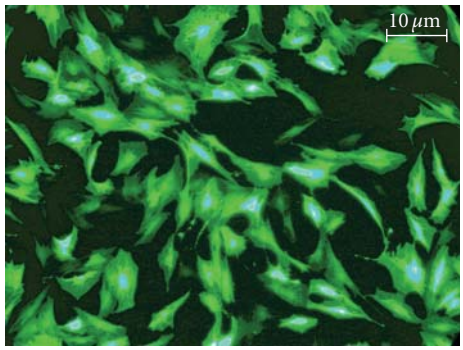

Figure 3: Efficient adenoviral-mediated transduction of ATSC. ATSCs were transfected with 100 pfu/cell of AdEGFP and fluorescent cells were observed after 48 hours: nearly 80% cells were EGFP-positive as shown in the figure.

(NRP1), and GTP cyclohydrolase 1 (GCH1), encoding soluble neurotrophins which are known to mediate neuronal growth, differentiation, migration, and neuroprotection [9, 14, 15]. The neuronal cadherin CDH2 belongs to the major transmembranar signalling complex cadherin/catenin that plays a key role in neuronal processes during early development. It is activated during neural circuit formation and maturation to mediate axonal outgrowth and arborisation [16, 17]. Moreover, nearly all genes in the list are implicated in developmental processes within the nervous system, such as neurogenesis, neuron differentiation, axonogenesis, axon guidance, nerve growth, and glia differentiation and migration (Table 1). The phosphoribosyl pyrophosphate synthetase 1 (PRPS1) and the phosphoglycerate mutase 1 (PGAM1) genes are implicated in metabolic pathways which are essential in neuronal function and maintenance (see function details and references in Table 1).

5.2. ATSCs Induce Neurite Outgrowth in PC12 and LAN5 Cells. In order to evaluate the effects of the supposed neurotrophic properties of ATSC, the capability of inducing visible changes in cell morphology of neural cells was first assessed *in vitro*. For this purpose, LAN5 cells were either cultured in ATSC-CM or cocultured with human ATSC for three days. Both cells cultured in ATSC-CM (Figure 2(b)) and those in coculture (Figures 2(c)-2(d)) displayed evident changes in shape and morphology, compared to those grown in standard culture medium (Figure 2(a)). The morphological changes consisted in the formation and elongation of neurite-like processes observed in discrete loci of the culture plate. The outgrown neurites seemed to establish contacts with both neural cells and ATSC in culture (Figure 2(d)).

In addition, the adrenergic PC12 cell line was cultured in presence of ATSC-conditioned medium (ATSC-CM) for four days. PC12 primed with βNGF and PC12 cultured in HDF-CM was used as positive and negative neuro-differentiation controls, respectively. The morphological analysis showed the extensive outgrowth and extension of neurite-like structures in both βNGF- and ATSC-CM-treated cells exhibiting essentially overlapping features (Figure 2(f)-2(g)), compared to cells cultured in standard medium (Figure 2(e)). Cells grown in HDF-CM showed clear morphological signs of distress, becoming small-rounded vacuolized cells, with a marked tendency to detach (Figure 2(h)).

Table 1: Selected ATSC-specific upregulated genes involved in neurospecific functions ($P < 0.01$).

Gene symbol	Gene bank	Gene name	Neurospecific functions				Process	References
			protection	development	trophism	metabolism		
SLC1A1	NM_004170.5	Solute carrier family 1, member 1	+				Protection against glutamate neurotoxicity	[18]
CDH2	NM_001792.3	Cadherin 2, type 1, N-cadherin (neuronal)		+			Pre-to-postsynaptic adhesion neuronal migration Axonogenesis synapse assembly	[19]
CELF2	NM_001025077.2	CUG triplet repeat, RNA binding protein 2	+	+			Motor neuron survival splicing control during development	[7]
VLDLR	NM_003383.3	Very low density lipoprotein receptor	+	+		+	Protection against hypoxia and glucose starvation Nervous system development lipid uptake in neurons and astrocytes	[20]
NRP1	NM_003873	Neuropilin 1	+	+	+		Cell survival axon guidance Migration and invasion	[15]
NGFB	NM_002506.2	Nerve growth factor, beta polypeptide		+			Neuron differentiation nerve growth	[9]
ENC1	NM_003633.2	Ectodermal-neural cortex	+	+			Anti-apoptotic nervous system development	[21]
GCH1	NM_000161.2	GTP cyclohydrolase 1	+		+		Protection from brain damaging events secreted by astrocyte	[14]
FGF2	NG_012449.1	Fibroblast growth factor 2		+	+		Neurogenesis migration	[22]
NDN	NM_002487.2	Necdin homolog (mouse)	+	+			Protects neuron from oxidant stress Neuron development glial cell migration	[23]
PRPS1	NM_002764.3	Phosphoribosyl pyrophosphate synthetase 1		+		+	Purine synthesis nervous system development	[24, 25]
PGAM1	NM_002629.2	Phosphoglycerate mutase 1 (brain)	+			+	Regulation of energy metabolism neuroprotection against Aß-toxicity	[26]

5.3. In Vivo Analysis of ATSC-Specific Neurotrophic Features. The functional significance of the ATSC-specific upregulation of genes involved in the neural lineage has been further investigated *in vivo* after transplantation of ATSCs in the neonatal rat brain. ATSCs were efficiently transduced with Ad.eGFP prior to *in vivo* transplantation (Figure 3).

Histological examination of ATSC-transplanted young rats sacrificed 7 days after transplantation showed clusters of eGFP-positive ATSCs, characterized by rounded morphology, localized in the wall of the lateral ventricle, near the needle tract, surrounded by GFAP positive astroglial endings (Figure 4(a) A–C). In particular, based on the results

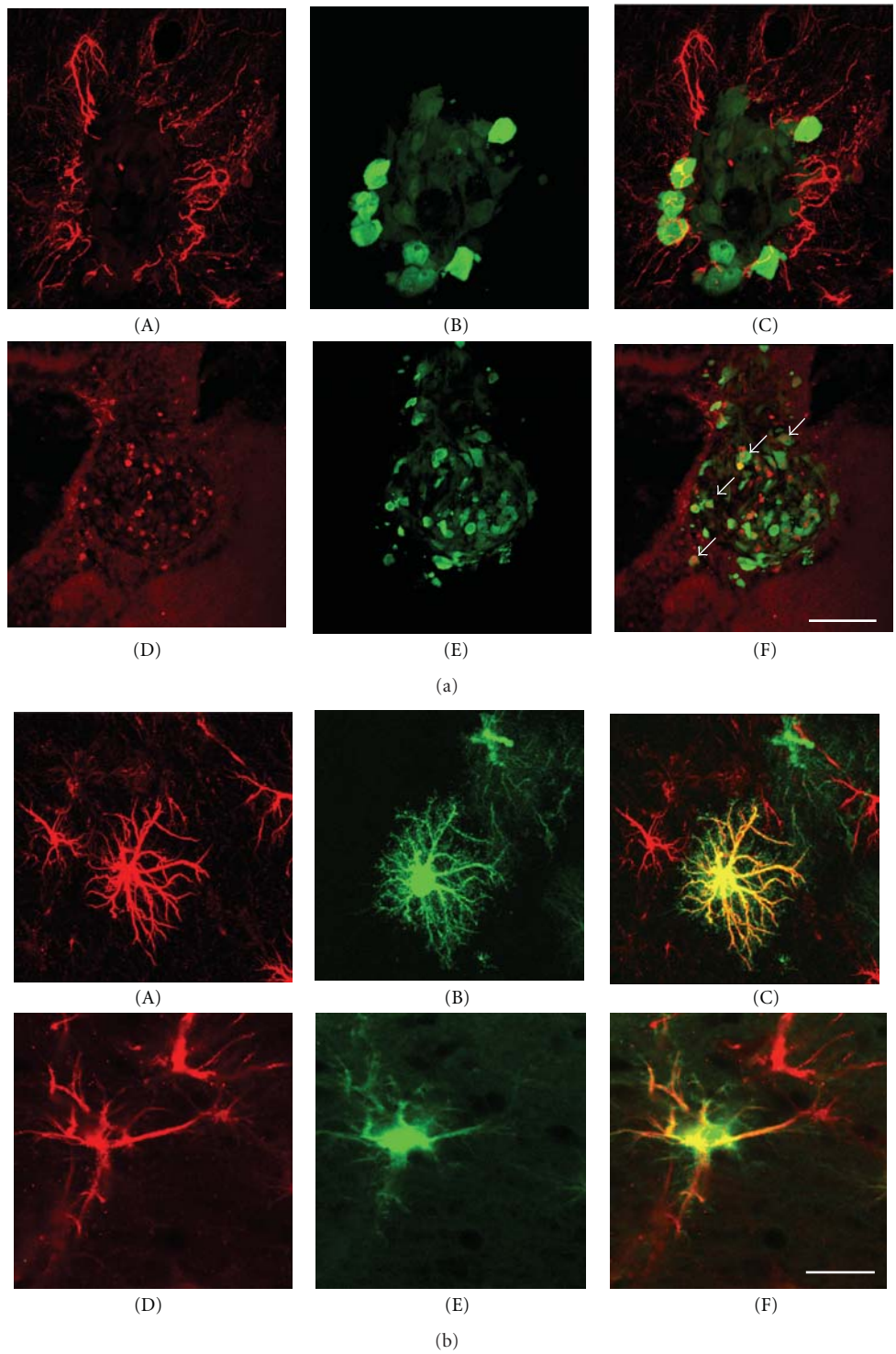

Figure 4: Engraftment of human ATSCs within newborn rat brain. (a) Confocal microscopy micrographs showing the engraftment of eGFP-positive (green; A, D) ATSCs within newborn rat brain 1 week after cell infusion. ATSCs exhibit a round morphology (A, C), are surrounded by GFAP-positive astrocytes (red; B, D), and express TRKA (red, B, D, arrows). (b) Engraftment and *in vivo* differentiation of human ATSCs within newborn rat hippocampus 2 weeks after implantation. Confocal images of GFAP (red; A) or TRKA (red; D) immunolabeled eGFP (green; B, E) expressing ATSCs. Engrafted cells express the astrocytic marker GFAP (yellow, C) and the TRKA receptor (yellow, F). Scale bars: (a) A–C 120 μm, (a) D–F 420 μm, (b) A–C 80 μm, and (b) D–F 60 μm.

observed *in vitro*, we assessed the expression of the anti-NGF-β receptor, as to further investigate the significance of the NGF/TRKA signaling pathway. ATSCs exhibited immunopositivity for the TRKA antibody 7 days after transplantation (Figure 4(a) D–F). At this time point, no co-localization with neuronal (Doublecortin, NeuN), astroglial (GFAP), or oligodendroglial (O4) markers were observed (not shown).

Histological examination of young rats sacrificed 15 days after transplantation confirmed the survival of ATSCs in the brain of injected animals. Grafted cells examined at this time point were mainly localized within the brain parenchyma, near the ventricular system and frequently in the hippocampus. They showed a bipolar or multipolar morphology with processes extending in various directions. Interestingly, confocal microscopy examination revealed that many of these eGFP- positive ATSCs coexpressed also the astroglial marker GFAP (Figure 4(b) A–C), while no colocalization between eGFAP and Doublecortin, NeuN, or O4 was found (not shown). Virtually all engrafted ATSCs expressed immunopositivity for anti-TRKA antibody (Figure 4(b) D–F)). Sham-operated animals exhibited only a mild GFAP-stained glial reaction around the needle tract (not shown).

6. Discussion

Different evidences indicated that transplanted MSCs promote endogenous repair of neurologically damaged areas and neural differentiation, via the release of soluble trophic factors and cytokines [27].

In particular, recent studies indicated that ATSC culture medium should contain neurotrophic factors, which were able to induce neuritogenesis in PC12 cells *in vitro* and protect brain from both hypoxic damage and glutamate neurotoxicity [28–30]. Nonetheless, only selected molecules have been dosed in ATSCs as possible neurotrophic candidates [28–31], while the expression of a wider panel of neuro-specific molecules has not been assessed in ATSCs so far.

The possible complete list of neurotrophic/neuroprotective factors specifically expressed by ATSC is proposed in this study, as a result of the *in silico* analysis of differentially expressed genes in MSC isolated from different adult tissues [3]. This revealed that ATSCs strongly and specifically express at least three neurotrophins: NFGB, NRP1, and FGF2. These secreted molecules reasonably represent the molecular background of ATSC-neurotrophic features. The *in vitro* assays in this study demonstrated that ATSCs could in fact induce neurite outgrowth not only in PC12, but also in human neuroblasts (LAN5 cell line). The induction of neuronal differentiation should be the result of the demonstrated presence of soluble secreted factors in ATSC culture medium [28] along with cell-to-cell contacts with neural cells *in vitro*. Thus, this event could be reasonably mediated by both NGFB, which promotes neuronal differentiation [9], and NRP1 that guides axon growth [15]. Also the nonneurospecific growth factor FGF2 could play a role in this event, being able to promote neurogenesis [22]. In addition, the adhesion molecule CDH2 that is expressed on the plasma membrane and is involved in axonogenesis and synapse assembly [19] could play a role in ATSC-mediated neuronal differentiation of LAN-5 cells. Although, the possibility that other factors participate in mediating this effects cannot be excluded.

Our data could also suggest that ATSCs neurotrophic function resides in a sort of astrocyte-like phenotype, as they specifically express genes belonging to the glial phenotype, including VLDR, FGF2, and NDN, according to GOA annotations. To this end, the necdin homolog (NDN) gene, involved in the NGFB signalling pathway, is particularly relevant, as it drives glial migration during nervous system development and is expressed in the cell projections [23]. Although the neural transdifferentiation capacity of MSCs has been largely debated, many recent studies emphasise the possibility of both bone marrow- and adipose tissue derived-undifferentiated stromal cells to differentiate along the neuroectodermal lineage to neuronal-like cells of the ectodermal lineage, mainly *in vitro* [32–42].

Recent data indeed assess the importance of cell-cell interactions along with the release of growth factors from the host tissue in ATSCs neural transdifferentiation [43]. In line with these observations, the results obtained *in vivo,* following cell implantation in the neonatal rat brain, indicate that ATSCs survive, migrate, and essentially differentiate toward an astroglial fate. Taken together, our observations suggest that ATSCs show a predisposition to the neural fate as they express a molecular phenotype resembling neural commitment *in vitro* and transdifferentiate along the neural lineage *in vivo*.

Recent reports evidence the successful implantation and migration of ATSCs *in vivo* using experimental models of rat brain ischemia, where they were able to promote functional recovery [44–47]. In addition, different groups reported the neural transdifferentiation of ATSCs transplanted in the injured spinal cord [48, 49], evidencing that, when detached from the physiological niche, they express ectoderm neural markers [50]. We may speculate that secreted soluble factors from neighbouring cells and physical reciprocal contacts with neural cells may cause/facilitate transdifferentiation processes, as also indicated by the expression of the NGF receptor TRKA by transplanted ATSCs. This evidence, reported in *in vitro* studies [51], could suggest a possible autocrine mechanism on ATSC, as they express NGFB *in vitro*, although the functional significance of this observation deserves further studies.

Taken together, the results obtained in this study seemed to indicate that ATSC neurotrophic features reside in their specific capability of expressing not only secreted neurotrophins/neuroprotective molecules, but also structural protein-coding genes, mimicking the astrocyte function in sustaining neurons metabolism and function in the central nervous system and being able to differentiate into astrocytes. These properties, along with their reported capacity to migrate in injured tissues, could suggest possible future applications of ATSCs in many diverse neurological contexts.

Author Contribution

Wanda Lattanzi and Maria Concetta Geloso contributed equally to this work.

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Review Article

The Use of Pluripotent Stem Cell for Personalized Cell Therapies against Neurological Disorders

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Although there are a number of weaknesses for clinical use, pluripotent stem cells are valuable sources for patient-specific cell therapies against various diseases. Backed-up by a huge number of basic researches, neuronal differentiation mechanism is well established and pluripotent stem cell therapies against neurological disorders are getting closer to clinical application. However, there are increasing needs for standardization of the sourcing pluripotent stem cells by establishing stem cell registries and banking. Global harmonization will accelerate practical use of personalized therapies using pluripotent stem cells.

1. Introduction

Infectious diseases have been life-threatening with the aid of newly emerging ones caused by environmental and sociocultural changes of human life. However, coping with infectious agents is being accelerated and accurate by scientific achievement resulting in antibiotics and vaccines development, so that importance of managing physical damages caused by external wound or physiological disorders of internal origin is being important in the present.

Approaches to cure the wound-mediated loss of physical function or genetic disorders had been focused on prevention from worsening of damage to deteriorate neighboring tissue or organs using medicinal and surgical efforts. Along the 20th century's era of organ transplantation, recent approaches of regenerative medicine using stem cell are emerging as a sensation in biomedical sciences in 21st century and provoking an innovative development in medicine.

It is expected that personalized regenerative medicinal approach using stem cells will be accelerated by continuous huge investment and research efforts throughout the world. Since human embryonic stem cell (hES) was first established in 1998 [1], researches to establish pluripotent stem cells, that is, ES cells, or isolate stem cells from various adult tissue or organs and to differentiate them into target tissues of therapeutic interest such as neuronal, cardiovascular, pancreatic, and hepatic lineages have been progressed and the possible outcomes are expected to be utilized as personalized therapeutics and as basic research tools for disease modeling. Although there are still challenges for practical application of stem cell therapies in clinical use in the present, however, the growing number of clinical trials for therapies using adult stem cells of various human tissue origin as well as hES cells is increasing public expectation for practical use of stem cell therapies in regenerative medicine (http://www.clinicaltrial.gov/). Here we reviewed what is current status of personalized therapies for neurological disorders using pluripotent stem cells and what is needed to further expand their application in practical use.

2. Pluripotent Stem Cells as Sources for Personalized Stem Cell Therapy

Pluripotent stem cells have infinite proliferative potential and capacity to differentiate into three germ layer-derived cell types of a body: ectoderm, endoderm, and mesoderm. Pluripotent stem cells comprise ES cells and, more recently, induced pluripotent stem (iPS) cells.

ES cells are derived from the inner cell mass of blastocyst [1]. ES cells can generate all type cells of three germ layer

origin while adult stem cells are thought to be limited in differentiation potential into various cell types of tissue origins. In comparison to adult stem cells which have limitation in *ex-vivo* proliferation, ES cells can be grown indefinitely in cell culture of optimized condition. With those natures, ES cells are beneficial as a source of cell replacement therapies for supplying a large number of cells needed for therapeutic application stably.

The iPS cells are reprogrammed from somatic cells by introduction of a number of ES cell-specific genes, mainly OCT4, SOX2, KLF-4, C-MYC, LIN28, and NANOG. The introduction of iPS cell by Shinya Yamanaka brought huge scientific interest into stem cell and regenerative medicine field because of biomedical and socioeconomical impacts of iPS cells [2–5]. Until now the technical improvement related to iPS cells generation is ongoing by using diverse cell types, different factors, and various methods [4–6].

Major advantages of iPS cells can be described by two aspects. First, the method of iPS cell establishment is free of ethical concern by which hES cells are critically screened before utilizing in research. Second, iPS cells are patient-specific source of pluripotent stem cell because the cells are originated from patients' somatic cells by introducing a number of ES-specific genes [2, 3, 7]. So to speak, patient-specific iPS cells are unlimited source of autologous stem cell therapy with perfect match of leukocyte antigen, and then we may overcome a hurdle of graft versus host interaction. Moreover, somatic cells from patients with genetic disorders have been reprogrammed to make iPS cells and then differentiated into disease-specific tissues for modeling, investigating disease pathophysiology and then drug discovering [8–10].

However, there are a number of reports for limitations of current technologies that hinders iPS cells into practical use. First, increase of tumor formation possibility is reported [11, 12]. Introduction of c-MYC protooncogene as one of reprogramming factors increases tumor formation. In addition, retro or lentiviral transduction of reprogramming factors causes not only epigenetic reconfiguration but also genetic modification which may lead to cancer formation [13, 14]. Second, it was reported that mouse iPS cells have immunogenicity to syngeneic recipient. This suggests that, although iPS cells are conceived to be immune-tolerated to autologous recipients, there are still hurdles to overcome to establish more ES-like iPS cells in terms of completeness [15].

Supported by the numerous research milestones for the achievement in pluripotent stem cells both hES and iPS cells, their clinical usage is being realized. A couple of clinical trials are undergoing (http://www.clinicaltrial.gov/); however, practical application of pluripotent stem cells is mainly being evaluated at the level of research and development. In order to solve remained controversy and problems many basic issues left to be done: to understand how to control stem cell proliferation and differentiation into specific cell types, to enhance survival in recipient, to prevent immune rejection and induce their integration into recipient cellular network, and to optimize the functional recovery of damaged tissue or organs in human disease. In addition, the use of pluripotent stem cells should solve the problems of the immune rejection of grafted stem cells and tumor formations in cell replacement therapy.

3. Historical Aspect of Personalized Therapies Using Stem Cells in Neurological Disorders

As above mentioned, stem cells have promised to revolutionize the future of regenerative medicine through the cell replacement therapies to treat a variety of deliberating diseases. Although using pluripotent stem cell for treatment is relatively new, convincing evidence has emerged the capability of various stem cell populations used for treatment of various diseases. The applications of stem cell therapies for treating neurological disorders are enormous. Mimicking the neural stem cell activity, the treatment of neurological disorders is based on the ideas that the replacement of damaged cells and the restoration of brain homeostasis can be achieved through transplantation of stem cell. Many laboratories have attempted stem cell treatment for central nervous system diseases, including spinal cord injury, stroke, amyotrophic lateral sclerosis, Parkinson's diseases, multiple sclerosis, and epilepsy [16–22].

The first nonhematopoietic bone marrow-derived multipotent stromal cells (BMSC) were reported in 1976 [23]. From the beginning, BMSCs were focused for cell therapy because they are easily obtained from human bone marrow aspirates, rapidly expanded in culture. Moreover, it was reported that BMSCs can be used autologously and do not form tumors after transplantation [24]. In recent report, the clinical trial for strokes with intravenous BMSC treatment long-term follow-up showed that the improved survival statics compare with control [25].

Fetal Neural Stem Cells (NSCs) can be derived from human fetal brain and capable of differentiating into neurons, astrocytes, and oligodendrocyte [26]. The fetal NSCs are generally isolated from abortus and their use is conceived less ethically controversial than hES cells. There is available evidence that supports efficiency of intrastriatal transplantation of human embryonic mesencephalic tissues in Parkinson's disease. These studies demonstrated that grafted dopamine neurons can survive, reinnervate the striatum, and restore dopamine release for up to 10 years despite an ongoing disease progress, which destroys Parkinson's disease patient's own dopamine neurons [27, 28]. However, a number of recent postmortem studies examined long-term fetal transplants in Parkinson's diseases have revealed that host pathogenic factors affect the transplants and their recipients [29–31]. The long-term clinical follow-up in six individuals who survived 9–16 years after the fetal neuronal tissue transplantation showed that the effect of transplantation is limited and three of them showed typical brain pathology of Parkinson's diseases at autopsy [29–31]. These indicate that the engrafted cells may be affected by the disease process of the recipients weakening the long-term clinical benefits as a therapeutic approach.

Over the last decade, there has been a rapid advance in research of pluripotent stem cells including ES and iPS cells. Many studies suggested that ES cells have the ability to generate variety of types of cells in neuronal lineages

including neural progenitor, neurons, oligodendrocyte, and astrocyte [32–37]. In addition, accumulating data have proven the therapeutic efficacy of hES cell-derived neural precursor cells (NPCs) in experimental model of neurological diseases [33, 34, 38, 39]. Recently a number of reports showed the use of iPS cells to treat several neurological disorders such as Parkinson's and Huntington's disease, spinal muscular atrophy, amyotrophic lateral sclerosis, and spinal cord injuries [4, 21, 40, 41].

The generation of iPS cells from a somatic cell having genetic disease patients offers the opportunity to disease modeling and possibility for treatment in personalized cell therapy by accelerating drug discovery. Patient-specific iPS cells from degenerative neurological diseases such as familial dysautonomia [8] and adrenomyeloneuropathy [42] opened possibility to comprehend disease mechanism and discover drug to moderate symptoms.

4. *In Vitro* Differentiation into Neural Lineage from ES Cells

Establishment of efficient and stable *in vitro* method for neural differentiation is an important preceding process for clinical application of pluripotent stem cells [43]. Characteristically, nervous system is composed of heterogeneous populations mixed with a variable cell types including neurons, astrocytes, oligodendrocytes, and their precursor cells [44]. To make these variable neural components during normal development, a part of ES cells undergoes neural induction and rostrocaudal and dorso-ventral patterning. Throughout these steps, human nervous system is assorted by forebrain, mid and hind brain, and spinal cord. After then temporal and spatial patterning is followed, resulting in differentiation of cortical progenitor cells into generate subtypes of neurons and formation of cortical structures with six layers. Each subtype of neurons has its own specific function and its damages and defects are associated with different types of diseases or disabilities, undoubtedly [45]. Many previous researchers have tried for efficient derivation of many mature cell lines from pluripotent stem cells. Unfortunately, *in vitro* differentiation process cannot be always reflected to *in vivo* differentiation steps in neurogenesis. Additionally transcriptional variability between the hES cell lines and also their differentiated neural cells is remained, reflecting the heterogeneity in the way the ES cells were established [46]. Here, we introduce and summarize the representative protocols and mechanisms of the neural differentiation from the pluripotent stem cells.

In vitro neural differentiation of pluripotent stem cells involves three main stages of neural induction, expansion of neural progenitor cells, and differentiation to neurons and glial cells [47, 48].

In earliest procedures for neural induction, hES cells were led to spontaneous differentiation into embryoid body and then neural progenitor cells appearing rosette-like structures were mechanically isolated [36]. With identification of various differentiation-related cellular mechanisms and pathways, the inhibition of bone morphogenetic proteins (BMPs) and/or SMAD signaling pathways is known to be necessary for efficient induction of neural induction of hES cells. BMPs are members of transforming growth factor-beta (TGF β) and activate the specific type I and type II receptors, leading to phosphorylation of their downstream SMAD [47]. Using this mechanism, Chambers et al. [49] have designed an efficient protocol for neural conversion from one ES and two iPS lines. They treated with recombinant Noggin and drug SB431542 for combined dual inhibition of SMAD signaling and identified expression of neuroectodermal markers, PAX6 and SOX2, in mRNA and protein levels [49]. Another study demonstrated that hES cells can differentiate into the Pax6+/Sox1+ neural rosette in the presence of noggin and supplemental addition of fibroblast growth factor (FGF) [50]. Additionally, inhibition of FGF signaling pathway led to impairment of neural induction, suggesting that FGF signaling can implicate in the neural specification stages independently of BMP signaling [51]. More recently, it is found that coincident blockade of Activin/Nodal and BMP pathways by SB431542 and dorsomorphin enhances the neural differentiation in variable pluripotent stem cell lines. An advantage of this protocol is that the differentiation efficacy is constant neural regardless of the divergence [48].

Following neural induction, neural expansion process should be announced. In this stage, the neural precursor cells in EBs are expanded in suspension culture with media usually containing mitogenic factors, such as bFGF or epidermal growth factor (EGF) [47, 48]. WNT signaling pathway is thought to be contributed for formation and maintenance of hES cell-derived neurosphere in this stage [52].

In the next stage, hES cell-induced and -expanded neural progenitor cells are differentiated into neural or glial cells. Plating of the precursor cells on the laminin, fibronectin or matrigel substrates promotes differentiation of the precursor cells to neurons and glia. In addition, absence of previously treated mitogenic factors helps their differentiation process into mature cell lines [47, 48]. A study found that the determination of the cell lineages is governed by combined activation of some signaling pathway including Rho/ROCK and PI3K/Akt pathways [47].

The final stage of the *in vitro* neural differentiation process is a neural specification that means terminal differentiation of immature cells into full matured cells with acquisition of their own typical cellular functions. Combined treatment of various cytokines in mid-stage neural cells makes various types of mature function-specific neural cells. It is very important to reveal how to differentiate to a specific neural cell lineage for replacement therapy using ES cells as a substitution of abnormal or malfunctioning cells. Selective loss of dopaminergic neurons in the substantia nigra is a characteristic of Parkinson's disease and many studies have developed the methods for differentiating from ES cells to mature dopaminergic neurons [36]. Both Shh and FGF8 are considered as important ligands for production of dopaminergic neurons. Biologic effects of these molecules in neurogenesis support the evidence of addition of Shh and FGF8 in the culture media that have been applied in most previous described protocols [36, 53, 54]. For differentiation into glial lineages, treatment of FGF2 followed by treatment

of PDGF, IGF, NT3, and Shh generates oligodendrocytic differentiation of hES cells [55]. Another glial cell types, mature astrocytes, can be produced from hES cells by using cyclopamine, Shh inhibitors, and human astrocytes mudium [56].

5. Epigenetic Regulations in Embryonic Development as well as Neural Differentiation

Epigenetic changes mean heritable alterations of gene expression in the absence of changes in DNA sequence. Epigenetic events include DNA methylation, histone modification, and nucleosome remodeling, leading to structural changes of chromatin and subsequent transcription regulation [57]. Polycomb group proteins (PcG) are representative regulators of epigenetic changes and they are conservative components from *Drosophila* to humans. Structurally they form distinct multimeric complexes, called Polycomb repressive complexes (PRCs), classified as two groups, PRC1 and PRC2. PRC2 is composed of EZH2, SUZ12, and EED, which acts as initiators of transcription inhibition [58]. PRC1 can recognize the trimethylates lysine 27 occurred by PRC2 through the chromodomain and maintain the gene repression status [59].

The expression of ES cell and their development-related genes are modified by epigenetic regulatory factors [60]. PcG is known to be an essential constituent for maintaining the properties of ES cells by regulation of the transcription factors OCT4, SOX2 and NANOG, all that are expressed in undifferentiated status and are associated with ES cell self-renewal and pluripotency [58]. As differentiation progressed, pluripotent genes including OCT4, SOX2, and NANOG are downregulated by methylation on the promoter region [61]. On the other hand, differentiation-related genes expressing in hematopoietic stem cells and neural progenitor cells are activated by demethylation of their promoter region [58]. PRCs also play crucial roles as inhibitors of differentiation. Using genetic ontology, a study demonstrated that the functions of PcG-binding genes in hES cells are involved in development, transcription regulation, organogenesis and neurogenesis. Hox, GATA, NeuroD, FOX, POU, and MYO are controlled by PcG and it means that PcG represses the expression of differentiation-promoting genes. This study demonstrated that SUZ12 occupies a subset of differentiation-promoting genes also bound by OCT4, SOX2, and NANOG in hES cells [58]. Another study revealed that when ES cells experienced spontaneous differentiation forming embryoid body (EB), PcG-binding genes are specifically activated in differentiated state or EBs [61]. PcG can closely and delicately regulate the expression of both pluripotent and differentiated genes to proper development.

In *in vivo* studies, PcG also is proven to be concerned in organogenesis and differentiation of pluripotent stem cells. PRC2 subunits (Eed, Ezh2, Suz12) knockout mice suffered a congenital defect in gastrulation [62–64]. EED or Suz12 knockout murine ES cells showed decreased H3K27 methylation as well as abnormally increased expression of differentiation-related genes, suggesting the epigenetic changes produced by Eed, Suz12 and histone methylation contribute the differentiation process [63, 65, 66]. Another study revealed that Suz12 knockout murine ES cells fail to achieve the primary neuroepithelial development [66]. Thus, epigenetic regulation is necessary for appropriate development and differentiation on pluripotent stem cells.

Neural differentiation from ES cells is also effected by epigenetic events, such as methylation or acetylation. Among the epigenetic events, H3K4 trimethylation and H3K9 methylation were found to play roles in repressing the pluripotency- and differentiation-associated genes during neurogenesis from hES cells, respectively [67]. Moreover, Bmi-1, one of the polycomb group proteins, allows maintaining the self-renewal and proliferation of neural stem cells. Bmi-1 knockout neural stem cells have a decreased clonogenic capacity and proliferative index, in which epigenetic change is implicated in maintaining stemness [68–71]. Another PcG, Ring 1B, is also concerned with maintenance of pluripotency and self-renewal in ES stem cell-derived neural precursor cells. Ring 1B deficient ES cell-derived neural stem cells show a decreased efficacy and size of neurosphere formation as well as increased neural induction [68–71]. EZH2 expression can determine the lineage of cell differentiation in the murine neural stem cells. Overexpression of EZH2 promotes the oligodendrocytic differentiation and concordantly impedes the astrocytic differentiation [72]. Therefore, epigenetics cannot only be an important factor to maintain pluripotency but also determine the cell fate. If we use this epigenetic effect on differentiation of ES cells, we will be able to establish more efficient and stable cell lines for treatment. In fact, recently reported studies found that histone deacetylase inhibitor, sodium butylate, has an effect for improvement of the neural differentiation induction from murine ES cells [73, 74].

6. Recent Advance of Personalized Therapies Using Pluripotent Stem Cells

Use of pluripotent stem cells in personalized therapies has made rapid progress to practical application accumulating growing evidence in different pathophysiological conditions in small and larger animal models. Clinical trials are a major step in the development of personalized stem cell therapies into practical application. As of 30, June, 2011, there are 3604 ongoing clinical trials of stem cells worldwide (http://www.clinicaltrial.gov/). Although the number of clinical trials using human pluripotent stem cells is only limited to two, however, recent approval of the human pluripotent stem cell clinical trials provides new prospects for cell replacement strategies in a broad spectrum of human neurological disorders. The Geron Corporation has initiated the first clinical trials of an ES cell-based therapy on human. This study will evaluate the drug GRNOPC1, ES cell-derived oligodendrocyte progenitors, on patients with acute spinal cord injury. Consequently, Advanced Cell Technology has secured FDA clearance to proceed with two separate Phase 1/2 clinical trials to test the safety of the hES cell-derived

retinal pigment epithelial (RPE) cellular therapy for Dry Age-Related Macular Degeneration and for Stargardt's Macular Dystrophy.

Although it is sure that the clinical trials of hES cell-derived therapy will strengthen possibility of personalized therapies using pluripotent stem cells in practical application, however, immunological diversity of human leukocyte antigens (HLAs) is a problem remained. As already mentioned, patient-specific iPS cells have many hurdles to get over for practical application to human body such as tumor formation and possible immune rejection mediated by aberration of genetic integrity. Expanding an allogeneic pool of in high-standard "clinical grade" hES cell lines established at utmost ethical mandate might be a way to accelerate clinical application of personalized pluripotent stem cell therapies by increasing chances to find HLA-matched cell source [75–77]. As an effort, there are increasing needs for pluripotent stem cell registry and banking worldwide and the number of them is being increased [78–81]. There are a number of well-organized pluripotent stem cell registries running based on government worldwide such as NIH Human Embryonic Stem Cell Registry (http://escr.nih.gov), European Embryonic Stem Cell Registry (http://www.hescreg.eu/), and Korea Stem Cell Registry (http://kscr.nih.go.kr/). The International Stem Cell Banking Initiative (ISCBI) is a global network of stem cell banks that aims to facilitate practical application of stem cell research by harmonizing stem cell banking on a global level [78, 79]. It is expected that the pluripotent stem cell-based personalized therapies will be strengthened by international collaboration, harmonization, and standardization for establishment, characterization, and qualification of the pluripotent stem cell as well as differentiation into functional tissues. Moreover, convergence of cutting-edge sciences such as systems biology and informatics approaches into pluripotent stem cell registries and banking will be needed for further enhancement of personalized therapeutic application of pluripotent stem cells.

Author's Contribution

Hye-Yeong Ha and Si-Hyong Jang are equally contributed.

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Review Article

Progenitors for the Corneal Endothelium and Trabecular Meshwork: A Potential Source for Personalized Stem Cell Therapy in Corneal Endothelial Diseases and Glaucoma

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Several adult stem cell types have been found in different parts of the eye, including the corneal epithelium, conjunctiva, and retina. In addition to these, there have been accumulating evidence that some stem-like cells reside in the transition area between the peripheral corneal endothelium (CE) and the anterior nonfiltering portion of the trabecular meshwork (TM), which is known as the Schwalbe's Ring region. These stem/progenitor cells may supply new cells for the CE and TM. In fact, the CE and TM share certain similarities in terms of their embryonic origin and proliferative capacity *in vivo*. In this paper, we discuss the putative stem cell source which has the potential for replacement of lost and nonfunctional cells in CE diseases and glaucoma. The future development of personalized stem cell therapies for the CE and TM may reduce the requirement of corneal grafts and surgical treatments in glaucoma.

1. Introduction

Rapid progress in stem cell research in recent years provides new hope for the treatment of various previously incurable diseases. The basic treatment principle is to replace lost or damaged cells with healthy ones derived from stem cells and/or stimulate endogenous regeneration via paracrine effects mediated by the transplanted stem cells [1, 2]. Stem cells can reasonably be categorized into three main types according to their origins: embryonic stem (ES) cells, induced pluripotent stem cells (iPSCs), and adult stem cells. The focus of this paper will concern a specific region of the eye, namely, the adult stem cells of the human corneal endothelium (CE) and neighbouring trabecular meshwork (TM), there will be considerable overlap in the techniques applied to the differentiation of cells and obstacles to be addressed before regeneration therapies are available.

2. Stem Cell Overview

Whilst ES cells have numerous advantages in research such as their unlimited capacity to self-renew and pluripotency allowing them to differentiate into any cell type in the body, the fear of teratoma formation, immune-rejection issues, and ethical concerns regarding the destruction of embryos have slowed their progress towards clinical trials.

The research field of iPSCs has rapidly gained momentum since the discovery by Takahashi and Yamanaka [3]. iPSCs afford an advantage in that an autologous approach may be possible and as such circumventing the ethical and immunological disadvantages of ES cells. However, there are major safety concerns that involvement of retroviral or lentiviral vector integration in iPSCs engineering may cause genomic disruption and oncogenesis [4–6]. Besides, after the reports proving iPSCs retained epigenetic memory from

the somatic cell of origin [7, 8], questions have been raised whether iPSCs are completely pluripotent like ES cells, as how far back they are reprogrammed will influence their directed differentiation potential. Despite the bright future iPSCs may have, extensive efforts and a measured scientific approach are required to guarantee safety and production quality, to understand more about the molecular signaling and pathways, and to find out reliable differentiation protocols of iPSCs before transplantation can be done in patients.

Adult stem or progenitor cells are also referred to as somatic stem cells. They reside in many adult tissues such as the bone marrow, skeletal muscle, heart, brain, skin, and limbus (summarized in [9]). Although they are not pluripotent, they still retain high plasticity. Their ethical superiority over embryonic stem cells and autologous origin avoiding immunological suppression after surgery makes them a popular stem cell source for small-scale clinical application [10, 11]. Adult stem cells are enriched in locations that are very close to the target tissue, as such, they may have more direct and faster access to the site of injury when compared with other stem cell types [9]. In addition, they have already undergone critical developmental stages, which render them comparatively easier to commit to the cell types desired [12, 13]. Understandably, difficulties such as their isolation, expansion efficiency due to limited cell numbers and integration and survival in the host tissue still remain to be unravelled. Adult stem cells, albeit with their own limitations, may be a relatively safer and more ethical alternative cell source for therapeutic use at present.

In the eye, the most successful stem-cell-based therapy to date has been the use of limbal epithelial stem cells to regenerate the corneal epithelium [14]. Apart from limbal epithelial stem cells, intensive research has been done on different niches of adult ocular stem cells, such as conjunctival epithelial and retinal stem cells, aiming for ocular repair and regeneration [13, 15, 16]. One niche of cells that has had relatively limited attention and may be of considerable clinical value (which is the focus of this paper) are the progenitor cells located in the transition zone between the periphery of the CE and the anterior extension of the TM, which is known as the Schwalbe's Ring region. In the recent decade, more and more evidence emerged to support the idea that these progenitors may be able to provide new cells for the CE, TM, or possibly both. This opens up a new prospect on research using these intriguing progenitors to treat CE diseases and glaucoma. In this paper, we will review the biological properties of CE and TM cells, summarize and discuss the evidence suggesting the presence of stem-like cells in the transition area, and, in addition, outline how these cells can be used for regeneration.

3. CE and TM: Structure, Function, and Embryology

3.1. Corneal Endothelium. Cornea is the transparent tissue located at the front of the eye which provides approximately two-thirds of the total ocular refractive power (Figure 1(a)). It consists of five layers: the multilayered epithelium, Bowman's membrane, the stroma, Descemet's membrane, and the endothelium. CE is on the posterior surface of the cornea facing the anterior chamber. It is composed of a single layer of regularly arranged hexagonal and pentagonal cells which are around 5 μm thick and 20 μm in diameter [17]. The crucial function of the CE is to maintain corneal transparency by regulating corneal hydration while allowing nutrients from the aqueous to diffuse back to the avascular cornea. The endothelium accomplishes this by a pump-leak model. It serves as a "leaky" barrier to permit selective permeability of the nutrients but prevents bulk fluid flow into the stroma. At the same time, it actively removes excess fluid from the stroma into the anterior chamber through ionic fluid pumps to prevent corneal swelling. In addition to the barrier and pump functions, the endothelial cells are responsible for the synthesis of Descemet's membrane, which is the basement membrane where the endothelium resides [18, 19].

3.2. Trabecular Meshwork. The anterior chamber of the eye is bordered anteriorly by the CE and posteriorly by the iris. At the periphery of the chamber, there lie the TM, scleral spur, ciliary body, and iris root, which form the anterior chamber angle (Figure 1(b)). The TM extends from an anatomical position called Schwalbe's line, which marks the end of Descemet's membrane, to the ciliary body and iris root at their junction. There is a specific cell population near the transition area (Schwalbe's line) between cornea and meshwork, and this will be discussed in detail in Section 5. Together with Schlemm's canal, the collector channels, and aqueous veins, the TM forms the major structure for aqueous humour outflow [17]. Aqueous humour is produced by the ciliary body and passes through the pupil into the anterior chamber. It subsequently leaves the eye through the TM into Schlemm's canal, then from there to the intrascleral plexus, and finally to the episcleral venous system [20].

The TM is a porous tissue comprised of three regions: the innermost uveal meshwork which is chord-like in structure, the deeper corneoscleral meshwork with flattened sheet-like trabeculae, and the juxtacanalicular connective tissue (also called cribriform layer or endothelial meshwork) which links the corneoscleral trabeculae with the inner wall endothelium of Schlemm's canal [17, 21]. The trabecular lamellae or beams contain collagenous cores surrounded by endothelial cells, and the lamellae are bridged by the TM cells [22]. The corneoscleral and uveal meshwork do not provide much resistance to aqueous outflow and Grant showed that aqueous outflow facility was not affected even if the inner parts of the TM were excised [23]. The outflow resistance resides primarily at the region near the juxtacanalicular connective tissue and the endothelial lining of Schlemm's canal [22]. Since the Schlemm's canal is shorter than the TM in the anteroposterior direction, the TM can also be divided into the anterior nonfiltering and posterior filtering portions.

3.3. Embryology. During embryogenesis, the neural ectoderm, the surface ectoderm, the neural crest, and, to a lesser extent, the mesoderm are involved in the development of

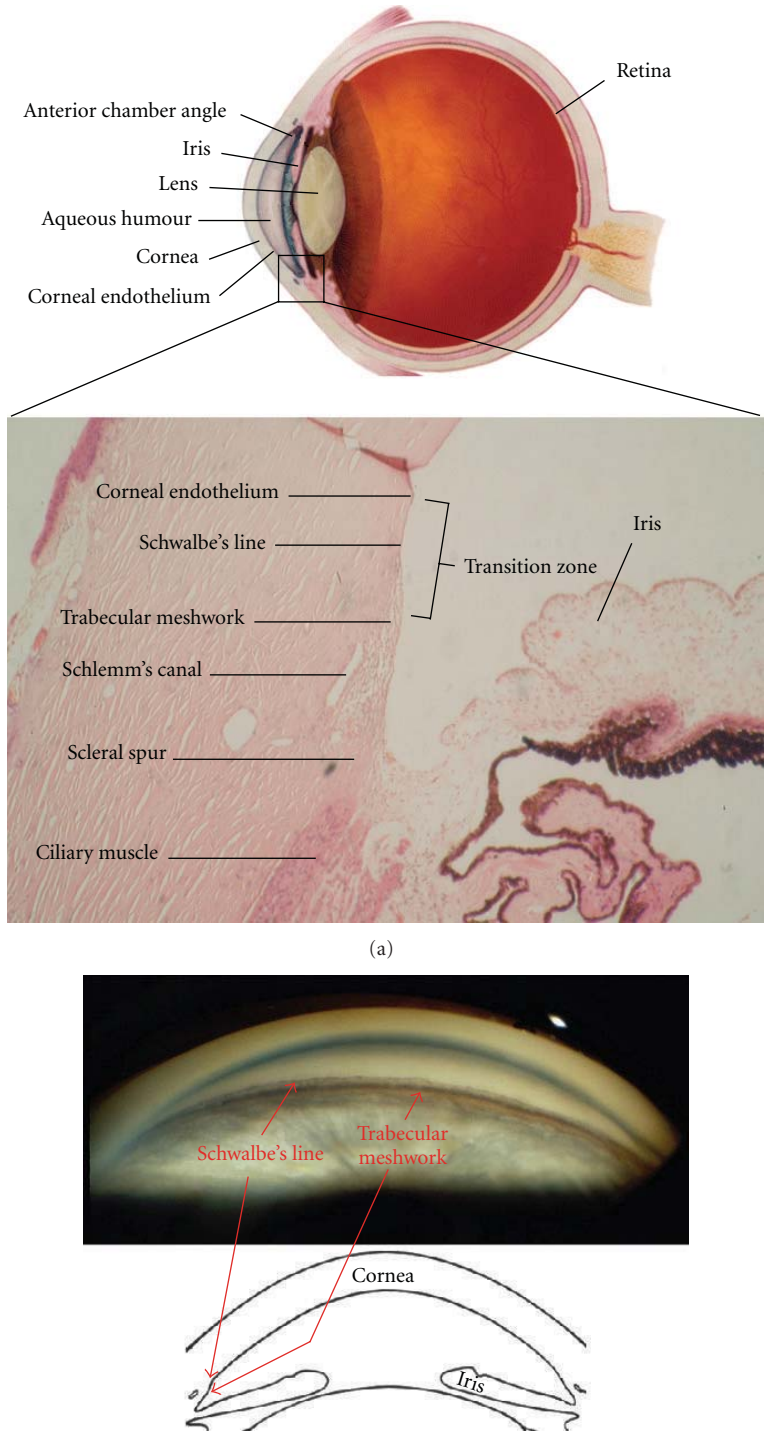


Figure 1: (a) Schematic diagram of the human eye and transverse section of haematoxylin and eosin stained chamber angle tissues (×100). (b) Anterior chamber angle as viewed in gonioscopy.

the eye. The CE and TM are both derived from the neural crest. They are formed from the first wave of neural crest-derived mesenchymal cells migrating between the surface ectoderm and the lens. The development of the cornea begins at approximately 33 days of postfertilization [24]. At around the 40th day, a double row of flattened cells posterior to the basal lamina of the corneal epithelium is produced by the mesenchyme and it develops into the monolayer of CE by the 18th week [18, 24, 25]. At this time, the CE extends nearly to the angle recess. This endothelial membrane covering the angle recess starts to regress at around 15 weeks of gestation [25].

The primitive TM is formed at around the fourth month. It consists of a triangular mass of undifferentiated mesenchymal cells. During the seventh month, these cells flatten and become slightly separated from each other, and the cavities are filled with extracellular fibers. The fibers are then organized to form the trabecular lamellae or beams. Some cells with a stellate phenotype form the juxtacanalicular layer of the TM. The complete morphogenesis of the TM is finished around birth [24–27].

These tissue developments require specific gene regulatory networks in which many transcription factors and molecular signals are involved. Although the detailed developmental networks are still not well defined, some contributing factors are known. Cvekl and Tamm performed a comprehensive review of the transcription factors that are associated with the anterior segment morphogenesis [26]. They include PAX6, PITX2, PITX3, FOXC1, FOXE3, LMX1B, and MAF, where PAX6 is the essential regulator for eye development in different organisms [26, 28, 29]. It is involved in controlling neural crest migration and thus has a critical role in early formation of the CE and TM [30, 31]. The CE did not develop in *Pitx2*$^{-/-}$ and *Foxc1*$^{-/-}$ mice and the TM was abnormally formed [32–35]. LMX1B was shown to have a direct link to the dysgenesis of the TM [36]. Whilst these transcription factors clearly have an important role, some other transcription factors also influence CE and TM development [37–41]. In addition, specific signaling molecules also play a key role in coordinating the anterior segment growth. This is borne out with transforming growth factor (TGF)-beta 2 knockout mice which developed a much thinner cornea with CE failing to develop [42, 43]. Moreover, heterozygous deficiency of BMP4 resulted in absent or hypoplastic TM and Schlemm's canal, and profound extracellular matrix deficiencies in the TM [44]. For the role of different growth factors during embryogenesis and differentiation of the eye, readers are referred to the review by Tripathi et al. [45].

4. Biological Properties of CE and TM Cells

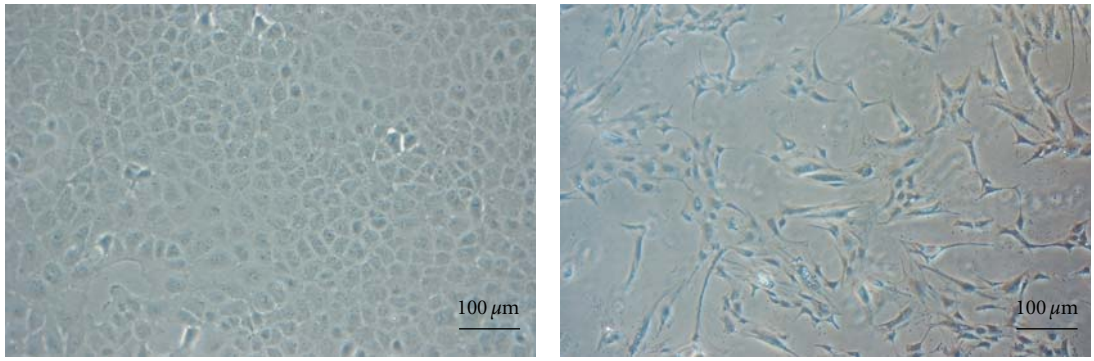
4.1. Cellular Characteristics and Markers Identification. CE cells adjoin one another with extensive interdigitations and are interconnected by tight and gap junctions. The tight junctions do not completely encircle the cells so that the endothelium can allow selective permeability for nutrients. The apical sides of the cells contain a band of actin filaments which helps maintain cell shape and barrier function and facilitate cell migration in wound healing [46]. Ultrastructurally, the endothelial cells have a large nucleus and contain numerous mitochondria, a prominent endoplasmic reticulum and Golgi apparatus, which are the characteristic features for cells metabolically active in transport, synthesis, and secretory function [18].

Although the CE is named "endothelium," it differs from the vascular endothelium. The CE is derived from the neural crest, whereas the vascular endothelium is from the mesoderm [25]. Additionally, the CE does not contain Weibel-Palade bodies nor express factor VIII, which are the typical vascular endothelium markers [47]. The CE displays an unusual combination of immunoreactions to antibodies against intermediate filaments vimentin and neurofilaments, as well as neural markers including neural cell adhesion molecule, neuron specific enolase, and S-100 protein [47–50]. These observations are not surprising when we trace back the origin of the CE. However, there have been controversies about the cytokeratin (CK) profile in the CE. CK8 and CK18 expressions in human CE cells were described in several studies [51–53] but not in others [54, 55]. Merjava et al. proposed that the variable results were due to different processing approach and antibody sensitivities [53]. The labeling of CK7 and CK19 in the CE is even more controversial [51, 55, 56]. To date, there has not been a specific marker for the CE. One can mainly distinguish them by their hexagonal morphology and evaluate their function using an Ussing chamber to measure the transport activity [57–59].

The TM cells bridge the intertrabecular spaces through cytoplasmic extensions, and adjacent cells are firmly connected to each other by desmosomes [22]. Electron microscopic studies revealed that gap junctions form the main intercellular connection between the TM cells [60]. The major actin distributions in the TM cytoplasm are straight stress fibers [61]. However, cross-linked actin networks (CLANs) have also been detected in human and bovine TM tissues [61, 62]. Similar to CE cells, there are no specific biomarkers to identify TM cells. It has been shown that the TM cells express vimentin, non-muscle actin, aquaporin-1, acetylated and acetoacetylated low-density lipoproteins, and the alpha-2 adrenergic receptor [49, 63–66]. The expression of myocilin in TM cells was increased after dexamethasone treatment [67]. Nevertheless, these proteins are also present in other cell types, making it difficult to use a single marker to identify TM cells. Some other potential TM markers including the matrix GLA protein and chitinase-3-like-1 were reported by other groups [68, 69].

Despite the lack of specific marker proteins, the TM cells possess some typical physiological characteristics. Rohen and Van der Zypen was the first to show that TM cells have phagocytic capacity [70]. It is believed that the phagocytosis helps remove debris in the circulating aqueous humour [71]. Besides, meshwork cells can synthesize a variety of extracellular materials including collagens, glycoproteins, and glycosaminoglycans (see [72] for review). The replacement and modification of the extracellular matrix compensates the gradual washout of materials during aqueous perfusion, so that the necessary outflow resistance is maintained. Moreover, the presence of contractile filaments in the TM cell cytoplasm indicates their contractility [73, 74]. It was found that substances that contract meshwork cells decrease the aqueous outflow facility and vice versa [75].

Both the CE and TM cells are exposed to continuous workload throughout lifetime, yet, they have limited proliferative capacity in situ to replace lost cells under normal circumstances [76, 77]. In the CE, the surrounding cells spread and slide to fill the gaps caused by cell loss. The endothelial cells are arrested in G1-phase of the cell cycle [76]. Bovine TM cells were also shown to be locked in



(a) (b)

Figure 2: (a) Corneal endothelial and (b) trabecular meshwork cells cultured *in vitro*.

G0/G1-phase [77]. Although both cell types can be grown in culture, they are contact inhibited [76]. The division rate of bovine TM cells decreased to negligible amounts when they were in contact and formed gap junctions [78].

4.2. Consequence of Cell Loss or Malfunction. There are approximately 4,000 CE cells per mm^2 at birth, but the cell density decreases with age at a rate of 0.6% per year throughout life [79]. The cell number is usually adequate to maintain normal corneal function for a lifetime. However, besides the factor of ageing, endothelial cell loss can also occur due to disease, trauma, and surgery. These may result in a higher cell depletion rate than normal, leading to endothelial failure and hence, loss of visual acuity. In order to maintain adequate corneal function, a minimum level of 400 to 700 endothelial cells per mm^2 is required and the cells need to be of uniform size and shape [18]. Hence, corneas for grafting need to be screened for endothelial health and cell numbers before they are accepted. Some ocular diseases are manifested by abnormal endothelial cells. Fuch's endothelial dystrophy is a corneal disease involving malfunction of the endothelial cells, in which irregular warts or excrescences of Descemet's membrane are secreted. The excrescences are collagenous secretions (known as guttata) deposited at the posterior surface of the membrane, causing disruption of the overlying endothelial cells and thus compromise endothelial function [18, 80]. Another disease attributable to aberrant CE is the iridocorneal endothelial (ICE) syndrome. The endothelium proliferates and migrates outward to the TM and across the angle onto the surface of the iris, which may progress to glaucoma, corneal decompensation, or both [81, 82].

The TM cellularity decreases with age as well. Alvarado et al. reported a cell loss rate of 0.58% per year [83]. This is comparable to that seen in the CE. It was estimated that there were 750,000 cells in the meshwork at 20 years of age but the number decreased down to around 400,000 by 80 years [84]. Other age-related changes in the TM include trabecular thickening, trabecular fusions, and alterations to the extracellular material in the juxtacanalicular meshwork; all of which would increase the aqueous outflow resistance and subsequently the intraocular pressure (IOP) [71]. Pathologically elevated intraocular pressure is the major risk factor in primary open angle glaucoma (POAG). Indeed, these age-related changes are intimately linked to the glaucomatous alterations found in POAG patients. The glaucomatous eyes were found to have significantly more cellular losses compared with age-matched normal eyes [85]. This is believed to precipitate the decrease in drainage facility. When cell loss is progressive, trabecular thickening and fusion may develop due to adhesions of the denuded portions of the trabeculae. Furthermore, accumulation of extracellular materials and meshwork cell hyperplasia in glaucomatous TM that are believed to obstruct the outflow pathway was also documented [71]. Hence, TM cells are essential to maintain a healthy meshwork for aqueous drainage.

4.3. Culture In Vitro. In spite of the restricted replication capacity *in vivo*, the CE and TM cells can be grown in culture under appropriate conditions. Figure 2 shows the *in vitro* culture of bovine CE and TM cells. It has been demonstrated that fibroblast growth factor (FGF) stimulates the proliferation of CE and TM cells [86–88]. Hepatocyte growth factor (HGF) is also a competent mitogen for both CE and TM cells in a dose-dependent manner [89, 90]. Culturing of CE cells on dishes coated with collagen type IV, laminin, or fibronectin favoured the formation of a typical hexagonal monolayer [86]. Hyldahl reported that the addition of insulin-like growth factor-1 (IGF-1) and epidermal growth factor (EGF) stimulates CE cells to initiate DNA synthesis [91]. Treatment of TM cells with platelet-derived growth factor (PDGF) can also increase their cell division. Besides, it enhances the phagocytic activity and promotes extracellular matrix secretion [92]. IGF-1 was shown to promote the incorporation of [3H] thymidine in TM cells, whereas vascular endothelial cell growth factor (VEGF) restrained cell growth [93]. Studies have revealed that TGF-beta, which is present in the aqueous humour, inhibits TM cell proliferation and suppresses S-phase entry of CE cells [94, 95]. Table 1 shows a summary of the mentioned growth factors effects on CE and TM cell proliferation *in vitro*.

Table 1: The influence of growth factors on CE and TM cell proliferation.

Growth factor	Corneal endothelium	Trabecular meshwork
EGF	++	++
bFGF	+	++
HGF	+	++
PDGF	++	+
IGF-1	+	+
TGF	−	− −
VEGF		−

Both the CE and TM cells are unique cell types in the eye. They do not normally replace themselves once they are lost in aging or diseases. To date, there has been no cell therapy for the treatment of CE and TM related diseases. A lot of unknown facts about the regenerative capacity of TM or CE still exist. Thus, the potential to repair or replace the CE and TM is an important area that needs to be explored.

5. Evidence of Stem-Like Cells in the Schwalbe's Ring and Their Therapeutic Implication

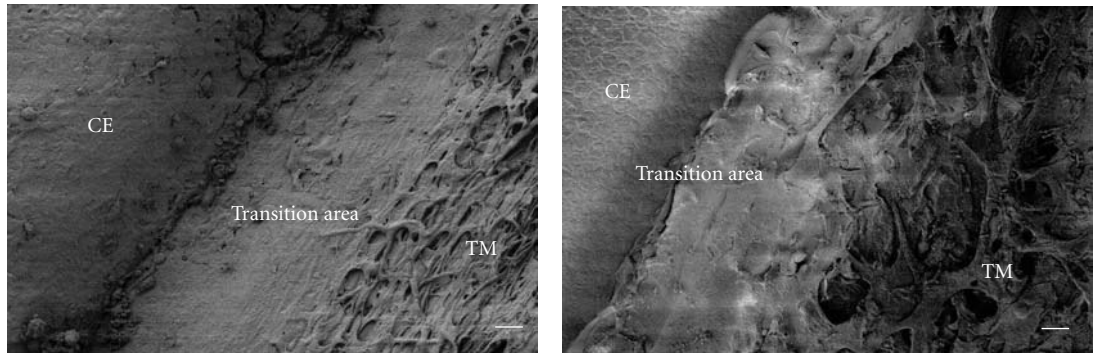
Stem cells are undifferentiated cells which can renew themselves indefinitely and produce one or more progenies through symmetric and asymmetric division, whereas progenitor cells have relatively limited self-renewal capacity and more restricted differentiation abilities. As mentioned earlier, stem/progenitor cells have been identified in various adult mammalian tissues. They are crucial for tissue renewal and regeneration. These adult stem cells have some key characteristics, including small cell size and high nucleus to cytoplasm ratio, high proliferative potential, slow cell cycle, and poor differentiation capacity with primitive cytoplasm [96]. They reside within a specialized microenvironment called niche, which offers protection and nourishment to the cells [97]. It is believed that adult stem cells have huge ethical and immunological advantages over embryonic stem cells as a future therapeutic option.

In the eye, accumulating evidence reveals that there is a population of stem-like cell located in the transition area between the periphery of the CE and the anterior nonfiltering portion of the TM (Figure 3). This transition region is referred to as Schwalbe's Ring. Schwalbe's line marks the peripheral termination of the Descemet's membrane and can be viewed clinically in gonioscopy (Figure 1(b)). In 1982, Raviola identified a population of unusual cells located just beneath the Schwalbe's line in rhesus monkey, which she called Schwalbe's line cells [98]. These cells are different from the typical CE and TM cells and have distinct ultrastructural features. As described, these cells "form a discontinuous cord, oriented circumferentially at the corneal periphery and deep to the CE lining of the anterior chamber." They morphologically resembled whorled multilamellar bodies of type II alveolar epithelial cells of the lung and were proposed to be secretory. Stone et al. found that these cells were immunoreactive to neuron-specific enolase, suggesting that they may have neuroregulatory function in the anterior segment [99]. Rittig and colleagues later reported intense staining of the enzyme hyaluronan synthase in Schwalbe's line cells, indicating their hyaluronan production ability [100]. Samuelson et al. documented Schwalbe's line cells in canine eyes as well [101]. In general, there seems to be a distinct cell population in the transition area, while their function is still unclear.

Not much attention was paid to Schwalbe's line cells until there was more evidence supporting the presence of stem/progenitor cells in this transition zone. The idea came primarily from the observation of an increase in TM cell division localized to the anterior nonfiltering portion of the TM after argon laser trabeculoplasty (ALT) [102]. ALT is a glaucoma therapy which aims at lowering the IOP. The principle of this laser procedure is not to make drainage holes through the TM, but to "blanch" the tissue which creates superficial burn restricted to the uveal meshwork [103]. The exact mechanism by which this treatment lowers IOP is not known, however, one of the possible mechanisms of action is the repopulation of the TM by stimulating cell division [104]. Indeed, several studies have shown marked tritiated thymidine incorporation into the TM cells following ALT in different species [102, 105, 106]. Acott and colleagues demonstrated a four-fold increase in TM cell division in human laser-treated explants compared with untreated controls [102]. They found that more than 60% of the cell division was initially localized to the anterior nonfiltering region of the TM and these proliferative cells migrated to repopulate the burn lesions afterwards. It appears that these cells are putative stem cells that are invigorated after ALT to repopulate the TM, possibly through the release of growth factors and cytokines. Due to their location at the insertion region into the cornea just beneath Schwalbe's line, Kelley have named them the "TM insert cells" [107].

Although ALT can lower the IOP successfully and, to some extent, repopulate the cell-deficient TM in glaucoma, uncontrolled repair process that occurred in the tissue may become a detrimental consequence. An abnormal corneal and/or trabecular endothelial cell sheet covering the anterior uveal meshwork was observed in some glaucoma patients after ALT [108, 109]. In some cases, they can grow extensively and block the aqueous outflow subsequently, leading to the failure of the surgery. Alexander et al. observed this aberrant endothelial membrane as well in normal human TM which was subjected to ALT [110]. They found that laser placed close to Schwalbe's line advanced the endothelial extension. It was believed that these repopulating processes after ALT involve migration of a specialized population of cells extending from the Schwalbe's line region [109, 110].

In addition to the observations in the TM, a significantly higher cell density at the peripheral CE also suggests that stem-like cells may be present in the peripheral transition region to provide differentiated CE cells [111, 112]. Otherwise, the cell density should be uniform all over the CE. It has been documented in the literature that at least some CE cells have the ability to divide under specific circumstances [113–116]. It was found that the peripheral CE cells retained higher replication competence than those in the central and this



(a) (b)

Figure 3: Scanning electron micrographs of (a) human and (b) bovine eyes, showing the transition between the peripheral cornea and anterior portion of the trabecular meshwork. There is a distinct transition area in the human tissue but the transition is more abrupt in the bovine one. The samples were coated with gold and imaged at an accelerating voltage of 4 kV and a working distance of 8 mm using a SE2 detector (Gemini LEO 1550 SEM). Scale bar = 20 μm.

was independent on the donor age [117]. Moreover, corneal grafts in hosts who retain the peripheral endothelium survive much longer than grafts in hosts with CE cell loss [118]. Persistent precursors from the host cornea may explain the enhanced long-term survival of grafts. Interestingly, Balachandran et al. reported in a case series that in spite of complete graft detachment after Descement membrane endothelial keratoplasty, spontaneous recovery of corneal transparency was observed in two patients [119]. They suggested that "endothelial transfer, migration, regeneration, or a combination thereof from either the donor or the recipient may explain the visual recovery." Schwartzkopff et al. later reported *in vivo* re-endothelialization following complete endothelial cell loss of the grafted donor cornea in rats and suggested that peripheral CE cells in recipients can support the regeneration [120]. These findings indicate that CE may have some sort of regenerative capacity under specific conditions, which is not consistent with the long-term belief that they do not divide *in vivo* [76]. In particular, the peripheral CE seems to be the regenerative zone in these conditions. As such, research of the Schwalbe's Ring region has become even more interesting, as the precursor cells in this transition area may be able to supply new cells for both the TM and CE.

In recent years, molecular marker studies supply more supportive data for the stem cell niche at the transition zone. Whikehart et al. detected telomerase activity, which is a stem cell maker, in the peripheral cornea [121]. They also observed bromodeoxyuridine (BrdU) labeling, which is a marker for cell division, in the TM and posterior limbus. The BrdU incorporation increased and extended into the CE in response to mechanical wounding. McGowan et al. showed that cells at the transition region express stem cell makers nestin, alkaline phosphatase, and telomerase [122]. Following corneal wounding, additional putative stem cell markers (Oct3/4, Wnt1) and differentiation markers (Pax6, Sox2) were observed. It was suggested that the putative stem cells in the transition area migrated to renew the wounded CE. To date, there has been no specific marker for this population of putative stem cells despite the observation of a different immunohistochemical profile in the CE, TM insert cells, and TM cells. Neuron-specific enolase was found to locate at the anterior but not posterior portion of the human TM [49]. Ankyrin G and human milk fat globule protein (HMFG, also known as breast antigen 46) were highly expressed in the insert cells. On the contrary, YKL-40 (also known as chitinase-3-like-1 or cartilage glycoprotein-39) had lower expression levels when compared to the CE and TM [107, 123].

Ideally, if the molecular signature of the stem-like cells is known, one can isolate and enrich the stem cell pool relatively easily using fluorescence-activated cell sorting (FACS) or magnetic immunosorting. However, the search of the specific stem cell signature will involve a laborious process and screening of a huge amount of putative markers. Hence, attempts have been made to isolate and propagate undifferentiated progenitor cells using a sphere culture protocol [124–126]. Precursors from human and rabbit CE have been successfully isolated using the sphere-forming assay and it was found that the peripheral CE contained significantly more precursors than the central region [127–130]. Mimura et al. proved that this culture assay selectively isolated younger progenies [131]. Huang and colleagues showed that bovine CE cells resembled bovine aorta in its content of endothelial colony forming cells [132]. Our sphere culture of primary peripheral bovine CE cells revealed the presence of undifferentiated precursor cells with self-renewal capacity and their potential to differentiate into neuronal lineages (Figures 4 and 5). Besides the CE, progenitor spheres were also isolated from human TM primary cultures [133]. It is likely that these isolated precursors from the CE and TM are from the transition zone in between them. It remains to be determined whether "Schwalbe's Line cells", "TM insert cells," and precursors having been isolated are the same cell type. For our convenience we have called the progenitor cells "PET cells" (Progenitors for Endothelium and Trabeculum) so not to presume until proven that we have the exact same cell population previously described.

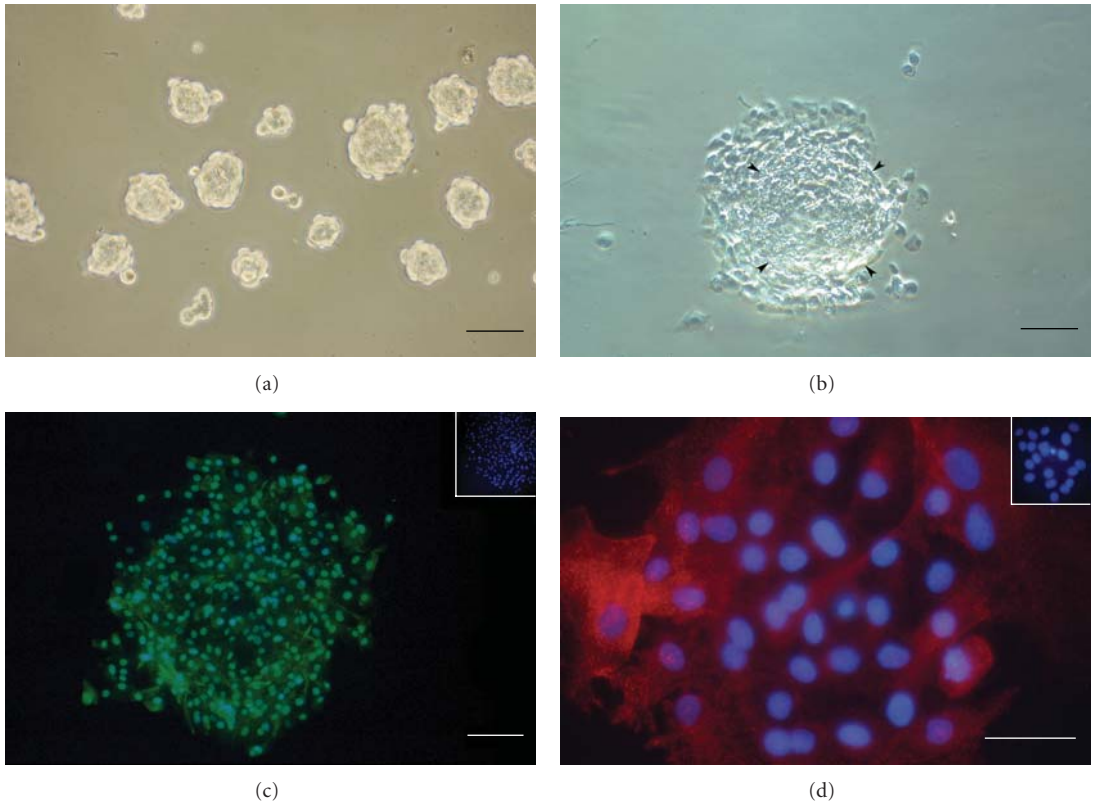


Figure 4: Sphere culture of bovine peripheral corneal endothelial cells. (a) Floating spheres on day 7 in defined serum-free media. Aggregation and development of dark cores can occur when spheres are left over the optimal culturing period of 5–7 days. (b) Cells migrating from an attached sphere on adherent substrate. The arrowheads show the contour of the sphere. (c) Nestin (green: undifferentiated cell marker) and (d) β-III tubulin (red: neuronal marker) staining were detected in the cells that migrated from the primary spheres. Nuclei were counterstained with DAPI (blue). Insets are negative controls with nonimmunized IgG. (a–c) Scale bar = 100 μm; (d) Scale bar = 50 μm.

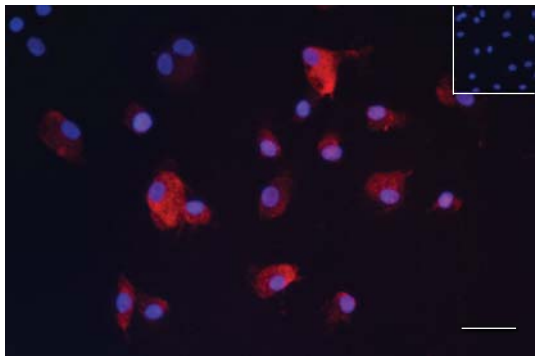


Figure 5: After 7 days of differentiation, cells derived from the spheres also expressed β-III tubulin. Nuclei were counterstained with DAPI (blue). The inset is a negative control with nonimmunized IgG. Scale bar = 50 μm.

The studies of stem cells in the eye have important implications in ocular health and disease treatment. Animal models using the isolated CE precursors for regeneration have been documented in several studies [59, 134, 135]. The PET cells within the transition area may be a promising cell source for replacing worn-out endothelium *in vivo* or boosting the number of endothelial cells *in vitro* on potential corneal graft materials. As mentioned in the previous section, ICE syndrome is manifested by abnormal proliferative CE cells that grow and cover the angle. It is tempting to speculate that the aberrant cells are metaplastic progenitors residing in the endothelium. Treatments can possibly be developed by targeting these cells. Besides, we know that the number of TM cells drops significantly in glaucoma patients, which precipitates the blockage of the aqueous outflow pathway. Thus, repopulating the cell-deficient TM using the PET cells may be a useful treatment to enhance drainage in glaucoma.

6. Summary and Future Directions

Recent progress in stem cell research provides an optimistic prospect on their use in regenerative medicine and tissue engineering. Specifically, advances in iPSCs and adult stem cells research raise hope for personalized cell replacement therapies. However, before iPSCs can be clinically applied, extensive efforts are needed to devise reliable production methods to address the safety concerns. In this paper, we summarized the accumulating evidence of the presence of putative stem cells in the transition zone between the peripheral CE and the anterior extension of the TM. We also

discussed the origin and biological properties of both CE and TM cells. Up to now, there has been no clear definition of the progenitor cells located in the transition area. We have called the putative stem cells "PET cells" as they have the potential to replenish both the CE and TM. It remains to be determined whether the previously described "Schwalbe's Line cells" and "TM insert cells", as well as the precursors having been isolated, are of the same cell type; if they are, exactly what proliferation and differentiation potential do they retain, why do they not seem to repopulate the TM in glaucoma or the CE in age and disease when these populations are sorely depleted and finally how can they be used therapeutically?

Further research is required to establish the protocol to regulate cell division and differentiation of the PET cells towards appropriate lineages for repopulation of the diseased CE and TM. We need to identify which factors and signals govern their division and differentiation. Another challenge is the specific biomarker identification of the PET cells, which would facilitate stem cell isolation and enrichment. Furthermore, a better understanding of the migration and settlement properties of the PET cells is also important for the use of possible bioengineered materials. CE and TM cell loss is central to a number of ocular conditions including corneal diseases and glaucoma. In spite of the challenges, PET cells represent an attractive therapeutic stem cell source for the regeneration of the CE and TM. It is hoped that future research will ultimately lead to the development of stem-cell-based therapies for CE diseases and glaucoma, which can reduce the requirement for corneal grafts and laser or surgical treatments in glaucomatous patients.

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Review Article

Experimental Limitations Using Reprogrammed Cells for Hematopoietic Differentiation

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We review here our experiences with the *in vitro* reprogramming of somatic cells to induced pluripotent stem cells (iPSC) and subsequent *in vitro* development of hematopoietic cells from these iPSC and from embryonic stem cells (ESC). While, in principle, the *in vitro* reprogramming and subsequent differentiation can generate hematopoietic cell from any somatic cells, it is evident that many of the steps in this process need to be significantly improved before it can be applied to human cells and used in clinical settings of hematopoietic stem cell (HSC) transplantations.

1. Introduction

The *in vitro* generation of hematopoietic stem cells (HSC) and mature hematopoietic cells from embryonic stem cells (ESC) promises to provide an alternative source of cells that could replace total bone marrow cells or HSC-enriched fractions of them. This is especially necessary in the case of human cells in clinical settings for HSC transplantations. In addition, studying hematopoiesis *in vitro* bypasses the need of donor cells, in particular to study hematopoietic disorders in human. ESC lines can be cultured long term and allow, in contrast to HSC, homologous recombination of DNA, that is, the insertion of exogenously modified genes into the appropriate sites in the genome. Thus, genetically altered, ESC-derived HSC might allow the proper genetic repair of defective cells of the hematopoietic system, including those of the innate and the adaptive immune system. However, for transplantations of human cells histoincompatibilities between the ESC-derived HSC and the transplanted host might be the cause of transplant rejections.

Since it has now become possible to generate ESC-like induced pluripotent stem cells (iPSC) from differentiated peripheral cells [1, 2], HSC as well as mature hematopoietic cells might in the future be generated from differentiated cells of a patient via iPSC. Somatic cells that are either mature, fully differentiated cells or are restricted in their ability to develop into a limited collection of cell types can be induced to become pluripotent, so that they exhibit higher differentiation capacity. This process is called reprogramming. It is not yet clear whether reprogramming will always equal dedifferentiation. The original, and most widely employed method to induce iPSC from somatic cells uses ectopic expression of the transcription factors Oct-4, Sox-2, and Klf-4, either with or without c-myc [1, 3–8]. However, concerns limiting clinical applications of patient-derived, that is, directly converted iPSC, include potential epigenetic differences between iPSC and ESC [9–18], and possible modifications of the genome by insertions and continued expression of the transcription factors that could affect the capacities of reprogrammed iPSC to properly differentiate. In our case of interest, we discuss some limitations to develop them into HSC and their differentiated hematopoietic cell lineages.

Several studies have improved the procedure of the generation of iPSC from a variety of different types of differentiated cells to find the most efficient method. In general, attempts to optimize both cell-intrinsic and exogenous factors to achieve optimal growth, survival, and differentiation requirements, first for the transfection phase and, thereafter, for the conversion from the differentiated cells to the iPSC have been made [1, 3–8]. Many studies exist showing that iPSC share the characteristic of ESC, that is, they can give rise to all cell types of a proper body, proven by the development

of chimeric animals and teratoma formation [1]. However, these qualitative analyses do not provide information about the quantitative efficiency of development. Thus, to investigate whether iPSC can replace ESC to study development and for clinical applications, efficiencies of development are needed.

Here, we summarize our experience with Oct-4/Sox-2/Klf-4-transduced mouse embryonic fibroblasts (MEF), mouse bone marrow-derived (MBM) hematopoietic progenitors, and mouse fetal liver-derived preB lymphocytes in the *in vitro* generation of iPSC that show varying levels of continued expression of the transduced transcription factors in iPSC and in differentiating hematopoietic cells. These levels of transgenic expression relate to the potency of the iPSC to differentiate subsequently *in vitro* to hematopoietic cells.

Hematopoietic development from ESC and iPSC is one of the best-studied differentiation programs. Culture systems have been developed that allow the differentiation of hematopoietic lineages *in vitro* from ESC and iPSC [19–27] which we have attempted to optimize for myeloid, T, NK, and B cells [28]. However, the efficient development and maintenance of *in vivo* reconstituting HSC from ESC and iPSC remains challenging. For a clinically relevant procedure of generating transplantable HSC, first, the best type of differentiated cell for conversion to iPSC with the best cell-intrinsic and extrinsic factors have to be found. Thereafter, improved methods need to be developed to generate and stabilize the pluripotent, long-term reconstituting potentials of transplantable HSC.

2. Reprogrammed Somatic Cells as New Sources for the Generation of Hematopoietic Cells

2.1. Step 1: From Differentiated Cells to iPSC. Somatic cells were first reprogrammed by somatic cell nuclear transfer [29–31]. Later, lineage-associated transcription factors were identified within a pool of 24 pluripotency-associated factors that had the potential to reprogram adult cells into pluripotent cells upon retroviral transduction [1]. Thus, transduction of mouse fibroblasts with Oct-4, Sox-2, Klf-4, and c-myc-generated iPSC by selection for *Fbxo15* activation that expressed pluripotency markers, generated teratomas upon subcutaneous injection, and contributed to different tissues upon blastocyst injection [1]. Transcription factor-based reprogramming has been optimized, so that c-myc was omitted and cells were selected with reactivation of *Nanog* and Oct-4 as well as by checking the ESC-like morphology [4, 6, 8, 32]. Facts, hypotheses, and unresolved issues of cellular reprogramming [33] and the maintenance and change of epigenetic memory in iPSC [34] have recently been discussed extensively. As summarized by Hanna et al. [33], gene expressions and biological characteristics of iPSC may be influenced by genetic backgrounds (different strains of mice, healthy donor-derived versus patient-derived iPSC), incomplete or heterogeneous iPSC formation, additional or alternate reprogramming factors, and transgene-expressing iPSC.

In our experiments, we have used the method of retroviral transduction with three vectors that constitutively express Sox-2, Oct-4, and Klf-4, respectively, and in which the transcription factor genes are not excisable, for example, by cre/lox-mediated deletion. We have generated iPSC lines from MEFs, and MBM. All of our iPSC lines express ESC-characteristic markers and form teratomas *in vivo* [28].

Continued transgene expression in our iPSC lines at different levels, even throughout differentiation to hematopoiesis *in vitro,* appeared possible. When this was measured, a remarkable difference became apparent. All MEF-iPSC lines showed expression patterns of the three transgenic transcription factors that were hardly above those of the corresponding endogenous genes, while all MBM-iPSC lines showed a markedly higher expression of Oct-4,Klf-4 and Sox-2. It appears that a higher threshold expression of the three factors is needed to reprogram MBM-iPSC than MEF-iPSC.

Distinct differentiated cells need different culture conditions, for example, different stromal cells or other cytokines (Figure 1). While MBM-derived cells do not grow well in the iPSC condition without IL-6 and SCF, MEF do. This may contribute to our observations that the efficiencies of establishing MEF-derived iPSC are higher than that of MBM-derived iPSC in our experiments. This indicates that the establishment of iPSC is more difficult if the original somatic cells from which the iPSC are intended to be induced do not fit iPSC media conditions on MEF and LIF.

The tissue culture conditions for the transduction and subsequent *in vitro* conversion to iPSC appear markedly different. Thus, when we consider the changes that MEF proliferating in medium alone, compared with MBM proliferating in medium substituted with SCF and IL-6 have to undergo after viral transduction to become iPSC MEF should find it easier to continue proliferation and survival in LIF-substituted media. Maybe the higher expression of the three transduced transcription factors is favourable for the more difficult conversion of MBM to iPSC. Thus, we suggest that the ability of cells to grow in "iPSC selection media" might influence their efficiency to reprogram.

2.2. Step 2: From ESC and iPSC to HSCs and Mature Hematopoietic Lineage Cells. For the differentiation of ESC towards several types of mature hematopoietic cells, two protocols have been developed—the formation of embryoid bodies (EB) that form in suspension culture and the cocultivation of ESC with stromal cells. In the first protocol, ESC are allowed to grow in suspension in the absence of feeder cells and LIF, differentiate spontaneously, and form spheroidal aggregates mimicking embryonic tissues, so called embryoid bodies [35–38]. Cells within developing EB can differentiate to mature cells, including hematopoietic lineage cells [39, 40]. Hematopoietic progenitor cells, which have the tendency to exist as mobile, nonaggregated single cells, must be freed by dissociating procedures from these EB aggregates.

In the second protocol, cocultivation of ESC with preadipocytic stromal cells allows a two-dimensional differentiation into hematopoietic cells without the formation of those complex aggregated structures and, thus, an easier, gentle isolation of progenitors of hematopoietic development [21, 27]. Furthermore, the use of the M-CSF-deficient stromal cell line OP9 avoids premature differentiation to myeloid

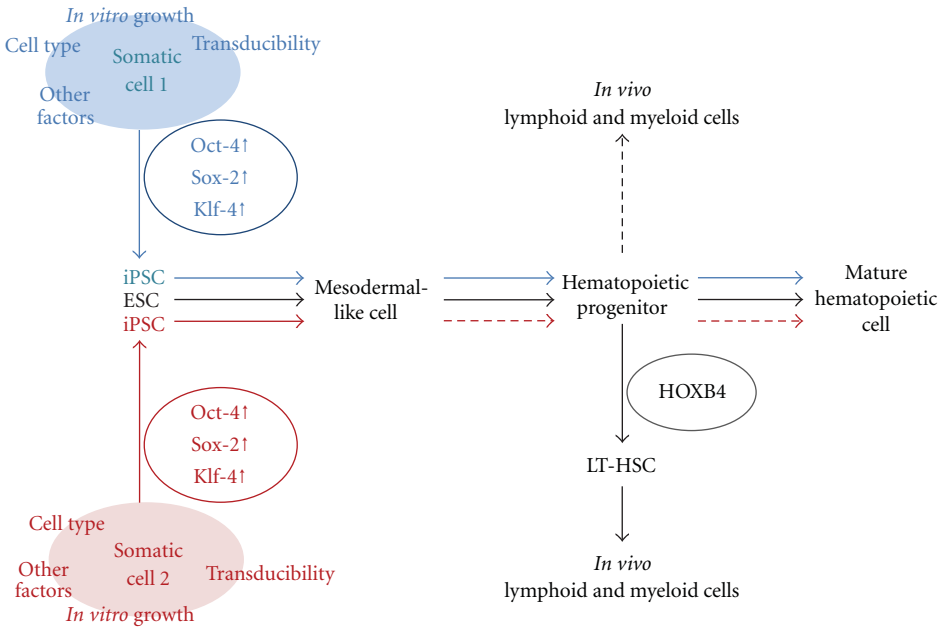

Figure 1: Overview of the current understanding of the efficiency to induce iPSC from different types of somatic cells, and subsequent development of iPSC into hematopoietic cells. Dashed lines implicate lower numbers of cells developing from the former cell type compared to full lines.

lineage cells and allows the development of T, NK, and B lymphoid cells [21]. In our *in vitro* differentiation experiments comparing ESC and iPSC [28]—the latter generated by retroviral transduction with Sox-2, Oct-4, and Klf-4—we did observe a reduced ability of iPSC-derived mesodermal-like cells to differentiate into hematopoietic progenitors in vitro. When Oct-4, Sox2, and Klf-4 were still highly expressed in the differentiating cells. The overexpression of Sox-2 appeared to be inversely related to hematogenic potency (data are summarized in Table 1).

In conclusion, our experiments suggest—as those of others [41]—that expression of virally transduced genes must be terminated before the induction of differentiation. The three different transcription factors appear to impede hematopoietic development to different extents. While Oct-4 and Klf-4 appear to be tolerated at continuously elevated levels to generate at least progenitors and precursors of T, NK, B, and myeloid cell development, levels of Sox-2 need to be downregulable for hematopoietic development. From these results, it appears that overexpression of the transgenic transcription factors inhibits development of Flk-1$^+$ mesodermal to CD45$^+$ hematopoietic progenitors. Constitutive expression has been shown by others not to affect the development of iPS cells into cells of the hematopoietic system [42, 43]. We would expect from our results that the transgenic expression of the three transcription factors in their iPSC lines should be as low as that of our MEF-iPSC lines.

If normal mouse or human somatic cells are used for the generation of iPSC the viral vectors should be deletable [44] without mutagenic consequences or should be introduced as proteins [45] or as synthetic modified mRNA [46].

3. Generation of HSC from ESC and iPSC Still Needs to Be Improved

Even if the procedures for the generation of iPSC will eventually be faithful and efficient enough to yield cells with the same differentiation potencies as those of ESC the subsequent efficient generation of transplantable, reconstituting HSC derived from ESC and iPSC cells still has been difficult until today. Murine iPSC can be used to generate new mouse strains in which bone marrow should, in most cases, become the source of normal numbers of long-term reconstituting HSC. In contrast, human iPSC, obviously, can not be used for such an *in vivo* development of HSC. Hence, the development of human HSC from ESC and iPSC must be attempted by differentiation in tissue cultures. The most successful method to obtain HSC *in vitro* from ESC is to transduce the cells with HOXB4 [23, 25, 47–54].

However, such retroviral modifications generate cells in which the "per cell" hematopoietic potency is still inferior to the same number of unseparated total bone marrow cells. Furthermore, retrovirally transduced cells carry the risk of mutations which might lead to malignant transformations, for example, leukaemia in the case of HOXB4 [55]. A few studies have reported transplantations of non-HOXB4-transduced cells resulting in long-term engraftment of both

Table 1: Differentiation of MBM- and MEF-derived iPSC lines in comparison to ESC lines. Numbers of cells indicate those developed from 4×10^3 undifferentiated cells (day 0). Expression levels represent amounts of mRNA determined by quantitative RT-PCR, normalized to GAPDH expression, and calculated as expression values of the respective genes in undifferentiated Bruce4 ES cells (day 0).

Cell line	Number of $Flk1^+$ cells on day 5	Number of $CD45^+$ cells on day 10	Sox-2 expression on day 5 relative to Bruce 4 on day 0	Sox-2 expression on day 10 relative to Bruce 4 on day 0	Oct-4 expression on day 5 relative to Bruce 4 on day 0	Oct-4 expression on day 10 relative to Bruce 4 on day 0	Klf-4 expression on day 5 relative to Bruce 4 on day 0	Klf-4 expression on day 10 relative to Bruce 4 on day 0
	$\times 10^5$	$\times 10^5$	$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$	$\times 10^{-2}$
J1 ES	2.7	5.5	1.3	0.2	31	0.2	480	1.7
Bruce 4 ES	3.2	23	0.5	0.04	41	0.08	32	0.04
MBM-iPS A	3.1	10	1.0	6.3	2600	120	3600	83
MBM-iPS C	2.2	0.1	18	1050	11000	2500	3700	700
MEF-iPS 1	2.6	7.3	16	21	12	56	3.2	5.8
MEF-iPS 5	4.0	18	45	71	0.65	70	22	5.6

the lymphoid and myeloid compartments, but none of them could reconstitute hematopoiesis in secondary transfers, [56–59]. The question remains which kind of progenitor is developed under these conditions.

It has been shown that yolk sac progenitors display minimal HSC potential [60–62]. In contrast, para-aortic splanchnopleura-derived cells can give rise to bone marrow reconstituting HSC which are capable of definitive hematopoiesis [60, 61, 63]. It might be that ESC differentiation *in vitro* generates only HSC capable of primitive, but not of definitive hematopoietic potency. That would explain the inability of ESC-derived hematopoietic progenitors to generate HSC with the capacity to develop into lymphoid cells upon transplantation. This possibility ignores the fact that ESC and iPSC can be differentiated into primitive, that is, erythrocytes expressing fetal-type haemoglobin, and definitive cells, that is, lymphocytes, *in vitro*. HOXB4 overexpression in hematopoietic cells derived from ESC and from yolk sac enables the detection of transplantable HSC [47] (Figure 1). Hence, HOXB4 works in two ways. One is to increase the number of transplantable HSC. The second is to make HSC transplantable by modifying the homing receptors. Therefore, the injection of hematopoietic cells from human ESC directly into the bone marrow results in the detection of repopulatable HSC [49]. In conclusion, we need to understand the molecular program that induces this switch in greater detail to induce the formation of long-term reconstituting HSC with definitive hematopoietic potential, as HOXB4 does, but without retroviral insertion.

Finally, nonhematopoietic cells provide niches in bone marrow for the proper hematopoietic differentiation that are yet to be defined, and that are missing in the culture conditions of differentiating ESC. Furthermore, long-term repopulating HSC that reside in the bone marrow are in a deeply quiescent (G_0) state and lose engraftment potential during their $S/G_2/M$ transit [64–67]. The present tissue culture conditions favor proliferation of HSC candidate cells. The development of conditions allowing cells to enter into and survive in the G_0/G_1 phase would be another important step towards establishing HSC *in vitro*.

4. Conclusions

Both stages of the *in vitro* development, first, from somatic, differentiated cells into iPSC and second, from iPSC into HSC are still so inefficient, even with murine cells, that the clinical use of human HSC derived from a patient's somatic cells are far from reality. It will need many more improvements at the various stages of reprogramming and differentiations of cells (Figure 1). Different somatic cell types represent different differentiation states, which have different growth abilities *in vitro*, different susceptibilities to be transduced by retroviral vectors and other yet unidentified factors, that make differently capable to become reprogrammed with different efficiencies. To allow effective reprogramming to iPSC, reversibly inducible or nonintegrative methods for reprogramming need to be used, since constitutive overexpression of reprogramming factors has been shown to interfere with differentiation. ES cells, and, to a lesser degree also, iPSC can be developed into all types of hematopoietic lineages in vitro. However, the reproducible generation of transplantable, engraftable HSC in vitro from pluripotent cells without overexpression of HOXB4 is still challenging (Figure 1).

Conflict of Interests

The authors have no conflicting financial interests.

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Review Article

Modeling Neurological Disorders by Human Induced Pluripotent Stem Cells

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Studies of human brain development are critical as research on neurological disorders have been progressively advanced. However, understanding the process of neurogenesis through analysis of the early embryo is complicated and limited by a number of factors, including the complexity of the embryos, availability, and ethical constrains. The emerging of human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs) has shed light of a new approach to study both early development and disease pathology. The cells behave as precursors of all embryonic lineages; thus, they allow tracing the history from the root to individual branches of the cell lineage tree. Systems for neural differentiation of hESCs and iPSCs have provided an experimental model that can be used to augment *in vitro* studies of *in vivo* brain development. Interestingly, iPSCs derived from patients, containing donor genetic background, have offered a breakthrough approach to study human genetics of neurodegenerative diseases. This paper summarizes the recent reports of the development of iPSCs from patients who suffer from neurological diseases and evaluates the feasibility of iPSCs as a disease model. The benefits and obstacles of iPSC technology are highlighted in order to raising the cautions of misinterpretation prior to further clinical translations.

1. Introduction

Neurodegenerative and neurodevelopmental diseases are the important causes of disability and death of humans. Although the rapid development of novel diagnostic methods and therapeutic approaches has been in progress, there is no emergence of an efficient way to prevent and cure these diseases. Due to the lack of a suitable disease model and an adequate number of biopsy brain samples, the genuine etiology and pathology of many nervous diseases are unidentified. Transgenic animals for disease modeling were developed, and many of them show disease pathology and response to treatment trials. For example, the Alzheimer's disease (AD) mice, which overexpress familial type AD-associated genes, amyloid precursor protein (APP), Presenilin1/2 (PS), and Tau, show clinical pathologies of AD, such as progressive memory loss, extracellular plaque, and neurofibrillary tangles [1]. Although animal model continues to produce key insights into disease mechanisms, these systems have limitations that could be potentially overcome by human cellular models of diseases. Many transgenic murines do not faithfully mirror the respective human pathophysiology. For example, a mouse model for Down syndrome (DS) fails to recapitulate the human cranial abnormalities, a common associated feature of trisomy 21 [2]. This may suggest that the mouse model for human trisomy 21 is not fully appropriate, and an alternative system is necessary for exploring disease mechanisms [3].

Human embryonic stem cells (hESCs) are isolated from the inner cells mass of blastocyst stage embryos which can be further differentiated into three embryonic germ layers: ectoderm, mesoderm, and endoderm [4]. Recent results indicate that the differentiation of hESCs in culture follows the hierarchical sets of signals that regulate embryonic development in the generation of the germ layers and specific cell types [5]. Establishment of *in vitro* differentiation systems that recapitulate normal development will form the foundation for dissecting molecular interactions. The ability

to access and manipulate populations representing early developmental stages in the hESC differentiation cultures provides a new approach for addressing questions of lineage commitment, such as neurodevelopment [6]. This system provides a model of human brain development that enables manipulations comparable to those carried out in other organisms such as *Xenopus* and zebrafish, but in human species context. In order to derive disease-specific hESCs, there are two conventional methods which are (1) the isolation of single blastomere from morula stage embryos which are entered into preimplantation genetic diagnostic (PGD) program and (2) the derivation of hESCs by somatic cell nuclear transfer (SCNT) [7]. PGD is a clinical procedure for screening fertilized embryos at morula stage in order to ensure disease-free embryos [8]. The embryos that are diagnosed as disease threatened will be either discarded or donated for research [9]. On the other hand, SCNT is an alternative approach to generate patient-specific hESCs. The nucleus from the somatic donor cells is transplanted into the enucleated oocytes by micromanipulator, leading to union of both components [10]. However, the success rate of hESC establishment form PGD and SCNT is considerably low and technicaly demanding. Moreover, SCNT is limited by the lack of oocyte donors and ethical issues; causing genomic reprogramming by nuclear transfer has not been extensively demonstrated in human [11–13].

Because of the multiple drawbacks of hESCs and the derivation approaches, another pluripotent cells could be derived from somatic cells by the forced expression of key pluripotent transcription factors of hESCs (OCT4, c-MYC, SOX2, and KLF4), and these cells were named as induced pluripotent stem cells (iPSCs) [14, 15]. After the discovery of iPSCs by Takahashi et al. in 2007 , the trend of disease modeling has intensively focused on iPSC technology as this technique could generate pluripotent cells from diagnosed living patients and be further differentiated into disease-relevant cell types for drugs screening and disease development monitoring [16]. Thus, this paper will discuss the current success of iPSC derivation from neurological disease patients which will ultimately lead to an answer of pathological causes and a novel pharmaceutical product to treat those diseases.

2. Insights: The Properties and Origins of Human Pluripotent Stem Cells

Primarily, hiPSCs have been characterized by following the characteristics of hESCs. These two pluripotent cell types display several similar properties, such as their morphology, self-renewal, differentiation capacity, cell surface antigens, and gene expression profile [16]. They are immortalized cells and could be differentiated into all primary embryonic germ layers, including gut epithelium, cartilage, bone, smooth muscle, striated muscle, neural epithelium, embryonic ganglia, and stratified squamous epithelium [17]. hESCs and iPSCs show the high expression of telomerase reverse transcriptase [18] as well as the stage specific embryonic antigens (SSEAs), which are SSEA-3, SSEA-4, TRA-1-60 and TRA-1-81. Interestingly, it was demonstrated that the expression pattern of these surface antigens is present in the ICM of human blastocysts, providing evidence that hESCs continue to resemble the cells in the ICM [19]. Self-renewal and pluripotency of hESCs and iPSCs are mainly controlled by intrinsic transcription factors. The best-studied intrinsic factors are Oct4, Nanog, and Sox2, which play essential roles in both mESCs and hESCs. The downregulation of these factors leads to hESC differentiation [20–22]. The significant roles of these transcription factors in pluripotency have been confirmed by the ability to reprogram human fibroblasts to become pluripotent cells [16]. As exhibiting pluripotency, when embryoid body is formed from hESCs and iPSCs, the cell aggregates show similar structure to early stage of human embryos which consist of all three embryonic germ layers [23]. hESCs can also form teratomas following the injection of cells into nude mice, which reflects their *in vivo* differentiation capability [24, 25]. Even if hESCs have been considered a cell resource for regenerative medicine, there are several controversial issues needed to be taken into account, including ethical constrains of embryo destruction, graft-versus-host disease, and the difficulty to obtain diseased-specific cell lines. Since the discovery of iPSCs by using dermal fibroblasts, scientists have extended to various origins of starting cells such as neural stem cells [26], adipose-derived mesenchymal stem cells [27], umbilical cord blood [28], and T-cell lymphocyte [29, 30]. For these reasons, iPSCs are suggested to become a new paradigm for generating patient-specific pluripotent stem cells to model neurological genetic diseases.

3. hiPSCs Breakthrough: The Systems for Neurological Disease Modeling

In clinical research, it is hard to obtain brain tissues from either live or dead patients for investigating diseases. This difficulty has limited the knowledge of human neurological abnormalities and pathology progression during the course of diseases [31]. Reprogramming of somatic cells from nervous disorder patients by using iPSC technology has provided an opportunity to generate disease-harboring pluripotent cells which can be differentiated into neural cells for studying disease development. Dermal fibroblast cells are widely used as starting cells for somatic cell nuclear reprogramming. The differentiation propensity of iPSCs toward specific cell types, such as neural cells, is marked by the epigenetic memory of starting cells [32]. The current established iPSCs derived from neurodevelopment and neurodegenerative disorder patients are summarized in Table 1, while the similarities and differences of ESCs and iPSCs are illustrated in Table 2. Categorizing by types of diseases, neurological disease-specific iPSCs could be divided into two major groups which are early-onset neurodevelopment diseases and late-onset neurodegenerative diseases. Neurodevelopment diseases are mostly triggered by abnormal gene expression, while neurodegenerative diseases typically resulted from both abnormal gene expression and environment factors. It is an advantage of iPSCs to mimic neurodevelopment disorders

Table 1: List of reported hiPSC disease models.

Disease	Target cell	Potential to be disease model		Drug test	Reference
		Successful differentiated into target cell type	Neuronal pathology		
Early-onset neurological disorders					
Fragile X syndrome	ND	ND	Loss of FMR1 expression	ND	[44]
Prader-Willi syndrome	Neurons	Yes	Imprint disorder	ND	[48, 65]
Rett's syndrome	Neurons	Yes	Loss of synapses, reduced spine density, smaller soma size	Yes	[31]
Familial dysautonomia	Neural crest cells	Yes	Loss of neural crest cells	Yes	[54]
Friedreich's ataxaia	Motor neuron	Yes	*FXN* gene repression	ND	[61]
Angelman's syndrome	Neurons	Yes	Imprint disorder	ND	[65]
Down's syndrome	Neuron	ND	ND	ND	[69]
Spinal muscular atrophy	Motor neurons	Yes	Loss of neuron formation, loss of *SMN* gene expression	Yes	[34]
Late-onset neurological disorders					
Amyotrophic lateral sclerosis (ALS)	Motor neurons	ND	Not shown	ND	[38]
Huntington's disease (HD)	Striatal neurons	Yes	Not shown	ND	[37]
Parkinson's disease (PD)	Dopaminergic neurons	Yes	Not shown	ND	[35, 36]
Alzheimer's disease (AD)	Cholinergic neurons	Yes	Increase ratio of Aβ42 to Aβ40	Yes	[87]

* ND: not determined.

Table 2: Summarizing the similarities and differences of ESC and iPSC for disease modeling.

	hESCs	iPSCs	Reference
Source	ICM	Adult somatic cell	[4, 15]
Basic pluripotent characteristics			
Alkaline phosphatase activity	Yes	Yes	[4, 15]
Pluripotent genes expression	Yes	Yes	[4, 15]
Pluripotent cell surface markers	Yes	Yes	[4, 15]
In vitro and *in vivo* multilinage differentiation	Yes	Yes	[4, 15]
Transcriptomic expression	Normal pluripotent gene expression	Pluripotent gene expression but not all genes similar to hESCs and depend on reprogramming technique	[36, 100]
Proteomic expression	Normal proteomic expression	Not all proteomic expression identical to hESCs	[101]
Disease modeling	From PGD diagnosed embryo in IVF clinic	From diagnosed adult patient somatic cell	[88, 102]
Disease-related expression characteristic	Yes	Yes	[88, 102]

since neural cells derived from iPSCs display an early stage of disease development [31, 33]. However, immature phenotypes of neurons derived from iPSCs hinder the applications of modeling for late-onset diseases [33]. It is important to note that although some neurons derived from iPSCs predominantly exhibited disease pathology and responded to pharmaceutical agents [31, 34, 35], a number of neural cells from certain diseased iPSCs did not show neuronal pathology, such as Parkinson's disease [35, 36], Huntington's disease [37], and amyotrophic lateral sclerosis [38]. Specific gene mutations or environmental stress inducers are needed in order to accelerate the pathology of those diseases [35, 36].

4. The Current Available iPSCs from Monogenic Early-Onset Neurological Disorders

4.1. Fragile X Syndrome. Fragile X (FX) syndrome is an X-linked dominant disorder which is the most common form of inherited mental retardation [39]. The cognitive, behavioral, and physical phenotypes vary by sex in which males are severely affected due to the X-linked inheritance of mutation [40]. This disease caused by no expression of the fragile X mental retardation 1 (FMR1) gene, resulting from untranslation of CGG triplet repeat expansion in the 5′ UTR region of the gene [41]. According to the loss of FMR1 protein, developmental retardation is significantly found in the cerebral cortex. Quantitative examination of human brain autopsy exhibits abnormal dendritic spine lengths, and shapes are more immature when compared with normal age match controls [42, 43]. Fragile X syndrome iPSCs (FX-iPSCs) were generated from 3 FX-affected males and compared the regulation of FMR1 transcription to human FX-ESCs. FX-iPSCs cloned demonstrated typical characteristic of pluripotent stem cells [44]. Surprisingly, although the mutant FMR1 gene is expressed in FX-ESCs, FMR1 gene expression remains inactive in FX-iPSCs. In addition, even somatic FX-fibroblasts were pretreated with demethylating agent 5-azacytidine prior to reprogramming; FMR1 gene remained transcriptionally silent in all FX-iPSCs clones. This hypothesized that other epigenetic mechanisms may affect the aberrant expression of FMR1 gene. This brought the attention that the differentiation of FX-iPSCs into neurons may not facilitate pathological study of fragile X syndrome [44]. Further examination of multiple factors, such as epigenetic factors, is required in order to improve FX-iPSC properties, in particular gene expression pattern [33].

4.2. Prader-Willi Syndrome (PWS). Prader-Willi syndrome (PWS) is a neurological disorder characterized by neonatal hypotonia, failure to thrive, hypogonadism and short stature, mild-to-moderate mental retardation, and compulsive hyperphagia in early childhood that leads to morbid obesity [45]. PWS and AS are closely related in which the imprinted genes on the proximal long arm of chromosome 15 are affected. PWS is affected by the lack of gene expression in paternal chromosome, 15q11-q13, while genes in maternal chromosome are repressed by DNA methylation [46]. The definition of etiology of this disease is unclear, but a deficiency of the paternally expressed *SNORD116* snoRNAs can result in a PWS or PWS-like phenotypes [47]. PWS-iPSCs were generated from fibroblast of diagnosed PWS patients by using retrovirus producing 4 exogenous transcription factors. PWS-iPSCs showed positive-to-standard hESC characteristics: cell surface antigens, endogenous hESC transcription factors, and teratoma formation. Moreover, the methylation status of proximal promoter, *OCT4* and *NANOG*, displayed extensive DNA demethylation in PWS iPSCs clone at a comparable level to hESCs. PWS-iPSCs displayed a normal number of chromosomes, but DNA segment translocation was observed from chromosome 15 to chromosome 4 [46, 48]. The expression of *SNORD116* analyzed by quantitative PCR demonstrated that the high expression of *SNORD116* was observed in normal fibroblast and normal iPSCs, but PWS fibroblasts and PWS-iPSCs showed low expression [48]. It is suggested that PWS-iPSCs did not display normal expression pattern of imprinted genes which are crucial for disease determination [48]. The results supported the hypothesis that genomic imprinting is not susceptible to nuclear reprogramming and refractory to acquired *de novo* alteration.

4.3. Rett's Syndrome. Rett's syndrome is an inherited neurological developmental disorder which is associated with X-linked gene inheritance encoding methyl-CpG-binding protein 2 (MeCP2) [49]. MeCP2 is located in the nucleus of many types of CNS neurons and functions as a transcriptional repressor by associating with chromatin remodeling complexes [50]. This disease is characterized by a variety of clinical manifestations, indicating developmental arrest and psychomotor repression. The patients have mental retardation, epilepsy, respiratory dysfunction, stereotypic hand movement, growth retardation, scoliosis, and spasticity [51]. RTT-iPSCs were generated from four distinct MeCP2 mutation fibroblasts, using retroviral reprogramming vectors. The resulting cells were pluripotent as WT-iPSCs. Immunocytochemistry results against trimethylated histone 3 lysine 27 (m3H3K27) were positively marked at the nucleus of some, but not all, undifferentiated RTT-iPSCs, similar to the control hESCs. This showed that the memory of the previous chromatin inactivation state had been erased. By immunostaining, RTT fibroblasts and RTT-iPSCs-derived neuronal population reduced MeCP2 protein levels. Moreover, the half reduction of MeCP2 protein is consistent with the random X-inactivation. It was noted that X-inactivation was reset in RTT-iPSCs and restored randomly during neuronal differentiation. This caused the variation of MeCP2 protein level, reminiscent of the brain of RTT patients [31]. After 8 weeks of neural differentiation, the number of VGLUT-positive glutamatergic neurons from RTT-iPSCs was less than that derived from either to WT-iPSCs or hESCs. This phenotype could be found when the WT-iPSCs knocked down the expression of MeCP2, while the overexpression of MeCP2 could increase the number of VGLUT1-positive neurons derived from RTT-iPSCs. Morphological analysis of RTT neurons demonstrated that the number of spine of RTT neuritis and cell soma sizes was reduced when compared to WT neurons. Interestingly, high concentration of aminoglycoside antibiotic, such as gentamicin, could increase MeCP2 protein and, consequently, the number of glutamatergic neurons [31]. This result suggested that RTT-iPSCs were providing the excellent disease modeling for RETT syndrome and could confirm the pathology of the disease.

4.4. Familial Dysautonomia. Familial dysautonomia (FD) or Riley Day Syndrome is an autosomal recessive disorder, characterized by the developmental loss of neurons from the sensory and autonomic nervous system [49, 50]. FD is caused by the mutation in a splice site of the I-kB kinase complex-associated protein (*IKBKAP*) gene, which causes

tissue-specific exon skipping and expression of a truncated mRNA transcript [52]. Reduced levels of normal IKAP protein are associated with a defect in cell motility [53]. FD-iPSCs were generated from 10-year-old female FD patients using lentiviral vector encoding 4 classical vectors [16]. Genetic defect, homozygous 2507+6T>C, was confirmed in FD-iPSCs by sequencing and IKBKAP analysis using RT-PCR. Gene expression profile of neural crest derived from FD-iPSCs showed that, among the 20 most deceased transcripts in FD neural crest precursors, many genes were involved in peripheral neurogenesis and neural differentiation. Moreover, the number of paxillin-positive cells was reduced in FD-iPSCs-derived neural crest progenitors, referring to the aberration of cell spreading and migration [54]. They have reported that plant hormone kinetin could reduce the levels of the mutant *IKBKAP* splice in FD cells [54–56]. Epigallocatechin gallate and tocotrinal were exposed to FD-iPSCs-derived neural crest precursors and showed dramatic reduction of the mutant *IKBKAP* splice form; however, the hormone did not show a significant increase in the expression of neurogenic markers or improve the migration behavior [54].

4.5. Friedreich's Ataxia. Friedreich's ataxia (FRDA), the most common inherited ataxia, is associated with a mutation of the *frataxin* gene on chromosome 9 [57, 58]. The incidence of mutations was often found in an unstable expansion of GAA repeats in the first intron. The level of GAA repeats is correlated with the downregulation of gene expression and the progression of disease severity [58]. The cardinal features of FRDA are ataxia of all four limbs, cerebella dysarthria, lack of reflexes, sensory loss, and pyramidal signs. In addition, frataxin insufficiency leaded to skeletal deformities, cardiomyopathy, and the risk of diabetes [57, 58]. Although mice models of this disease are available, they do not fully recapitulate gene silencing and frataxin protein level [59, 60]. FRDA-iPSCs were established by retroviral transduction with 4 classical transcription factors [61] and showed typical characteristics of pluripotent cells as well as retaining the marked repression of *FXN* mRNA. FRDA-iPSCs still repeated the GAA expansion in both parental pathogenic alleles. Furthermore, GAA repeat lengths in FRDA-iPSCs change overtime in culture. The role of mismatch repair (MMR) enzyme *MSH2* was investigated in FRDA-iPSCs. mRNA expression and western blotting analysis of FRDA-iPSCs showed large increases in MSH2 level when compared with donor fibroblast. The FRDA-iPSCs have been expected to provide a valuable modeling to study repeat instability mechanism [61]. Differentiations to disease-specific cell types, such as neurons, cardiomyocytes, and pancreatic beta cells, for studing effect of abnormality are required. Moreover, the novel drugs or therapeutic protocols are expected to develop by using this FRDA-iPSCs modeling.

4.6. Angelman's Syndrome. Angelman's syndrome (AS) is clinically delineated by the combination of seizure, absent speech, and hypermotoric and ataxic movements [62, 63]. AS patients exhibit a predisposition toward apparent happiness and paroxysms of laughter, and this finding helps to distinguish AS from others involving severe developmental handicap [63]. AS is a severe genetic disorder, caused by mutation or deletion of the maternally inherited *UBE3A* gene in chromosome 15. This gene encodes an HECT (homologous to E6-associated protein C terminal) domain E3 ubiquitin ligase [62]. The combination of epigenetic silencing of paternal allele and gene inactivation of maternal allele of *UBE3A* leads to chiefly incomplete loss of UBE3A protein selectively in most neurons in the brain [64]. Recently, AS-iPSCs lines were established from fibroblasts of two AS patients, who carried maternally inherited deletions of chromosome 15q11-q13 [65]. The methylation imprinting was assessed and showed similar patterns to patient's fibroblasts. Only an unmethylated paternal allele was observed in AS-iPSCs. Neurons and astrocytes derived from AS-iPSCs were matured *in vitro* and exhibited train of action potential and excitatory postsynaptic current. The levels of *UBE3A* expression were significantly reduced in both of AS-iPSCs- and AS-iPSCs-derived neurons, while normal iPSCs or iPSCs-derived neurons maintain the level of *UBE3A*. There is evidence suggesting that brain-specific *UBE3A* repression is mediated by a snoRNA [47, 66, 67]. Northern blot hybridization used to assess expression of snoRNAs, *SNORD 116* and *SNORD 115*, demonstrated that *SNORD 116* is expressed in both iPSCs and iPSC-derived neurons derived from normal or AS individuals, whereas *SNORD115* expression is restricted to iPSC-derived neurons. It is suggested that the neuron-specific repression of *UBE3A* may occur relatively late during neurogenesis, coincident with the upregulation of *SNORD 116* and *SNORD115* during neural differentiation. This finding proposed that the methylation imprinting is difficult to be reprogrammed, and the epigenetic status is resistant to a global erasure [65]. This AS-iPSC model recapitulates the tissue-specific pattern of *UBE3A* imprinting; thus, it provides an important tool to address the timing and mechanisms controlling epigenetic status of *UBE3A* during human neural development. In addition, AS-iPSCs-derived neurons will also be a useful system for the characterization of the physiological abnormalities of the disease at a cellular level [65].

4.7. Down's Syndrome. Down's syndrome (DS) is a developmental disorder, caused by trisomy of chromosome 21. The key manifestations of the disease are mental retardation, craniofacial abnormalities, and clinical defections of several systems such as heart, gut, and immune system [68]. However, the trisomy of chromosome 21 in mice did not result in disease symptoms which means that mouse is not a suitable system to model AD pathology [2]. DS-iPSCs were established from DS patient fibroblasts by using either four (*Oct4*, *SOX2*, *KLF4*, and *c-MYC*), or three (without *c-MYC*) reprogramming factors. DS-iPSCs showed the characteristics of trisomy 21 anomaly by chromosomal G-banding analysis, but none of differentiation studies have been conducted [69]. DS-iPSCs are not only attractive for the investigation of DS development, but also interesting for other DS-related diseases such as AD, which is a frequently coincident disease in DS patients.

4.8. Spinal Muscular Atrophy. Spinal muscular atrophy (SMA), an autosomal recessive disease, is one of the leading genetic causes of infant mortality due to the specific loss of alpha motor neurons in the spinal cord [70, 71]. Clinically, SMA is caused by the homozygous deletion of *survivor motor neuron 1* and 2 (*SMN1* and *SMN2*), and the disease severity spans a broad spectrum, based on the onset period [71]. *SMN2*-derived pre-mRNA transcripts could be alternatively spliced, causing a single nucleotide difference, a silent cytosine-to-thymine (C-T) transition within exon 7, from the normal mRNA. This single nucleotide change restricts the length of SMN2 protein and translates to a dysfunction protein, named SMN Δ7. Patients with high copy number of *SMN2* producing more full length of SMN by *SMN2*, are observed in milder form of the disease [72]. SMA-iPSCs were established from a type I SMA patient and his unaffected mother. iPSCs were generated from primary fibroblasts with lentiviral constructs encoding *Oct4, SOX2, NANOG*, and *LIN28.* SMA-iPSCs showed pluripotent characteristics like hESCs and were not different from WT-iPSCs. iPSC and fibroblast SMN RNA were analyzed. RT-PCR analysis showed that SMA-iPSCs and SMN fibroblast have lower levels of SMN RNA than WT-iPSCs and WT fibroblast. Moreover, qRT-PCR result confirmed the significantly reduced level of full-length *SMN* transcript in SMA-iPSCs, 32–39% reduction compared to wild type. SMA-iPSCs were differentiated into motor neurons which are pathological specific cell types. Interestingly, motor neurons derived from SMA-iPSCs group showed significantly fewer number and reduced size than motor neurons derived from WT-iPSCs. However, there was no difference in total number of Tuj1-positive neurons in either WT or SMA groups. This result suggested that SMA has a specific influence on motor neuron, and the disease phenotype selectively hinders motor neuron production and/or increases motor neuron degeneration at later time point. Neuron and astrocytes derived from SMA-iPSCs significantly increased the level of intranuclear gems, intranuclear form of aggregated SMN protein, after valproic acid and tobramycin, when compared to untreated group [34]. The results indicated that neural cells derived from SMA-iPSCs responded to drug screening and are able to be used as the disease model for further disease investigation.

5. The Established iPSCs for the Polygenic Late-Onset Neurological Disorders

5.1. Amyotrophic Lateral Sclerosis. Amyotrophic lateral sclerosis (ALS) is a progressive disease, characterized by the degeneration of upper, in layer V of the motor cortex, and lower motor neurons, brain stem, and anterior horn of spinal cord. The loss of motor neurons, especially in spinal cord, leads to progressive paralysis and denervation atrophy of striated muscles [73]. Inheritance in familial ALS (FALS) is typically autosomal dominant [74]. The mutation in only one gene, named *Cu/Zn superoxide dismutase* (*SOD1*), results in classical inherited ALS [75]. iPSCs were developed by using skin fibroblasts from 82- and 89-year-old FALS patients, who are heterozygous of the rare L144F ($Leu^{144} \rightarrow Phe$) dominant allele of *SOD1*. FALS-iPSCs showed normal characteristic of pluripotent stem cell like hESCs. FALS-iPSCs were differentiated to mature motor neurons, HB9 and ISLET1/2-positive cells, astrocytes, and GFAP-positive cells. The abnormality of motor neurons and differentiated cells from FALS-iPSCs has been described [38]. Thus, the expressions of disease characteristics as well as the pathological anomaly of motor neuron needed further investigations, such as persistent of SOD1 mutation in developed iPSCs.

5.2. Huntington's Disease. Huntington's disease (HD) is characterized by the loss of brain striatal neurons that results from the expansion of a CAG repeat, translated into glutamine and produced mutant huntingtin protein [76]. The pathological change in HD brain is evident by the massive loss of medium spiny neurons (MSNs) in the striatum and loss of neurons in the cortex which results in chorea, dementia, and eventually death [77]. HD-iPSCs line was made from HD patients with 72 repeat CAG nucleotides [37, 69]. Every cell derived from HD-iPSCs, including HD-NSCs and striatal differentiated neurons, contained 72 CAG repeats. HD neural stem cells (HD-NSCs) and striatal neurons could be generated from these HD-iPSCs, but the population of striatal neurons, DARPP-32-positive cells, were dramatically low, approximately 10% of the total neurons. Caspase activity was evaluated after the withdrawal of growth factors for 24 hours. In HD-NSCs, caspase 3/7 activity was stimulated, but not in WT-NSCs [37]. This suggested that HD-iPSCs and their differentiated striatal neurons are a suitable model for HD, and this could be further supported by the comparison with another alternative system in nonhuman primate ESCs [78]. However, the level of huntingtin protein in HD-iPSCs has not yet been demonstrated.

5.3. Parkinson's Disease. Parkinson's disease is the second most common neurodegenerative disease after Alzheimer's disease [79]. Currently, there have been identified 6 causative genes in which their mutations are associated with the high incidence of PD. These six genes include α-synuclein, parkin, UCH-LI, PINK1, DJ-1, and LRRK2/dardarin [80]. The manifestations of PD are resting tremor, bradykinesia, rigidity, and gait impairment. The motion difficulty of PD patients is attributed to the loss of dopaminergic (DA) neurons within the substantia nigra (SN), causing the dysfunction of the basal ganglia. The motor symptoms and survival rate of PD patients could be relieved by synthetic dopamine replacement [81]. PD-iPSCs were generated from idiopathic PD patients with either 3 (without c-Myc) or 4 transcription factors by using lentivirus flanked with *loxP* sites. Thus, these exogenous genes could be later removed by Cre recombinase in order to fabricate the factor-free PD-iPSCs. Not only establishing the pluripotent state, the factor-free PD-iPSCs also showed a close global gene expression profile to hESCs. DA neurons could be generated from PD-iPSCs at a comparable efficiency to non-PD-iPSCs or hESCs [36]. This was suggested due to the short time span of cultured neurons since the onset period of PD patients is approximately over 50 years old. In order to accelerate the

disease pathology, the exogenous stimuli, such as an exposure to oxidative stress, neurotoxin, or overexpression of PD-related genes (*α-synuclein* or *LRRK2*), may be needed to supplement the culture systems [36]. On the other hand, fibroblasts from monogenic mutation of PD were also reprogrammed to iPSC state. These fibroblasts carried *p.G2019S* mutation (called G2019S-iPSCs) in the *leucine-rich repeat kinese-2* (*LRRK2*) which is the most common PD-related mutation [35]. DA neurons derived from G2019S-iPSCs showed not only the increased expression of key oxidative stress-response gene and α-synuclein protein, but also the high sensitivity to caspase 3 activation and cell-death-causing agents. Moreover, the treatment of cell death inhibitor, ROCK inhibitor, or Y-27632, did not protect G2019S-iPSC-derived DA neurons from hydrogen peroxide or MG-132-mediated caspase 3 activation [35]. The results emphasized the possibility to generate the late-onset neurodegenerative disease models by using disease-related exogenous stimuli and mutant cell lines. However, the solid protocol of disease-specific cell type differentiation and long period maintaining differentiated cell *in vitro* are needed to develop further investigation.

5.4. Alzheimer's Disease (AD). Alzheimer's disease (AD) is known as the most common neurodegenerative disorder in aged people. The AD's patients show progressive memory retardation and cognition disturbance. The pathology of this disease is neuronal loss in the cerebral cortex accompanied by massive accumulation of amyloid fibril forming senile plaque and hyperphosphorylated tua protein forming neurofibrillary tangle (NFT) [82]. The amyloid fibril is mainly composed of β-amyloid (Aβ) peptide, the 40 and 42 amino acid form (Aβ40 and Aβ42), which is derived from proteolytic cleavage from the amyloid precursor protein (APP) by β- and γ-secretase enzyme activity [83–85]. Accumulation of Aβ plaque, mainly Aβ42, in the brain parenchyma is the initiation of AD pathogenesis and it leads to the formation of NFT which enhances degeneration of neurons [84]. Presenilin 1(*PS1*) and presenilin 2 (PS2) genes encode the major component of γ-secretase which mutated in autosomal-dominant familial Alzheimer's disease [86]. Mutation in the *PS1*, *PS2*, and *APP* genes is reported the most of familial early-onset cases of AD with high level of Aβ42 production and greater fibrillary amyloid deposits [86]. Recently, iPSCs from fibroblasts of FAD with the *PS1* mutation A246E and the *PS2* mutation N141I were established by retrovirus transduction with the five factors *Oct4*, *SOX2*, *Klf4*, *LIN28*, and *NANOG* [87]. All PS1 and PS2 iPSC clones demonstrated typical characteristics of pluripotent cell when compared with both normal 201B7 iPSC line [15] and the sporadic PD-derived iPSC line which was reprogrammed by the original methods [15]. Both PS1 and PS2 lines were induced to differentiate into neurons. Comparing with a normal control, they showed no difference of the efficiency to generate neurons; however, the ratio of Aβ42 to Aβ40 was significantly elevated in PS1 and PS2 iPSCs-derived neurons. Surprisingly, neither abnormal tau protein accumulation nor tangle formation was detected in FAD-derived neurons. This may result from the short culture period (2 weeks) for tauopathy formation. Not only produced the Aβ which is the pathological hallmark of AD, FAD-derived neurons also responded to γ-secretase inhibitor (compound E) and γ-secretase modulator (compound W) in order to decrease the ratio of Aβ42 to Aβ40. These data highlighted that both PS1 and PS2 iPSC-derived neurons respond to drug treatment as expected and could be useful for a novel drug screening for AD treatment [87]. Nevertheless, neural specific differentiation protocol and long-term culture methods of mature neurons are necessary to enhance dominant disease pathologies.

6. The Perspectives of Disease Modeling by Human Induced Pluripotent Cells

One of the most interesting aspects of iPSC technology is the possibility to develop autologous cells for cell replacement therapy. The patient-specific pluripotent cells could be differentiated into desired cell types in the unlimited cell number manner which, ultimately, could be transplanted into the patient's own body. However, techniques of reprogramming somatic cells are necessary to be nonviral, nononcogenic, and nongenetic modification. Moreover, diseases which are related to imprinting genes and epigenetic anomaly, such as FX [44], AS [65], and PWS [48, 65] are difficult to be completely reprogrammed and reset their epigenetic memory [32], which means iPSC technology needs further development in order to overcome these issues. Importantly, the differentiation protocols of desired cell types must be robust and efficient in order to produce high purified specific cell types. For these reasons, the use of iPSCs for cell replacement therapy is not yet ready for clinical applications at this moment [88].

The other applications of iPSCs are the generation of pluripotent cells from developmental or degenerative disorder patients for disease modeling and drug discovery. According to the lack of ideal animal models and inaccessibility to biopsy brain samples from live patients, generation of human pluripotent stem cells has opened an opportunity to investigate disease development *in vitro* [31]. Ultimately, if iPSCs could be generated in individualized manner, the most effective drug for each patient could be validated [33, 89]. To date, there are 4 technical challenges if we want to use iPSCs as a disease model. Firstly, the transgene-free iPSCs should be produced in order to minimize or eliminate genetic alterations in the derived iPSC lines. It has been reported that the gene expression features of factor-free PD-iPSCs were closely similar to hESCs [36]. There are many other factor-free methods available for iPSC establishment, such as episomal vector [90, 91], synthetic-modified mRNAs [92], recombinant proteins [93, 94], or miRNA [95, 96]. Secondly, the solid differentiation protocols of iPSCs into disease-specific cell types must be robust and efficient [35, 87]. The production of specific cell type in a clinical scale is very difficult, resulting from a short time span of cells in culture conditions. Moreover, specific cell-sorting methods have to be developed for purifying only

disease-related cell types for further investigation. Thirdly, the *in vitro* disease-relevant phenotypes must be formed. The most important feature for disease-modeling system is the appearance of disease phenotype; however, some of the neural cells derived from patient iPSCs do not show clinical disease phenotypes after the differentiation [36, 44, 69], in particular iPSCs derived from neurodegenerative diseases with long latency, such as Parkinson's and Huntington diseases. The possibility to overcome this challenge would be the attempt to accelerate the appearance of pathological phenotypes by the exposure of disease stimulators effects, such as oxidative stressors, hydrogen peroxide, or MG-132 [35, 36, 87]. Lastly, the disease-relevant phenotypes could be generated by human-animal chimeras. Some diseases may not be practical to *in vitro* model since the pathological onset appears in elderly patients with complex pathophysiology. Chimeras provide long-term access to complex and changing environmental context for hiPSCs. Many types of late-onset diseases, such as Alzheimer's and Parkinson's diseases, are multifactorial disorders, caused by both genetic and environmental factors. These chimeric animals will provide the *in vivo* model for long-term modeling in various types of environmental factors which will provide the close systems to human diseases [97, 98], such as triple knockout of amyloid precursor protein (APP), APLP1, and APLP2 chimeric mice which show a mixed population of triple knockout APP neuron in WT back ground brain [99]. In conclusion, the iPSC technology is the powerful technique which allows scientists to investigate the neurological disease development and screen pharmaceutical compounds. Several diseases of the nervous system remain to develop disease-specific iPSCs. In the near future, iPSC technology will facilitate stem cell biologists and neuroscientists to unravel disease mechanisms and discover the new therapeutic approaches for neurological disorders.

Conflict of Interests

The authors declare no conflict of interests.

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Review Article

Toward Personalized Cell Therapies: Autologous Menstrual Blood Cells for Stroke

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Cell therapy has been established as an important field of research with considerable progress in the last years. At the same time, the progressive aging of the population has highlighted the importance of discovering therapeutic alternatives for diseases of high incidence and disability, such as stroke. Menstrual blood is a recently discovered source of stem cells with potential relevance for the treatment of stroke. Migration to the infarct site, modulation of the inflammatory reaction, secretion of neurotrophic factors, and possible differentiation warrant these cells as therapeutic tools. We here propose the use of autologous menstrual blood cells in the restorative treatment of the subacute phase of stroke. We highlight the availability, proliferative capacity, pluripotency, and angiogenic features of these cells and explore their mechanistic pathways of repair. Practical aspects of clinical application of menstrual blood cells for stroke will be discussed, from cell harvesting and cryopreservation to administration to the patient.

1. Introduction

Cell therapy has established itself as an important field of research with considerable progress in the last years. Several disorders, including those of inflammatory, traumatic, degenerative, and autoimmune nature, are listed as potential targets for stem cell application. While the bone marrow leads the investigations, other sources of stem cells have been explored, searching for cells with higher plasticity and tissues with facilitated harvesting [1]. Disposable tissues, such as the amniotic fluid, placenta, and, more recently, menstrual blood, are being investigated as potential sources of stem cells for therapy [2–4]. The immature phenotype, high proliferative potential, and immunomodulatory effects of these cells suggest them as powerful tools for repair.

Neurovascular diseases are the third leading cause of death in the United States and the first cause of chronic disability [5, 6]. Aging of the population and changes in lifestyle, especially in developed countries, contribute to the progressive increase in the incidence of these disorders, more specifically stroke [7]. On the other hand, treatment is limited, and the only approved therapeutic agent for ischemic stroke is tissue plasminogen activator (tPA). More limiting, however, is the time window for tPA application, restricted to up to 3

hours after symptom onset [8]. As result, a report from 2008 estimated that only 1.8 to 2.1% of all stroke patients had been treated with tPA in the United States [9]. It is clear, therefore, that therapeutic alternatives are warranted for the remaining stroke-affected patients which, excluded from tPA benefits, are exposed to the chronic consequences of the disease.

Menstrual blood cell injections are proposed as a restorative therapy after stroke, aiming to provide functional improvement and, therefore, decrease disability of the affected patients. Migration to the site of injury, immunomodulation, and secretion of neurotrophic factors are their main footholds as therapeutic agents. When compared to bone marrow-derived cells, menstrual blood cells present more immature phenotype and behavior, albeit maintaining the characteristic adult stem cell safety [4–10]. Experimental studies have demonstrated benefits of menstrual blood cell administration, with tissue repair and functional improvement, not only in the central nervous system, but also in the heart and ischemic limbs [10–13]. Cell differentiation, although demonstrable *in vitro*, is still heterogeneously reported in the literature, and its relevance to the final outcome is not yet established. Of more importance may be the endogenous pathways of repair, which are also stimulated by the administrated cells.

This paper aims to characterize menstrual blood cells, describe their possible mechanisms of repair in stroke, and, finally, discuss practical aspects of autologous, thus personalized, application. At this time, however, the use of menstrual blood cells for autologous transplantation is restricted to the female population, regarding autologous use.

2. The Interaction between Stem Cells and Inflammation in Stroke

The injury that follows stroke includes the infarct core, which harbors the tissue that evolves almost instantly to death due to ischemia, and the penumbra area, which surrounds the core and that maintains viable, yet nonfunctional, tissue [14]. While the lesion in the core is mostly irreversible, the penumbra area may be rescued, and its fate depends upon severity of the ischemia and time elapsed before restoring blood flow [15]. Treatment with tPA is the first attempt to rescue the penumbra area [16]. Earlier destruction of the clot, restoring blood flow, significantly improves functional outcome of patients. However, application of tPA after the 3-hour time window increases the risk of hemorrhagic transformation of the ischemic stroke, therefore limiting its use to a small, fortunate group of patients that are able to reach the emergency room and complete neurological triage in time to be treated [17]. Therefore, secondary strategies to promote restoration of the penumbra area are strongly warranted.

Inflammation is settled shortly after the ischemic insult and plays a dual role in stroke [18]. Microglial activation leads to the inflammatory events, resulting in blood-brain-barrier leakage, edema, hemorrhage, and leukocyte infiltration [19, 20]. The migration of attracted cells from the systemic circulation amplifies the inflammatory response, that culminates with release of cytokines, nitric oxide and free radicals, further microglial and astrocytic activation, all of which contribute to the worsening of the neurotoxic environment. Therefore, while inflammation is important to promote clearance of debris, scavenge excess neurotransmitters from the extracellular space, control water and ion homeostasis, and increase the production of neurotrophic factors, it may also promote secondary damage to the nervous tissue [21]. Additionally, the scar tissue generated by the reactive astrocytes, encapsulating the infarcted area and protecting the remaining healthy brain from further damage, interferes with the repairing mechanisms, such as migration of stem cells and angiogenesis. In summary, inflammation is necessary and beneficial in the initial hours after stroke, however, as time progresses, interventions are needed to restrain the aberrant immunological response and thus protect the brain from further damage.

Cell therapy modulates the inflammatory response at the same time that stimulates repairing pathways. The results are improved when the cells are applied according to the dynamics of the inflammatory response. Therefore, interventions in the first 24 hours following stroke are mainly neuroprotective [22] and aim to restore vessel permeability. Treatment with tPA is indicated in this phase, therefore preventing further neuronal death. Thereafter, inflammation settles itself, with maximum intensity during the first week after stroke, during which the stem cells have their best applications, promoting restoration of the penumbra area. Injected intravenously, stem cells migrate to the site of injury and opportunely interact with the inflammatory environment, modulating its deleterious effects and at the same time maximizing its beneficial aspects [23]. Changes in the immunological profile towards a less inflammatory response, increasing of cells with regulatory function, decreasing of inflammatory cytokines and reversal of astrocytic reactivity are described as modulatory effects of stem cells [24, 25]. Moreover, cytokines and chemokines secreted by the damaged tissue can stimulate the differentiation of the injected cells into cell types of interest for repair [26]. Still in the inflammatory context, stem cells secrete neurotrophic factors, which in turn stimulate neuronal survival and endogenous repairing pathways [23, 25]. Once inflammation decreases, after approximately one month after stroke, according to animal studies, cells are no longer attracted to the damage site or are stimulated to differentiate in situ [27]. However, repair can still be promoted through local implantation of predifferentiated cells.

The use of undifferentiated cells through intravenous route seems to be the best approach for clinical translation since it is minimally invasive and possesses multiple therapeutic pathways. On one end of the spectrum of differentiation, embryonic cells have the advantage of high plasticity, trophic support, and proliferation [28, 29]. However, the risks of uncontrolled proliferation and teratogenicity, among other problems associated with this cell type, make them prohibitive as therapeutic agents. On the other end are the adult stem cells, which still maintain a certain variability in the scale of maturity, according to their source. Therefore, bone marrow-derived stem cells present lower proliferative capacity and less telomerase activity than stem cells derived from adipose, dental pulp, and endometrial tissue. These,

in turn, present more immature characteristics, expressing embryonic markers and differentiating *in vitro* into cell types from all three germ cell layers [1, 30].

3. Characterization of Endometrial-Derived Cells

The presence of stem cells in the endometrium was first described over 30 years ago [31]. The monthly shedding of the superficial layers suggested that cells with high proliferating capacities were present in the tissue. The endometrium is composed of epithelial cells, identified in the superficial layers of the tissue, and extending toward the interface with the myometrium, through the tubular glands. The remaining endometrium consists of stromal cells, smooth muscle cells, endothelial cells, and leukocytes [32]. Functionally, the endometrium can be divided in an upper layer, named functionalis, which contains mostly glands loosely held together by stromal tissue, and in a lower layer, basalis, consisting of dense stroma and branching glands. The functionalis is eliminated monthly, as menstruation, and the basalis persists and gives rise to the new endometrium, under hormonal influence. Against the initial belief that the stem cells were exclusively part of the basalis layer of the endometrium, and were not eliminated with menstruation, Meng et al. [33] detected stem cells in the menstrual blood. Several other studies followed, confirming the discovery [4, 34, 35] and consolidating menstrual blood as a possible source of stem cells.

Epithelial and stromal cells isolated from the endometrium or from menstrual blood and cultured *in vitro* show clonogenicity and proliferative capacity, but the epithelial cells soon lose part of their phenotypic markers and need a feeder layer to survive [33, 36]. Patel et al. [4] published a detailed study, in which stromal stem cells isolated from menstrual blood (MenSCs) were expanded *in vitro*, and showed clonogenic properties and multipotentiality. They also demonstrated that MenSCs expressed markers of pluripotency, such as Oct-4, SSEA-4, and c-kit, which are frequently found in more immature cell types, including the embryonic stem cells.

Recently, Allickson et al. [34] published a study about the proliferative properties of human menstrual blood-derived cells. Agreeing with previous studies, the cells presented high proliferative rates and immature phenotype, expressing embryonic cell markers, which remained unaltered after 20 culture passages. Interestingly, the cells demonstrated resistance, since they were able to be processed up to 96 hours after collection, high viability after processing, and longevity, as some of the cultures were able to be subcultured 47 times before senescence.

4. Experimental and Clinical Applications of Endometrial-Derived Stem Cells

Cui et al. published in 2007 the first report of an *in vivo* application of endometrial-derived cells. In this study, the investigators evaluated the effects of both endometrial cells and menstrual blood cells in a murine model of Duchenne muscular dystrophy. Fusion of the injected cells to myoblasts was observed *in vivo* and *in vitro*, followed by the production of human dystrophin by the treated muscle [35]. Shortly after that, Hida et al. [10] described their experience in differentiating menstrual blood-derived stromal cells *in vitro* into spontaneously beating cardiomyocyte-like cells. When menstrual blood cells were injected in the ischemic tissue of myocardial infarct rat models, functional improvement was noted, differently than what was observed when bone marrow stromal cells were used. Additionally, they compared the phenotype and proliferative characteristics of endometrial gland mesenchymal cells and menstrual blood mesenchymal cells, concluding that they are very similar populations of cells, the latter possibly deriving from the former. Finally, the authors also described cell engraftment and transdifferentiation into cardiac tissue, which seems to be a unique characteristic of this transplantation site, since other studies transplanting endometrial-derived cells into different tissues failed to detect expressive differentiation [11].

Regarding neurovascular disorders, Borlongan et al. [11] reported the results of menstrual blood cell transplantation in experimental stroke. Stromal-like menstrual blood stem cells were isolated, expanded, and selected for CD117, a marker associated with high proliferation, migration, and survival [37]. *In vitro* studies showed that the expanded cells maintained expression of embryonic-like stem cell phenotypic markers, such as Oct4, SSEA-4, and Nanog, even when cultured up to 9 passages, as an evidence of the safety and reliability of these cells, and some were induced to express neural markers (MAP2 and Nestin). Moreover, when added to cultured rat neurons exposed to a hypoxic insult, the menstrual blood cells provided neuroprotection, and when applied to rat stroke models, less neurologic deficit was observed on functional tests, irrespective of the injection site, that is, systemic or local administration into the striatum. However, analysis of the tissue, after animal sacrifice, revealed that although human cells were detected in the rat brain, some migrating to areas other than the injected, they did not show signs of differentiation, expressing their original markers. The observation suggests that, at least in the brain tissue, cell differentiation is not the main mechanism of repair.

Wolff et al. [13] reported the use of endometrial-derived cells in a Parkinson's disease mouse model. Endometrial-derived stromal cells were differentiated *in vitro* into dopaminergic-like cells, which expressed nestin and tyrosine hydroxylase (TH), an enzyme that participates in dopamine synthesis. Labeled human endometrium-derived cells and predifferentiated dopaminergic-like cells were transplanted into the striatum of the animals. Endometrial-derived stromal cells were able to migrate to the substantia nigra and also showed signs of *in vivo* differentiation, acquiring neuronal phenotype and expressing human TH. Taken together, these observations demonstrate the therapeutic potential of these cells to functionally restore the damaged tissue. They also reinforce the idea that cells do not need to be predifferentiated before transplantation and that more immature, less-differentiated cell types migrate easily to the inflammatory sites. In contrast to the observations of

Borlongan et al. [11], widespread cell differentiation was suggested by this study.

The only clinical study yet published evaluated the safety aspects of endometrial-derived stromal cells administration [38]. Four patients with multiple sclerosis were treated with intrathecal injections of 16 to 30 million cells, and one of the patients also received an additional intravenous injection. No adverse events were registered and, in the short followup of 12 months, the authors reported functional stabilization.

Endometrium-derived cells present a strong angiogenic potential that contributes to the experimental investigations of vascular growth and remodeling and, perhaps, even for designing clinical therapeutic studies, as these cells might be applied to cardiovascular diseases. The angiogenic behavior was already predicted by the observation of high levels of VEGF and its receptors in the tissue and is probably associated with the function of the cells in the endometrium, that is, rapid proliferation and implantation of the embryo [39]. In a pilot experimental study, Murphy et al. [12] demonstrated that intramuscular injections of endometrial-derived cells in hind limbs of rats were able to prevent the formation of necrotic ulcers after ligation of the femoral artery and its branches. The authors and others propose to investigate the angiogenic properties of these cells in chronic limb ischemia patients and, more recently, severe skin burns, using the cells associated to intelligent artificial films [40].

In summary, the available evidence regarding menstrual blood-derived cells favors their future application in clinical studies. In comparison to stem cells from other sources, especially those from the bone marrow, menstrual blood-derived stem cells have the advantage of presenting a more immature phenotype, through the expression of embryonic-like surface markers. Their immature behavior is confirmed by *in vitro* differentiation studies in which menstrual blood-derived cells originate diverse tissue types from all three germ layers [4, 33]. Moreover, they seem to have a higher proliferative capacity, above 30 population doublings, when compared to stromal cells from other sources, such as the bone marrow and dental pulp, which are limited to approximately 20 population doublings [1]. Additionally, cultured menstrual blood cells maintain longer telomerase activity than bone marrow-derived cells [4, 34], indicating delayed senescence. These observations may reflect higher regenerative and differentiation potentials *in vivo*, yet to be confirmed by comparative studies between cells from different sources. Whether endometrial-derived stem cells provide repair through cell differentiation or through paracrine effects, stimulating endogenous repairing pathways, is a point still to be established. The available evidence suggests that the type of tissue and possibly the nature of the injury may determine the repairing mechanisms.

5. Personalized Therapy: Practical Aspects

Personalized medicine is a rapidly expanding field offering patient-specific therapies to treat disease. Autologous cell transplantation may be a form of personalized medicine that could afford many benefits in the clinical setting, such as the elimination of graft versus host disease. Transplantation of autologous stem cells may circumvent the need for immunosuppressants, which can cause many deleterious side effects to the patient. Increased availability is another advantage of autologous stem cell transplantation. Ethical controversy surrounding the collection of a patient's own stem cells is greatly reduced, thus increasing the accessibility of these cells. Unlike embryonic and fetal stem cells, harvesting autologous stem cells from the patient does not harm another organism. Embryonic stem cells carry an increased risk of tumor formation, a characteristic not exhibited in autologous adult stem cells. Increased safety and decreased ethical controversy make autologous stem cells an appealing therapeutic option for neurological disease. The debate currently surrounding not only embryonic stem cell retrieval but also stem cell usage in general has sparked issues with the usage. Obtaining cells from a patient's own body would circumvent the ethical controversy as it is not harvesting viable cells from one individual donor to another individual recipient, but it is entirely for the same patient. Additionally, these cells would not be taken in ways possibly deemed as "therapeutic cell cloning," such as the current debate following embryonic and fetal stem cells.

Menstrual blood cells are a promising source of repair. Their immature behavior warrants migration, immunomodulation, secretion of growth factors, and, in some cases, differentiation. These properties, coupled to the angiogenic potential of the cells, make them attractive for restorative approaches following ischemic stroke, as already demonstrated by translational research [11]. Moreover, these cells are optimal candidates for autologous therapy, following the current trend to cryopreserve biological products intended for future use. Although stromal cells have low immunogenicity due to the lack of MHC class II expression [41], therefore enabling allogeneic application, autologous use is still preferred. Some advantages are guaranteed lack of immunogenicity, reverting in longer cell survival and no induction of local inflammatory reaction, safety, and diminished risk of ethical conflicts.

The one-week time window that follows stroke seems the best opportunity for cell therapy. While inflammation is at its best degree, there is enough time to stabilize the patient, complete physical examination and laboratory tests, discuss the therapeutic options with the patient and family, and, finally, apply the cells. Unfortunately, the time window is still short for expansion of autologous cells *in vitro*, as the procedure usually requires a few weeks to obtain minimum number of cells. Menstrual blood cell banking, thus, seems to be the best strategy and may become a strong competitor to the already available umbilical cord blood banks.

Safety is one important concern regarding the use of menstrual blood. Since the blood is collected through the placement of a silicone cup inside the donor's vagina, therefore exposed to microbial contamination, some precautions are required, such as refrigeration of the collected specimens and use of antibiotics [34]. Additionally, the cells should be processed, expanded, and stored in a certified facility, under strict rules of cleanliness and safety. Additional safety measures include phenotypical verification of the cells after expansion, ensuring that they maintain the original markers

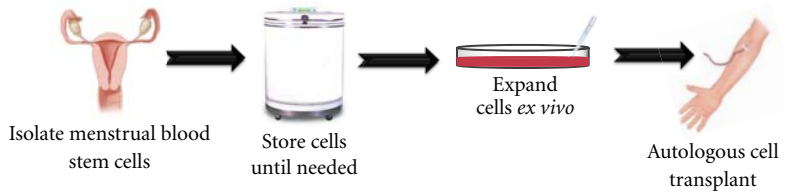

Figure 1: Isolation and transplantation of autologous menstrual blood-derived cells. Menstrual blood cells can be collected and stored prior to injury or the development of a neurodegenerative disease. At the time of injury, the cells can then be thawed and expanded ex vivo. Once the ideal number of cells has been reached, the autologous menstrual blood-derived cells can be transplanted into the patient.

and cytogenetic evaluations, excluding chromosomal aberrations and microbiological tests.

A hypothetical scenario would be a woman, in postmenopausal age, recently affected by an ischemic stroke. Due to the limited timeframe between the beginning of symptoms and final diagnosis, she would be part of the majority of patients that are excluded from tPA treatment. After further examinations, evaluation of the extension of the infarct and prognostic evaluations would be completed, estimating the degree of future, long-term disability. Finally, given the information that the patient has menstrual blood cells cryopreserved and ready for use, the therapy would be considered. After patient or familial consent, the cell banking facility would be contacted by the responsible physician, settling date and time for the infusion. The cells would be transported cryopreserved, being thawed at bedside and immediately infused intravenously, under physician supervision and cardiorespiratory monitoring. A second peripheral venous line would be available for emergency medication, if needed. After infusion, the patient would stay in the hospital during at least 24 hours, for adverse reaction monitoring, especially due to the possible toxic effects of dimethyl sulfoxide (DMSO) used in cell cryopreservation and to remaining traces of antibiotics in the cell suspension (Figure 1). After discharge, periodical evaluations would evaluate the patient's progress, establishing goals for the complimentary treatments such as physical and speech therapies, nutrition, and, finally, ability to resume work.

For a stroke-affected patient, the slightest improvement in neurological function can be decisive for self-sufficiency and, even, for ability to work, which are ultimately translated into financial independence. Therefore, investing in cell banking as a safety measure against possible future events may be a wise and even profitable step. While cell banking is already widely accessible for umbilical cord blood, only recently has it also become available for menstrual blood cells. Women in child-bearing age may donate multiple samples of menstrual blood, enabling storage of large amounts of cells for future use. As a further possibility, the cells could be expanded and differentiated into specific tissues and be ready for eventual transplantation use [42]. An efficient banking system for menstrual blood cells would require an organized and updated registration system, enabling prompt identification and rapid retrieval of the cryopreserved cells, just in time for therapeutic application.

6. Conclusions

The rescue of the penumbra area after stroke is decisive for functional outcome and a great opportunity for cell therapy [16]. Stem cells promote functional restoration especially through modulation of the activated immune system and secretion of trophic factors [25]. Although cell differentiation is observed in the experimental setting, its importance to the final outcome of the treatment is still undefined. Menstrual cells combine characteristics that are convenient for clinical application and, in parallel with cells derived from other disposable tissues, may have a role in the future investigations. Cryopreservation of autologous cells for future use may be a prudent strategy to those patients at risk of being affected by stroke. Obviously, menstrual blood as a source of autologous cells is limited to women as the target patient population. Despite the potential challenges still to be resolved, these cells represent important therapeutic tools that may improve the disease outcome, decreasing the mortality and morbidity of stroke patients.

Disclosure

CVB and PRS serve as consultants, and PRS is a cofounder of Saneron-CCEL Therapeutics, Inc., and CVB, PRS, and JGA have a patent application in this area, owned jointly by Cryo-Cell International, Inc. and Saneron-CCEL Therapeutics, Inc. Cryo-Cell International, Inc. provided the foundational menstrual stem cell technology in the patent applications of M. A. Walton and JGA wholly owned by Cryo-Cell International, Inc.

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Research Article

CNPase Expression in Olfactory Ensheathing Cells

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A large body of work supports the proposal that transplantation of olfactory ensheathing cells (OECs) into nerve or spinal cord injuries can promote axonal regeneration and remyelination. Yet, some investigators have questioned whether the transplanted OECs associate with axons and form peripheral myelin, or if they recruit endogenous Schwann cells that form myelin. Olfactory bulbs from transgenic mice expressing the enhanced green fluorescent protein (eGFP) under the control of the 2-3-cyclic nucleotide 3-phosphodiesterase (CNPase) promoter were studied. CNPase is expressed in myelin-forming cells throughout their lineage. We examined CNPase expression in both in situ in the olfactory bulb and *in vitro* to determine if OECs express CNPase commensurate with their myelination potential. eGFP was observed in the outer nerve layer of the olfactory bulb. Dissociated OECs maintained in culture had both intense eGFP expression and CNPase immunostaining. Transplantation of OECs into transected peripheral nerve longitudinally associated with the regenerated axons. These data indicate that OECs in the outer nerve layer of the olfactory bulb of CNPase transgenic mice express CNPase. Thus, while OECs do not normally form myelin on olfactory nerve axons, their expression of CNPase is commensurate with their potential to form myelin when transplanted into injured peripheral nerve.

1. Introduction

The only example of successful regeneration from peripheral neurons into the central nervous system (CNS) is within the olfactory system, where axons regenerate throughout life from the nasal mucosa into the olfactory bulbs of the brain. A specialized glia cell, the olfactory ensheathing cell (OEC), spans the CNS-peripheral nervous system (PNS) junction and is thought to bridge the gap to allow peripheral axons to penetrate the brain. Indeed, transplantation of cultured OECs leads to enhanced regeneration and remyelination of injured peripheral nerve [1, 2].

A large body of work supports the proposal that transplantation of OECs into various spinal cord injury and demyelination models can promote axonal regeneration, remyelination, and functional recovery [2–12]. Yet, some investigators have questioned whether the transplanted OECs form peripheral myelin, or if they recruit endogenous SCs that form myelin [13, 14]. These events are not mutually exclusive in that transplanted OECs could both facilitate SC invasion into the spinal cord and as well as myelinate axons. It is important to note that Franklin et al. [11] demonstrated myelination in the spinal cord by an OEC cell line, strongly suggesting that OECs can indeed remyelinate axons [9].

Although OECs do not form myelin on fine caliber olfactory nerve fibers during normal development, numerous studies have shown that OECs can remyelinate both CNS [15–18] and PNS [1, 2] axons in a variety of lesion models. This discrepancy between the normal developmental fate OECs *in vivo* and their differentiation when transplanted into demyelinated regions has raised the question of whether the myelination observed in OEC transplanted lesions is due

to contamination of OEC preparations with Schwann cells, oligodendrocyte precursor cells (OPCs), or even neural stem cells [13, 19, 20].

The enzyme 2′,3′-cyclic nucleotide 3′-phosphodiesterase or CNPase is expressed in both oligodendrocytes and SCs and is considered a marker for myelin-forming cells, although it is also found in other cells, including lymphocytes and photoreceptors as well as some neurons in long-term culture [21]. CNPase is both membrane bound and linked to microtubules and is the third most abundant myelin protein in the CNS, representing 4% of CNS myelin proteins. The role of this enzyme is not yet clear, although over expression mutations suggest that CNPase plays a role in myelin compaction [22, 23]. CNPase is the earliest myelination-specific protein expressed by oligodendrocytes and is expressed in both myelinating and nonmyelinating oligodendrocytes and SCs. CNPase is therefore considered to be marker for the potential of cells to produce myelin, rather than an indication of actual myelination and evidence of CNPase expression by OECs would therefore provide strong support for the idea that OECs are capable of forming myelin.

Studies using immunostaining with antiCNPase antibodies yielded ambiguous and conflicting results for CNPase expression by OECs from the olfactory bulb and olfactory neuroepithelium. CNPase staining was observed on some, but not all presumptive OECs in explant cultures from the olfactory bulb [24], but not on presumptive OECs in dissociated cultures from the nasal epithelium cultured on astrocyte feeder layers [25]. Immunostaining of developing olfactory bulb focused on CNPase staining of oligodendrocytes and did not report CNPase staining of OECs [26]. It is not clear therefore whether CNPase is expressed by OECs only in specific environments, or whether levels of CNPase may be too low to reliably detect with standard antibody staining protocols.

The recent development of a transgenic mouse in which an enhanced green fluorescent protein (eGFP) is linked to expression of CNPase [21] has provided an opportunity to evaluate CNPase expression by OECs in a variety of environments. Since the use of a reporter gene eliminates problems with both false positive and false negative antibody staining, GFP transgenic mice would allow detection of CNPase expression without the need to optimize staining protocols to specific tissue or culture conditions. In this study we have examined CNPase-linked eGFP expression by OECs in the olfactory bulb and in dissociated cell culture. The results indicate that OECs express CNPase in the outer nerve layer of the olfactory bulb as well as in culture, thus indicating that OECs express an important enzyme required for myelination. This provides further evidence of the intrinsic capability of OECs to myelinate axons upon transplantation.

2. Methods

2.1. Isolation and Characterization of OECs from CNP-EGFP Mouse. The CNP-EGFP mouse has been described previously [15, 27]. Freshly isolated OECs were obtained as reported previously [16, 28, 29]. Olfactory bulbs were removed from 4- to 8-week-old transgenic mice expressing GFP-CNPase and dissected free of meninges. The caudal one-third of the bulb was removed and discarded along with as much white matter as possible to isolate the outer nerve layer. Tissue was minced finely with a pair of scalpel blades (#10) on plastic culture dishes, and nonadherent tissue was washed from culture dishes and incubated for 25 min in collagenase A (0.75 mg/mL; Roche, Indianapolis, I, USA), collagenase D (0.75 mg/mL; Roche), and papain (12 U/mL; Worthington, Lakewood, NJ, USA) in calcium-free complete saline solution with trace cysteine for 25 min at 37°C on a rotary shaker in a CO_2 incubator. The tissue suspension was then centrifuged for 7 min at 300 ×g, and, the supernatant was discarded. The pellet was resuspended in 2 mL of Dulbecco's modified medium (DMEM, Invitrogen, Carlsbad, Calif, USA) with 10% fetal calf serum (FCS) using gentle mechanical trituration; first with a 5 mL culture pipette and then with two fire-polished silicone-coated pasture pipettes with successively reduced diameters. The volume of media was immediately increased to 20 mL, and undissociated pieces of tissue were allowed to settle for 2 min before transferring the cell suspension to another culture tube and centrifuging as before. Cells were washed twice, resuspended, and preplated for 1 h in a culture flask at 37°C in a CO_2 incubator. Nonadherent cells were gently washed off with DMEM, and the cells were centrifuged and resuspended three times in DMEM. Then cells were counted and concentrated to 3.0×10^4 cells/μL just prior to transplantation. P75NGFR- and S100-positive cells were counted in short-term cultures made from cell suspensions used for transplantation to assess purity of the cells. Over 95% of the cells were positive for p75NGFR and S100.

2.2. Immunostaining. To identify OECs, immunostaining for p75NGFR, a characteristic marker for OECs, was performed on cultured OECs. The cells were preincubated in normal goat blocking serum prior to incubation with the primary rabbit anti-p75NGFR monoclonal antibody (1 : 1000; Chemicon, Temecula, Calif, USA) followed by incubation with a fluorescein isothiocyanate- (FITC-) conjugated IgG (1 : 2000, Molecular Probes, Leiden, The Netherlands) secondary antibody for p75NGFR. Photographs were taken on a Spot RT Color CCD.

2.3. Immuno-EM. CNPase transgenic mice were deeply anesthetized (50 mg/kg sodium pentobarbital, i.p.) and perfused transcardially with PBS followed by 4% paraformaldehyde/0.02% glutaraldehyde in phosphate-buffered saline (PBS). Olfactory bulbs were excised, postfixed overnight in 4% paraformaldehyde, and embedded in 3% agar for vibratome sectioning. Free-floating sections (thickness 150 μm) were incubated in 2% normal goat serum for 30 min and then in rabbit anti-GFP antibody (1 : 2000; Chemicon) overnight at 4°C. The sections were incubated overnight with an anti-rabbit biotinylated secondary antibody (Sigma, St. Louis, Mo, USA) and then incubated for 1 h using a Vectastain Elite ABC kit (Vector Laboratories, Burlingame, Calif, USA). The sections were postfixed with 1% osmium tetroxide for 4 h, dehydrated in graded ethanol,

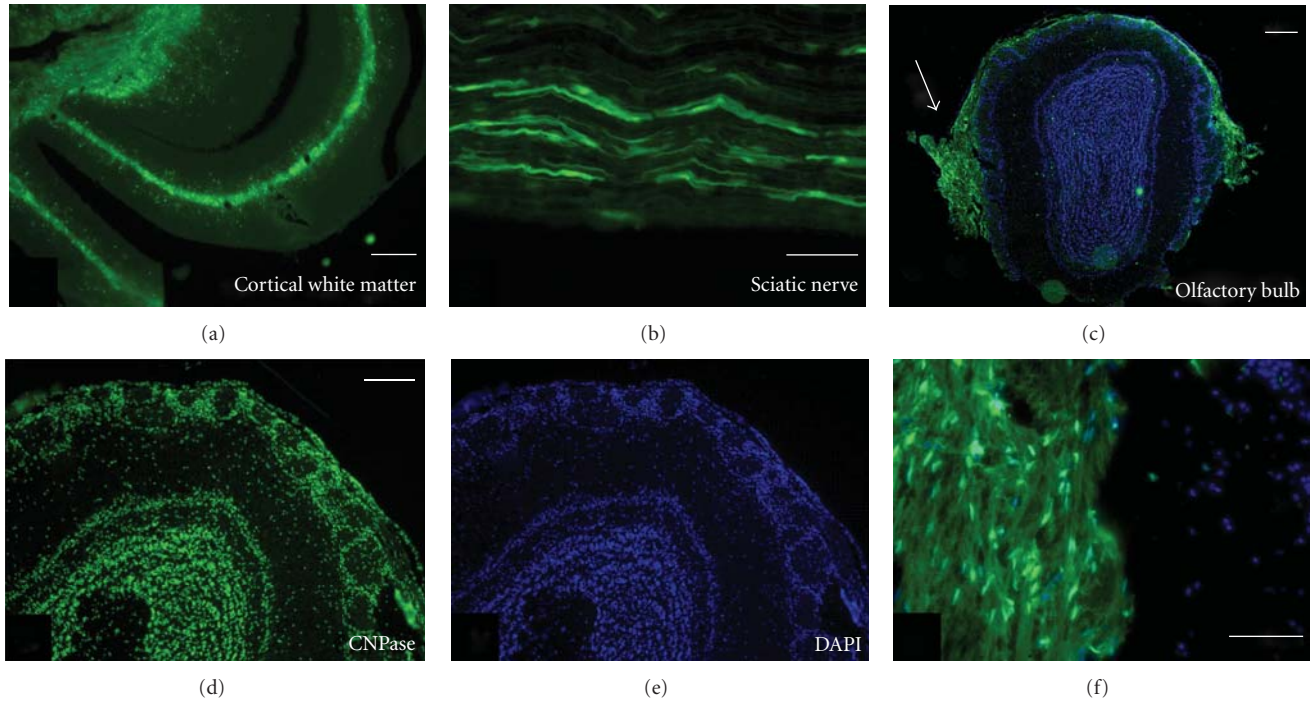

Figure 1: CNPase expression is characteristic of myelinating cells. (a) In the transgenic mouse where GFP is under the control of CNPase, GFP expression can be observed in cortical white matter (a) and sciatic nerve (b). The GFP is present in oligodendrocytes in white matter of the CNS and Schwann cells in the peripheral nerve. (c) Cross-section of the olfactory bulb from the CNPase mouse showing GFP expression in the olfactory nerve (arrow) and the outer nerve layer of the olfactory bulb (OB) where OECs are present as the only glia cell type. (f) Higher power image of the olfactory nerve from (c) showing GFP expression in the nuclei and cytoplasm of OECs in the outer nerve layer. (d) and (e) Immunohistochemistry for CNPase in a wild type mouse OB showing CNPase expression in deep white matter and outer margins of the bulb. Note that the round glomeruli are devoid of CNPase. Nuclei have been counterstained with DAPI (blue) in (c), (e), and (f). Scale bars: (a) = 8 μm, (b) = 12 μm, (c) = 500 μm, (d) = 150 μm and pertains to (d) and (e), (f) = 3 μm.

and embedded in Epox-812 (Ernest Fullam, Latham, NY, USA). Ultrathin sections were cut as described above but were not counterstained.

2.4. Induction of Nerve Crush Lesion and eGFP-OECs Transplantation Procedure. The Veterans Affairs Connecticut Healthcare System Institutional Animal Care and Use Committee approved all animal protocols. Experiments were performed in accordance with National Institutes of Health guidelines for the care and use of laboratory animals. Adult Sprague Dawley rats (200–225 g) were used for these experiments ($n = 12$). The rats were anesthetized with ketamine (75 mg/kg i.p.) and xylazine (10 mg/kg i.p.). The sciatic nerve was surgically exposed in anesthetized rats and injured by nerve crush lesion with fine microforceps for 40 seconds. This procedure completely transects all axons within the nerve and the animals showed signs of complete nerve transection [30]. The lesion site was standardized at the level of the piriformis tendon in the thigh. Cultured eGFP-expressing OECs from rat were detached from the culture flasks and resuspended in culture medium and adjusted to a concentration of 30,000 cells/μL. 2 μL of the cell suspension or vehicle alone (sham control) was injected 5.0 mm by using a Hamilton microsyringe caudally and distally into the crush lesion site. The animals survived for 5 weeks at which time they were intracardially perfused with 4% paraformaldehyde in phosphate buffer followed by removal of nerves for histological analysis.

3. Results

GFP expression in the cortex of the CNPase-eGFP transgenic mouse is strong in oligodendrocytes of cortical white matter (Figure 1(a)). Expression is also observed in Schwann cells of peripheral nerve (Figure 1(b)). Sections through the olfactory bulb in the CNPase-eGFP transgenic mouse indicate intense CNPase expression in the outer nerve layer of the olfactory bulb, the site where OECs are localized and interior regions of the bulb which are rich in oligodendrocytes (Figure 1(c)). Additionally, CNPase was strongly expressed in the olfactory nerve as it enters the olfactory bulb (Figure 1(c); arrow). A higher power image of the olfactory nerve from Figure 1(c) shows GFP expression in the nuclei and cytoplasm of the OECs (Figure 1(e)). Immunohistochemistry for CNPase of the olfactory bulb shows staining in deep white matter as well as in the outer nerve layer (Figure 1(d)).

3.1. Colocalization of p75NGFR with CNPase in OECs in the Olfactory Bulb. The low affinity NGF receptor, p75NGFR, is expressed by OECs and Schwann cells. Cells in the olfactory

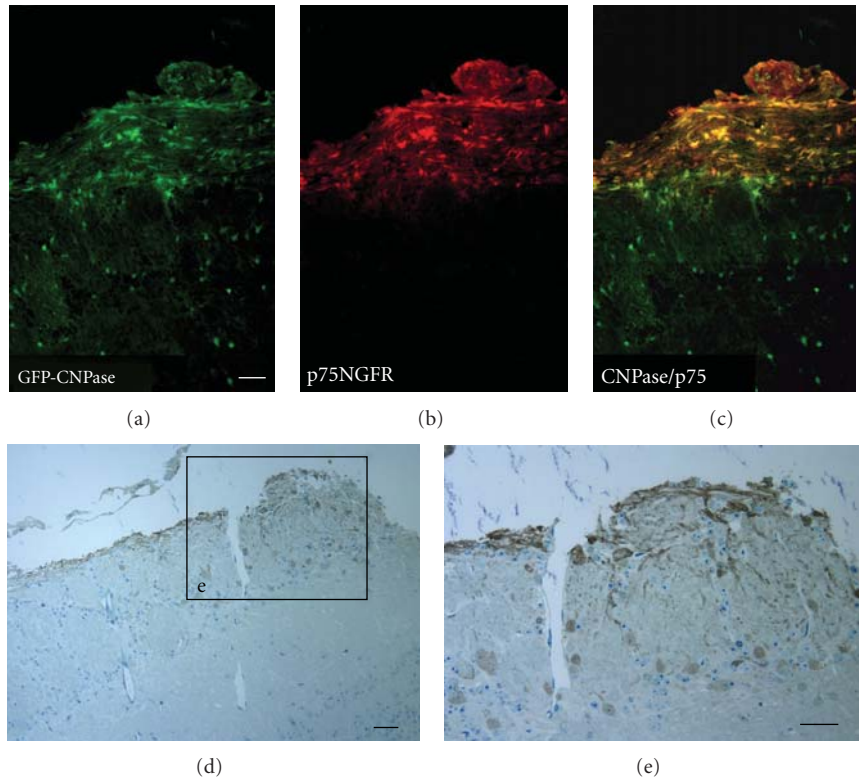

Figure 2: Colocalization of p75NGFR/CNPase and Immuno-EM. (a) GFP expression in the CNPase mouse in the olfactory nerve as it enters the OB and in scattered cells in the deep OB white matter. Immunostaining for p75 colocalizes with GFP in the olfactory nerve, but not in deep white matter (a–c). Immunoperoxidase staining for GFP in toluidine blue plastic sections (1 um) shows that the GFP was present in OECs in the outer nerve layer (d, e). Scale bars: in (a), pertains to (a–c) = 10 μm, (d) = 7, (e) = 10 μm.

nerve layer of eGFP-CNPase transgenic mice showed colocalization of GFP expression with the p75NGFR receptor (Figures 2(a)–2(c)). OECs can readily be identified in olfactory nerve as glial cells ensheathing large numbers of nonmyelinated olfactory nerve fibers and are distributed in the outer nerve layer of the olfactory bulb. For more detailed cellular localization of CNPase, immunoperoxidase staining of the olfactory bulb with a GFP antibody was performed and semithin plastic sections counterstained with toluidine, blue were obtained for more precise localization of eGFP in the OECs of the olfactory bulb and nerves. The eGFP (CNPase expressing cells) was localized in structurally well-defined OECs in the outer nerve layer of the olfactory bulb (Figure 2(d); higher magnification in Figure 2(e)). The cytoplasmic processes of the OECs wrapped bundles of nonmyelinated axons projecting within the outer nerve layer (Figure 2(e)). Thus, coexpression of p75 and CNPase within OECs in the olfactory bulb argues for the remyelination potential of OECs.

3.2. Cultured OECs from the CNPase Transgenic Mouse Maintain Their GFP Expression. The CNPase expression observed *in situ* was maintained in culture when cells were prepared for cell transplantation (Figures 3(a)–3(d)). Confirmation of OEC identity was established by p75 (Figure 3(b)) immunostaining characteristic of OECs. OECs are the only cells expressing p75NGFR in the olfactory bulb. Dissociated OECs derived from the olfactory bulb and maintained in culture for 4 days had both intense p75NGFR immunostaining and eGFP-CNPase expression (Figure 3(d)).

3.3. OECs Transplanted into Injured Peripheral Nerve Remyelinate Regenerating Axons. OECs prepared from olfactory bulb and transplanted into transected peripheral nerves remyelinate the regenerated axons [2]. The transplanted OECs, shown in green, aligned longitudinally with the regenerated axons (Figure 4(a)). The sciatic nerve crush model (axonotmesis) used completely transects all axons within the nerve. The images for Figure 4 were obtained several millimeters distal to the crush site indicating that regenerated axon were remyelinated by the transplanted OECs. Images of the sciatic nerve at 5 weeks post-OEC transplantation demonstrated an abundance of eGFP-OECs distributing along the injured nerve (Figure 4(a) with inset in (a)). eGFP can be seen in the nuclei and cytoplasm around regenerating peripheral nerve axons. The transplanted eGFP-OECs are longitudinally oriented and associated with neurofilament- (NF-) stained axons (inset Figure 4(a)). Longitudinal sections of a group of regenerated axons demonstrated GFP-OECs surrounding the axon (Figure 4(b)), and importantly, the remyelinated axons have nodes of Ranvier (Figure 4(b)) with appropriate sodium channel Nav1.6 expression (Figure 4(c)) flanked by the Caspr immunostained paranodes (Figure 4(d); overlay is shown in Figure 4(e)).

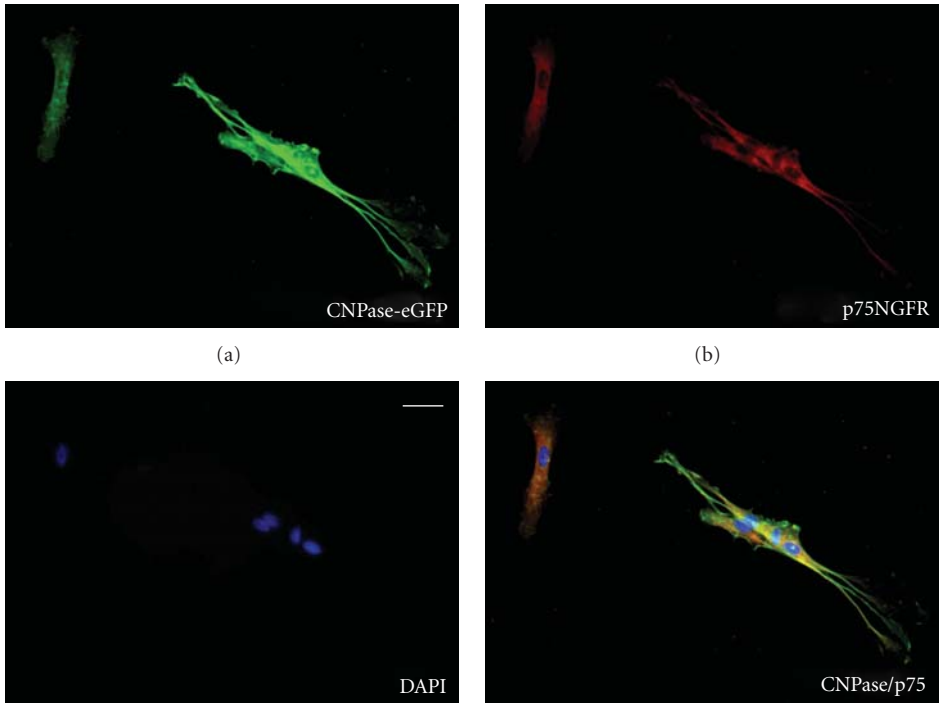

Figure 3: Cultured OECs from the CNPase transgenic mouse maintain their GFP expression. (a–d) Immunostaining of cultured olfactory bulb OECs derived from the eGFP-CNPase mouse indicates colocalization with p75. Nuclei stained blue with DAPI. Note the small cluster of spindle-shaped OECs and a more flattened OEC (upper left) both colocalize GFP (CNPase) and p75. Scale bar: (a) = 5 μm.

4. Discussion

Here we demonstrate that OECs in the outer nerve layer of the olfactory bulb express CNPase, the universal marker for myelinating cells. CNPase expression in OECs is maintained in highly purified cultures and OECs transplanted into injured peripheral nerves remyelinate regenerated nerve fibers. A difficulty in comparing results regarding the remyelinating potential from OEC transplantation studies from various laboratories is that differences are present in the age of the animals used for cell harvesting, purification procedures, and lesion models into which the cells were transplanted. OECs used in the present study were prepared relatively acutely from the outer nerve layer of the adult olfactory bulb; a CNS area rich in OECs *in vivo* [18]. The degree of cell purity (>95%) in our cell suspension as assessed using p75NGFR/S100 immunostaining was about the same as in other studies where immunopanning techniques were used [14, 31] or where OECs were prepared from embryonic tissue [32]. Mitotic inhibitors and stimulators of cell proliferation and differentiation were used in those studies. In our cell preparation method from adult tissue, we did not use mitotic inhibitors nor did we stimulate proliferation and differentiation *in vivo*. Contamination by SCs, which are also p75/S100 positive, in our cultures would be problematic in the interpretation that adult OECs are able to form peripheral-like myelin. However, one would expect at best a very minor contamination of SCs possibly associated with blood vessel innervation [14] or meningeal cells [32]. Such minor contamination could not account for the vast majority (>95%) of our cells displaying a p75NGFR/S100$^+$ phenotype in relatively acute cell suspensions.

Using transgenic mice which express GFP only in cells which express CNPase, we were able to show that OECs from the outer nerve layer of the olfactory bulb express CNPase and that OEC preparations isolated from this tissue using our isolation methods also express CNPase. This expression is an important prerequisite to demonstrate the myelination potential of OECs.

Transplantation of OECs prepared from adult olfactory bulb into various traumatic spinal cord injury and nerve injury models have demonstrated improved functional recovery. Histologically, axonal regeneration, remyelination, and neuroprotection have been reported following OEC transplantation [33]. However, Li et al. (2007) report that while OECs remyelinated regenerated spinal cord axons that they did not remyelinated regenerated optic nerve axons [34], while Schwann cells did remyelinate some optic nerve axons. Reason for this difference is uncertain. We demonstrated that the migration properties of OECs and SCs are different in the X-irradiated spinal cord: OECs migrate extensively in both gray and white matter and SCs do not [35]. Indeed, a number of unique properties have been described for OECs to distinguish them from SCs [36]. While several groups point out unique properties following *in vivo* transplantation of OECs as compared to SCs such as the formation of cellular tunnels which provide a permissive environment for axonal regeneration and greater mobility

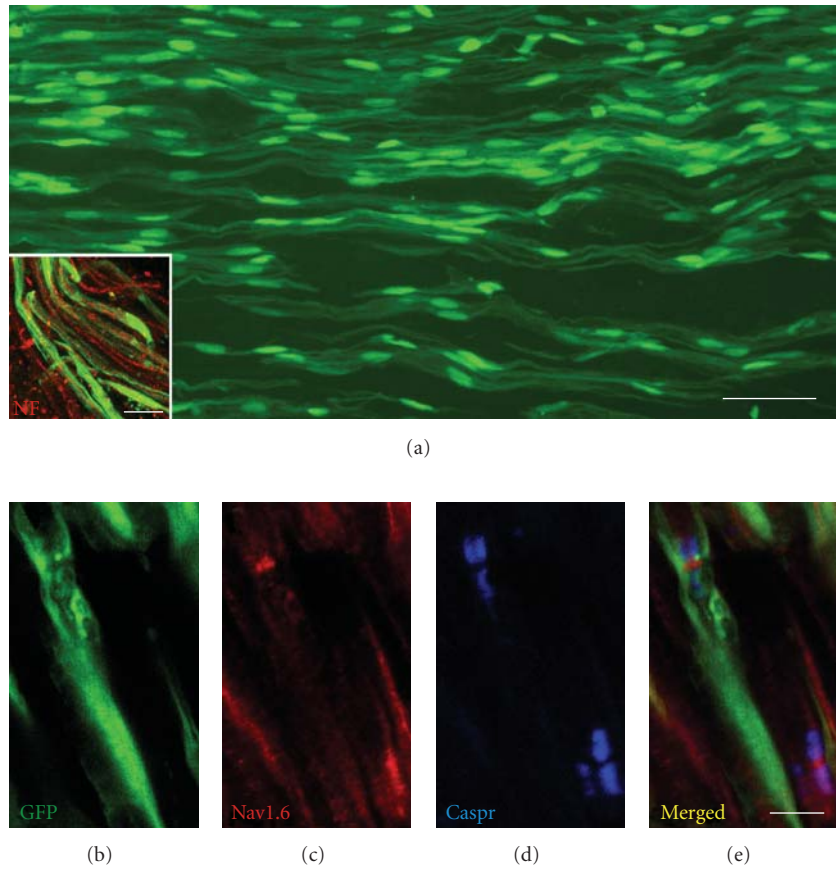

Figure 4: OECs transplanted into injured peripheral nerve remyelinate regenerating axons. (a) GFP can be seen in the nuclei and cytoplasm around regenerating peripheral nerve axons. The inset indicates that the GFP elements are longitudinally oriented with neurofilament- (NF-) stained regenerated axons. (b–e) Longitudinal section of a group of regenerated axons with GFP cells (b) surrounding the axons and sodium channel Nav1.6 (c) being flanked by the Caspr immunostained paranodes (d) indicating that the transplanted OECs can remyelinate regenerated axons which form appropriate sodium channels at the newly formed nodes of Ranvier (overlay in (e)). Scale bars: (a) = 50 μm (a), (b–e), and (a) inset in (a) = 5 μm.

in astrocytic regions [36] others suggest that the functional benefits of OEC transplantation may result from recruitment of endogenous SCs by the OECs [13, 19].

Currently, a number of clinical studies are underway exploring the potential clinical utility of OEC transplantation in spinal cord injury patients [37–41]. Better understanding of the ability of transplanted OECs to improve functional outcome and direct comparison to transplanted SCs in CNS injury will have an impact on the direction of future research directed toward clinical applications.

5. Conclusion

The results of this study indicate that OECs in the outer nerve layer of the olfactory bulb express CNPase, a universal marker for myelinating cells. CNPase expression in OECs is maintained in highly purified cultures and colocalizes in cells with p75 receptor expression. Moreover, in culture the OECs maintain strong eGFP-CNPase expression. OECs transplanted into injured peripheral nerves remyelinate regenerated nerve fibers which formed nodes of Ranvier with high density of sodium channels between the myelin segments formed by the transplanted OECs. These data demonstrate that OECs share the molecular machinery of CNPase expression with oligodendrocytes and Schwann cells indicating a third dominant myelinating cell type within the nervous system. These results encourage ongoing work with OECs as a therapeutic tool in peripheral nerve repair, in CNS trauma and demyelinating diseases.

Abbreviations

CNPase: 2′-3′-cyclic nucleotide 3′-phosphodiesterase
CNS: Central nervous system
DMEM: Dulbecco's modified medium
eGFP: Enhanced green fluorescent protein
FCS: Fetal calf serum
FITC: Fluorescein isothiocyanate
Nav1.6: Sodium channel subtype 1.6
NF: Neurofilament
OECs: Olfactory ensheathing cells
ONL: Outer nerve layer

OPC: Oligodendrocyte precursor cells
PBS: Phosphate-buffered saline
p75NGFR: P75 nerve growth factor receptor
PNS: Peripheral nervous system.

Acknowledgments

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Review Article

Mesenchymal Stem Cell-Based Tissue Engineering for Chondrogenesis

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In tissue engineering fields, recent interest has been focused on stem cell therapy to replace or repair damaged or worn-out tissues due to congenital abnormalities, disease, or injury. In particular, the repair of articular cartilage degeneration by stem cell-based tissue engineering could be of enormous therapeutic and economic benefit for an aging population. Bone marrow-derived mesenchymal stem cells (MSCs) that can induce chondrogenic differentiation would provide an appropriate cell source to repair damaged cartilage tissues; however, we must first understand the optimal environmental conditions for chondrogenic differentiation. In this review, we will focus on identifying the best combination of MSCs and functional extracellular matrices that provides the most successful chondrogenesis.

1. Introduction

Tissue loss or degeneration caused by congenital abnormalities, disease, or injury is of great consequence given human tissue's limited intrinsic potential for healing [1]. In particular, articular cartilage shows little or no intrinsic capacity for repair in response to injury or disease, and even minor lesions or injuries may lead to progressive damage and joint degeneration. Currently, frequent treatments, such as surgical intervention, to repair articular cartilage are less than satisfactory and rarely restore full function. One strategy for repairing articular cartilage degeneration via tissue engineering technologies is to create constructs of cells placed or injected onto or with matrices [2]. The underlying principle of tissue engineering (Figure 1(a)) involves the utilisation of biocompatible and mechanically conductive scaffolds, productive cell sources, and inductive molecules for the optimal differentiation and proliferation of the cell type of interest [3]. In this method, constructs of autologous, allogeneic, or xenogeneic cells seeded in scaffolds, that is, synthetic extracellular matrices designed to support cell growth and tissue development, are implanted at a repair site in the body to promote the differentiation and maturation of the cell type of interest (Figure 1(b)) [4]. In practice, tissue-specific cells are often seeded into the scaffold *ex vivo* prior to transplantation, and with time, the cells synthesise a new extracellular matrix (ECM) as the scaffold produces new, properly functioning tissue.

For this reason, the appropriate selection of cells and materials as scaffolds is one of the most important factors for successful, cell-based cartilage tissue engineering because the reconstruction and regeneration of damaged tissues occurs via an ordered pathway of cellular events affected by biological and mechanical factors [5, 6]. For clinical applications, autologous or allogeneic cell grafts are generally used. Autologous grafts are ideal, but they are often limited by the availability of donors [7]. Moreover, xenogeneic grafts are frequently subject to rejection as antigens present may elicit an immune reaction in the recipient and are further limited by pathogens found in the donor tissue. However, the use of allogeneic grafts is clinically routine due to the development of immunosuppressive drug therapies, such as cyclosporine, FK506, and rapamycin. Stem cells have the potential to be applied as a prepared allogeneic graft, thereby avoiding the need for tissue harvesting of prospective recipients, an extraordinary therapeutic advantage for many cell types. They have the capacity for self-renewal and the ability to generate differentiated cells. Recently, the field of stem cell biology has attracted more attention because of the isolation of human embryonic stem cells (ESCs), and the

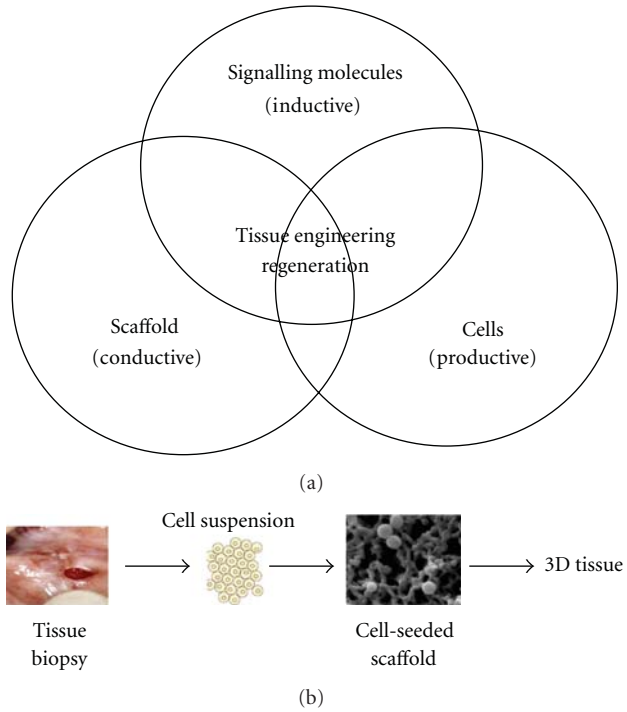

Figure 1: Tissue engineering strategy. (a) Basic principles of tissue engineering. (b) General methods of cell culture using a scaffold.

suggestion that adult stem cells may have a broader potential, that is, plasticity, than was previously thought [8].

ESCs derived from totipotent cells of an early mammalian embryo can proliferate indefinitely and can give rise to virtually any cell type. Therefore, the use of ESCs to replace damaged cells and tissues promises future hope for the treatment of many diseases. However, many countries now face complex ethical and legal questions as a result of the research to develop these cell therapies [9]. To circumvent these problems, many attempts have been made to isolate adult stem cells from mammalian tissues [10]. In particular, the adult bone marrow contains mesenchymal stem cells (MSCs), which contribute to the regeneration of mesenchymal tissues, such as bone, cartilage, muscle, ligament, tendon, adipose, bone marrow stroma, and other connective tissues [11, 12] and may be obtained from patients using minimally invasive techniques such as a bone marrow biopsy.

In this review, we will focus on the use of synthetic polymeric scaffolds in articular cartilage therapy and discuss the strategies for specific targeting. In particular, we will describe the potential use of MSCs to deliver these scaffolds.

2. MSCs for Cartilage Repair

In cell-based tissue engineering fields, selection of the source cells is required for consideration of several criteria, including ease of access and availability, a capacity for differentiation, and a lack of minimal immunogenic or tumourigenic ability. For cartilage repair in cell-based tissue engineering applications, source cells have included committed chondrocytes, ESCs, and adult stem cells. Each cell type has its limitations and advantages due to its intrinsic biological properties. However, chondrocytes have shown limited redifferentiation capability after *in vivo* expansion in clinical trials and in tissue engineering applications. Moreover, ESCs and their unwanted differentiations, such as tumour formations, are associated with ethical and legal concerns and are thereby an unsuitable cell source in basic research and clinical applications, despite the infinite pluripotentiality of ESCs. However, adult stem cells derived from various adult tissues have emerged as promising cell sources [11].

Among the adult stem cells, specifically multipotent adult stem cells, MSCs are considered to be the cell type of choice for cell-based cartilage tissue engineering because of (1) the ease with which they can be isolated and expanded and (2) their multilineage differentiation capabilities [13]. The isolation of these cells from adult tissues raises opportunities for the development of novel cellular therapies without the ethical considerations associated with ESC usage. Because of their multipotentiality and capacity for self-renewal, unlike ESCs, MSCs may represent units of active regeneration for damaged cartilage [14].

Although MSCs have shown great promise in cartilage repair and regeneration, several requirements should be examined to allow them to effectively differentiate into chondrocytes and maintain this differentiated phenotype prior to implantation or delivery. These would involve the methods and materials for culture conditions of MSCs to repair or restore full functions of damaged cartilage. Upon proper culture conditions containing certain exogenous factors, MSCs can be directed towards chondrogenic differentiation. Growth factors that promote chondrogenesis or demonstrate a chondrogenic effect both *in vivo* and *in vitro* include bone morphogenetic proteins (BMPs), transforming growth factor-β (TGF-β), and insulin-like growth factors [15–17]. BMPs are secreted molecules of the TGF-β superfamily of growth and differentiation factors that were originally detected in and purified from demineralised bone [18]. BMPs have been shown to function as key regulators in cartilage and bone development [19–22] and to function in repair and remodelling of the adult skeletal system [23–25]. These findings also provide crucial insights into cartilage repair and regeneration as the progression of osteoarthritis is always accompanied by damage to the subchondral bone and the formation of osteophytes. Despite many advances in proper culture conditions for MSCs, most of the methods are limited to a two-dimensional (2D) culture, and most of them provide little information about the proper chondrogenic induction of MSCs in three-dimensional (3D) culture.

3. MSC-Based Cartilage Tissue Engineering

The 3D culture system for cell differentiation and proliferation may improve our understanding of the structure-function relationship under both normal and pathological conditions. With regard to cell-based cartilage tissue engineering, successfully reconstructed cartilage tissue formation

would be structurally reunited with the peripheral cartilage and would suggest biomechanical properties necessary for permanence and efficacy under 3D environmental conditions [26, 27]. It is currently accepted that 3D behaviours of specific cells, including MSCs, are quite different from 2D behaviours, indicating that 3D *in vitro* culture systems can mimic the *in vivo* situation more closely than 2D cultures [28–30].

3.1. Design of ECM. The successful outcome of cell-based cartilage tissue engineering using a 3D culture of MSCs ultimately depends on the design of synthetic artificial ECMs for the proper differentiation of MSCs into chrondrocytes [31] because specific stem cells alone face obstacles in the construction of cartilage formation. For the development of viable cartilage formation, synthetic ECMs should be designed considering a number of requirements, namely, mechanical properties such as a capability to withstand the large contact stresses and strains of an articulating joint, allow functional tissue growth, and provide appropriate cell-matrix interactions to stimulate tissue growth [32, 33]. One challenge for these solutions is the delivery of stem cells to the targeted tissue without cell loss. Delivery of stem cells alone may not be sufficient to restore damaged tissues as a result of enormous cell loss after delivery. Furthermore, little is known regarding the optimal delivery strategy for stem cells. Increasing the efficiency of MSC delivery and targeting the infused cells to specific tissue locations could have a large impact on the therapeutic uses of MSCs to treat diseases [12]. The delivery of MSCs can be achieved using injectable matrices, soft scaffolds, membranes, solid load-bearing scaffolds, or immunoprotective macroencapsulation. Thus, to expand their clinical potential, next generation therapies will depend on smart delivery concepts that make use of the regenerative potential of MSCs, morphogenetic growth factors, and biomimetic materials.

Other challenges are associated with the biomaterial scaffolds designed to guide tissue growth and differentiation. These biomaterials must meet several criteria to maximise the chances of a successful repair, including biodegradability and/or biocompatibility, facilitating functional tissue growth, and appropriate biomechanical properties [34–36]. Biomaterials used for cartilage tissue engineering can have the form of cell-entrapped scaffolds with nano- or microstructures [30, 37, 38].

3.2. Biomaterial Scaffolds. Polymercross-linked scaffolds have been used for cell entrapment in cell-based tissue engineering applications, due to their 3D networks, tissue-like water content, structure stability, and biocompatibility [30, 40]. There are a number of candidate scaffolding materials that include natural polysaccharides and proteins, such as alginate and collagen, and synthetic polymers, such as polyethylene glycol (PEG), poly(ε-caprolactone) (PCL), polyglycolic acid (PGA), polylactic acid (PLA), and poly (D,L-lactide-co-glycolide) (PLGA) [41]. Although tissue-engineered constructs are well designed for 3D culture, maintaining the chondrogenic phenotype is problematic when culturing MSCs alone on the scaffolds, that is, without other local factors *in vivo*. To overcome these problems, a number of novel biomaterials, innovative cell culture techniques, and newly discovered growth factors should be utilised according to directions from cell-based tissue engineering applications. In particular, growth factors are local factors that are key regulators for proper differentiation of MSCs in research and clinical applications.

For long-term retention of cells *in vivo*, the cells should be contained within the inner structures of the scaffold. With a proper fabrication method, porous scaffolds can help cells to penetrate into the scaffold when implanted into the body [42, 43]. One of the basic problems from a scaffold design point of view is that to achieve significant strength, the scaffold material must have sufficiently high interatomic and intermolecular bonding, but must have at the same time a physical and chemical structure which allows for hydrolytic attack and breakdown. For example, PCL as the scaffold material degrades relatively slowly and possesses an appropriately high bulk stiffness to facilitate MSC differentiation toward skeletal lineages [44]. The PCL scaffold was used after loading TGF-β3 physically complexed with chondroitin sulfate (CS) [45], because it was designed to maintain an interconnected pore network for at least 6 months, and the neocartilage would have sufficient time to mature without biomechanical overload [46].

In addition to porous scaffolds, the biofunctional constructs have been developed for better chondrogenesis of MSCs. In the application of cartiliage formation in the research field, for instance, fully thermoreversible gelling polymers have attracted considerable attention for use as scaffold materials to hold cells *in situ* [47]. Na et al. developed poly(*N*-isopropylacrylamide) (PNIPAAm)-based hydrogel scaffolds to investigate their capability to deliver a mixture of MSCs and growth factors for the better induction of chondrogenic differentiation [48, 49]. These thermoreversible hydrogel scaffolds can revert from solid to liquid state and from liquid to solid state without abrogating their intrinsic properties. These scaffolds were completely soluble in aqueous solutions at temperatures below their lower critical solution temperature (LCST), but they solidify at temperatures above their LCST, forming a hydrated gel [49, 50]. However, its clinical application is limited due to its nonbiodegradability. Therefore, the design of biodegradable scaffolds, which have biofunctions as well as mechanical strength for effective chondrogenesis of MSCs, is required to overcome the issues. The ideal scaffold has sufficient strength to protect cells from compression and shearing forces, while still having injury site anchoring potential and porosity to allow nutrient and differentiation factors to diffuse through it. The scaffold must also degrade at a rate that optimizes cellular growth and tissue regeneration. Such ideal scaffolds have not yet been designed. The optimal time point for evaluation of a scaffold-based treatment is also critical, which depends on the scaffold, cells, and tissue in question [51].

Biodegradable micro- and nanocomposite materials that can provide the appropriate strength, integrate the desirable biological cues, and provide for the controlled sequential delivery of multiple growth factors would help fulfill the

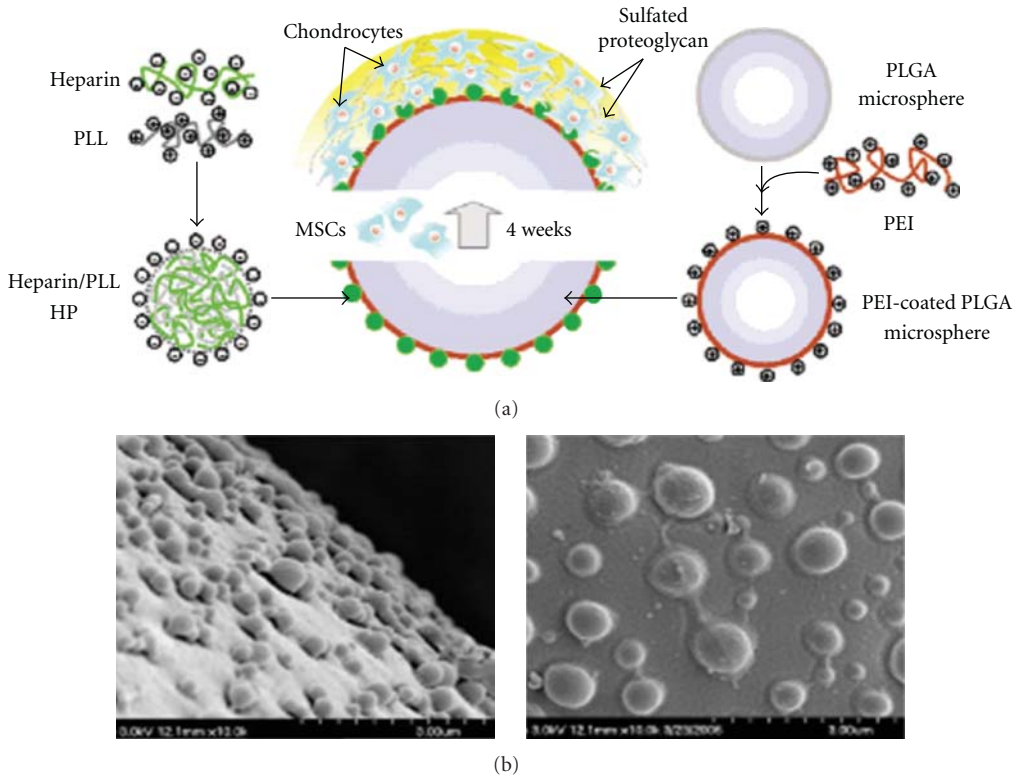

Figure 2: (a) Diagram of heparinized nanoparticles coated onto PLGA microsphere for stem cell delivery. (b) The SEM images of PLGA microspheres (upper (left) and bottom (right)) fabricated with heparin/poly(l-lysine) nanoparticles [37].

promise of regenerative medicine. Also the development of relevant scaffold design using suitable biomaterials and incorporation of appropriate biomolecules and the selection of cell types plays a vital role in tissue repair. Recent conceptual advances, which have taken advantage of new and practical techniques for size distribution and stabilisation control, have created novel routes for the synthesis of nanoparticle-based materials, in which nanoparticle building blocks can be spatially ordered in a controlled manner [37]. Polyionic complexed nanoparticles are composed of heparin and poly(l-lysine) as a stem cell-delivery system, as depicted in Figure 2(a). Heparin-functionalised hydrogel supported MSC viability and induced chondrogenic differentiation. Negatively charged heparin is widely used in the biomaterial field because it can interact with a variety of proteins that have heparin-binding domains, including various growth factors that enable the growth factors to cross-link their receptors. Additionally, poly(l-lysine) has been commonly used as a model cationic polymer to fabricate the polyionic complexes with anionic polymers. Heparin/poly(l-lysine) nanoparticles formed a polyelectrostatic layer-by-layer assembly and were sequentially immobilised on PLGA microspheres as microcarriers of MSCs. The heparin/poly(l-lysine) polyelectrolyte complex is highly distributed on the PLGA microspheres. PLGA is known as the scaffold material to easily form nano- or microstructured particles which can entrap cells or load small molecules due to its excellent biocompatibility, degradability, and processibility [52]. The specific binding activity of heparin in the bioconjugate is not reduced in the immobilisation process results, which may be due to the presence of heparin within the outer shell of the nanoparticles on the surfaces of the PLGA microspheres (Figure 2(b)). As shown in the SEM images, the heparin/poly(l-lysine) electrolyte complex is heavily distributed on the PLGA microspheres.

In another example using the nanoconstructs composed of fibrin hydrogels containing MSCs mixed with heparinised BMP-2, the bioactivities of entrapped MSCs mixed with growth factors were maintained for long term [30]. Moreover, PLGA microspheres can be used as cell delivery vehicles for controlled release of cells mixed with small molecules, which can help MSCs enhance their bioactivities. Park et al. reported the dual delivery of TGF-β3 and dexamethasone from transplanted PLGA constructs *in vivo* to engineer inflammation-free and cartilage-associated tissue [38].

Although PLGA has proved to be an excellent material for cartilage tissue engineering due to its biodegradable properties, mechanical strength, and ease of fabrication into a considerably complex formation, the principle usage of PLGA as a delivery vehicle has problems as it does not offer a desirable environment for cell adhesion due to its limitation of binding sites mediated by biological recognition and high hydrophobicity. Based upon an early fundamental step in which positive cell-substrate interactions enable cell proliferation, migration, and differentiation on the surface of materials, many studies have focused on modifying the matrix surface in an effort to increase cell-substrate interaction for cell delivery [53–55]. Binding

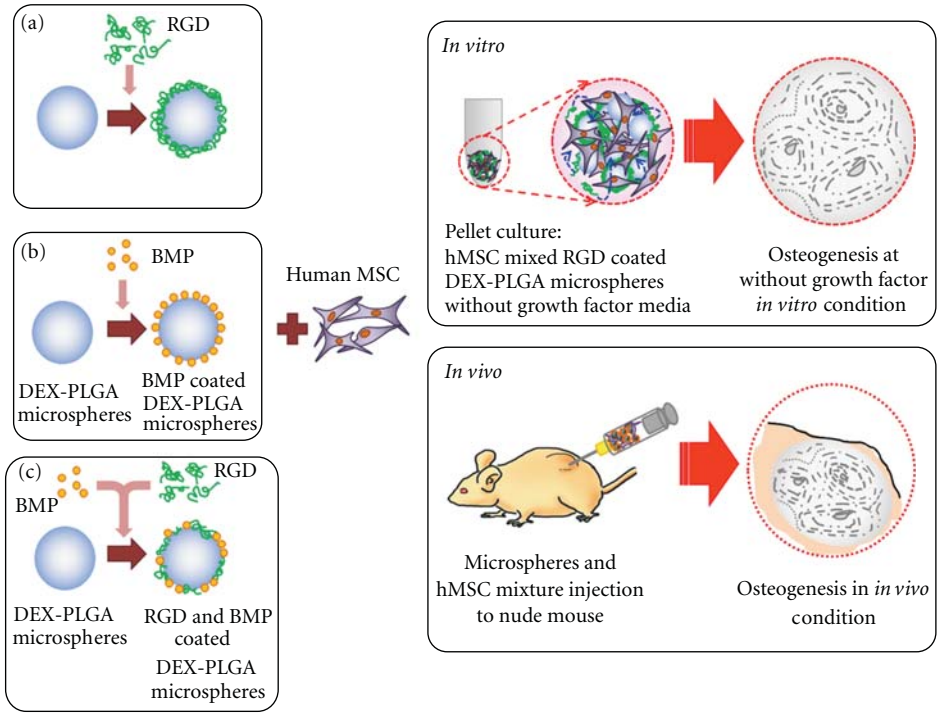

Figure 3: Schematic diagram of RGD-modified PLGA microspheres containing growth factors and dexamethasone. Using a layer-by-layer (LBL) technique, positively charged, PEI precoated PLGA microspheres coated with negatively charged RGD molecules were fabricated for hMSC delivery and regeneration of injured tissues. The combination of growth factors, DEX, and RGD was an effective scaffold for cell delivery and differentiation of embedded hMSCs [39].

sites in fibronectin, osteopontin, collagens, fibrinogen, and thrombospondin that contain the tripeptide Arg-Gly-Asp (RGD) are easily recognised by mammalian cells. The RGD sequences of the adhesive proteins are recognised by a structurally related receptor family, that is, integrins, which bind to RGD on the surface of cells, allowing cells to adhere [42, 56, 57]. The incorporation of bioactive motifs such as RGD may be the best adapted strategy to enhance cell adhesion [58–60]. As 3D scaffolds have a larger surface area and highly interconnected porous structures with suitable porosity and pore size, modification of the scaffold surface to improve the interaction between cells and the surface would have a greater potential for tissue engineering [39, 61–65]. A promising strategy is to immobilise RGD peptides on scaffold surfaces by evaluating embedded MSC behaviours, including attachment, cellular distribution, signal transduction, and survival on the modified surface. For instance, PLGA microscaffolds conjugated with RGD peptides were constructed as an MSC-delivery vehicle (Figure 3). The regulation of stem cell differentiation by adhesion molecules and growth factors has the potential to enable the formation of therapeutic vehicles for the delivery of MSCs that are easily fabricated, less expensive, and more easily controlled than currently available delivery systems. The embedded MSCs easily adhered onto PLGA microspheres mediated by the RGD peptide, proliferated well onto the scaffolds and differentiated to perform distinct functions [66].

Culture methods are deeply considerable to improve the chondrogenetic potential of MSCs, because MSCs markedly decrease with the increase of passage number. If cultured in a medium that is not supplemented with factors facilitating the maintenance of plural differentiation potential, MSCs can hardly differentiate into chondrocytes after repeated passages. Some strong inductive signals for chondrogenesis are required to differentiate the passage-cultured MSCs into chondrocytes [67]. Coculture of MSCs with mature chondrocytes is a strategy that both provides inductive signals and solves the cell source problem. Coculture techniques of MSCs and autologous chondrocytes are frequently used to improve induction of the chondrogenic differentiation of MSCs instead of including growth factors in the MSC culture [68, 69], because the chondrogenic differentiation of MSCs induced by growth factors exhibits some defects, including an instability of the chondrocyte phenotype and a lack of ECM secretion [70]. In the 3D hydrogel constructs, Coculture with autologous chondrocytes and MSCs can show a significantly higher number of specific lacunae phenotypes [68, 71]. Chondrocytes express soluble growth factors that can help MSCs selectively promote chondrogenesis, and this selective effect is not mimicked by an exogenously added growth factors.

In addition, transfection of MSCs with growth factor genes has been proposed and practiced. However, the outcomes are not completely desirable not only due to the damage caused by the invasive procedure of transfection but also because long-term overexpression of a growth factor may result in undesirable changes in the transfected cells. To overcome these challenges, effective chondrogenesis in

MSCs can be achieved by coculturing them with autologous chondrocytes transfected with growth factor genes [72]. This gene transfected Coculture system can avoid directly transfecting MSCs, but instead transfers growth factor genes to their Cocultured chondrocytes.

4. Final Remarks and Further Researches

The clinical need for cartilage repair technologies is unmistakable. Many people over the age of 40 suffer from degeneration or injury of their cartilage, leading to a reduced workforce and increased medical expenses. Thus, improvements in cartilage repair using a cell-based tissue engineering approach will greatly benefit public health and the economy. Personalised cell therapy for cartilage repair using cell-based tissue engineering technologies would provide clinically practical methods for producing a cartilage tissue equivalent. A number of biomaterials are available as scaffolds, and research continues to help us understand more details about how tissues develop and which cell type should be applied. These studies have provided details of how tissues grow *in vitro* and *in vivo*, but clinical applications depend on working with surgeons and the translation of these materials and technologies to *in vivo* models that are more relevant to patients. When cell-based cartilage tissue engineering technologies are applied to new animal models, we attempted to find better functional compositions for successful applications than were observed in previous studies. Although stem cell-based cartilage tissue engineering systems may demonstrate success even in animal models, there are a number of new challenges when the technologies are applied to humans. Further research on *in vivo* application must address immunological issues, integration of host and stem cell-based engineered cartilage, and the variability of tissue development in an *in vivo* environment, depending on surrounding disease processes, age, or physical activity. Therefore, interdisciplinary studies are not only necessary but crucial before cell-based cartilage tissue engineering can reach its full potential in cartilage repair and regeneration.

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Research Article

Feasibility of Treating Irradiated Bone with Intramedullary Delivered Autologous Mesenchymal Stem Cells

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Background. We aimed to explore (i) the short-term retention of intramedullary implanted mesenchymal stem cells BMSCs and (ii) their impact on the bone blood flow and metabolism in a rat model of hindlimb irradiation. *Methods*. Three months after 30 Gy irradiation, fourteen animals were referred into 2 groups: a sham-operated group ($n = 6$) and a treated group ($n = 8$) in which ^{111}In-labelled BMSCs (2×10^6 cells) were injected in irradiated tibias. Bone blood flow and metabolism were assessed by serial ^{99m}Tc-HDP scintigraphy and 1-wk cell retention by recordings of ^{99m}Tc/^{111}In activities. *Results*. The amount of intramedullary implanted BMSCs was of 70% at 2 H, 40% at 48 H, and 38% at 168 H. Bone blood flow and bone metabolism were significantly increased during the first week after cell transplantation, but these effects were found to reduce at 2-mo followup. *Conclusion*. Short-term cell retention produced concomitant enhancement in irradiated bone blood flow and metabolism.

1. Introduction

Radiotherapy has been proven to successfully treat local and regional neoplasic lesions but it may adversely impact on normal tissues [1]. High vulnerability to irradiation was already documented in various bone tissues (pelvis, sternum, vertebra, clavicle, femoral head, and mandible) [2] with subsequent deleterious effect on the bone metabolism and healing leading thereafter to infection, atrophy, pathological fractures, and osteoradionecrosis. For instance, the incidence of osteoradionecrosis after conventional radiotherapy ranges from 0.9% to 35% [3], with an increased risk when doses given to the mandible exceed 60 Gy [4]. Thus, irradiation of the mandible represents the most devastating radiotherapy-induced complication and might sometimes lead to surgical resection [5].

Since vascular ischemia is one of predictors of postir-radiation degeneration, the inception of angiogenesis by implantation of bone marrow mesenchymal stem cells (BMSCs) might represent a therapeutic approach for rehabilitating the irradiated bone tissue. Such potentiality was already documented in diverse ischemic pathologies such

as hindlimb ischemia [6] or myocardial infarction [7, 8]. Previous data regarding the role of BMSCs in the bone reconstruction have outlined their active contribution in the bone formation when seeded on various scaffolds [9, 10]. In a dog model of mandible segmental defect, the feasibility of bone reconstruction using morphologic and 3-D beta-tricalcium phosphate scaffold seeded with autologous BMSCs was highlighted by both bone formation and bone vascularization [10].

Experiments with BMSCs in the treatment or the prevention of radio-induced damage were reported on intestine [11, 12] and skin [13–15] using systemic [14–16] or local [11, 13] delivery. Little is known however about the effect of BMSCs in irradiated bone tissue, and especially, the bioavailability and biodistribution of these cells within the targeted areas since their in vivo monitoring is now mandatory to further understand their benefice.

The study was designed to explore, in a rat model of hindlimb irradiation, the feasibility of rehabilitating irradiated tibial bone tissue by intramedullary implanted BMSCs. The assessment of BMSCs' retention and distribution were conducted up to 7 days following transplantation using ^{111}In-oxine-labeling technique. Therapeutic effect on bone perfusion and metabolism was determined by serial ^{99m}-technetium hydroxymethane diphosphonate (^{99m}Tc-HDP) planar scintigraphy.

2. Materials and Methods

2.1. Animals. This study was conducted in 14 Wistar rats (initial body-weight of 410 g–460 g). All experimental procedures were in accordance with our local ethical committee and with the regulations of the Animal Welfare Act of the National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication no. 85-23, Revised 1996).

Three months after experiencing a hindlimb irradiation with a monodose of 30 Gy a ^{99m}Tc-HDP scintigraphy was performed. Thereafter, animals were referred into 2 groups: a control sham-operated group ($n = 6$) and a treated group ($n = 8$) in which ^{111}In-labelled BMSCs (2×10^6 cells) were intramedullary injected in irradiated tibial diaphysis; BMSCs being harvested before irradiation were cultured until passage 4, and their mesenchymal phenotypes were evidenced by flow cytometry.

To evaluate changes in bone blood flow and metabolism, serial ^{99m}Tc-HDP planar scintigraphy was scheduled at 3 months after irradiation and at 2 months after the cell therapy. The early cell retention after the cell therapy was assessed by additional dual recordings of ^{99m}Tc/^{111}in activities done at 2 hours, 48 hours, and 168 hours after the cell injection.

2.2. Irradiation Procedures. Irradiation of the hindlimb was performed under general anesthesia as previously described [17]. Briefly, the animals were placed in prone position upon a thick polystyrene phantom and their hindlimb was immobilized by adhesive tape. The focus skin distance was 70 cm, and the field size was 20×30 cm. The collimating block was positioned on a 0.5 cm thick acrylic platform to shield the body and only irradiated the exposition of the left hindlimb without the pelvis. Radiation with ^{60}Co was delivered in a vertical beam from a Theratron 780C X-ray machine delivering gamma rays of 1.25 MeV energy and dose rate of 1.4 Gy/min.

2.3. Noninvasive Imaging Procedures

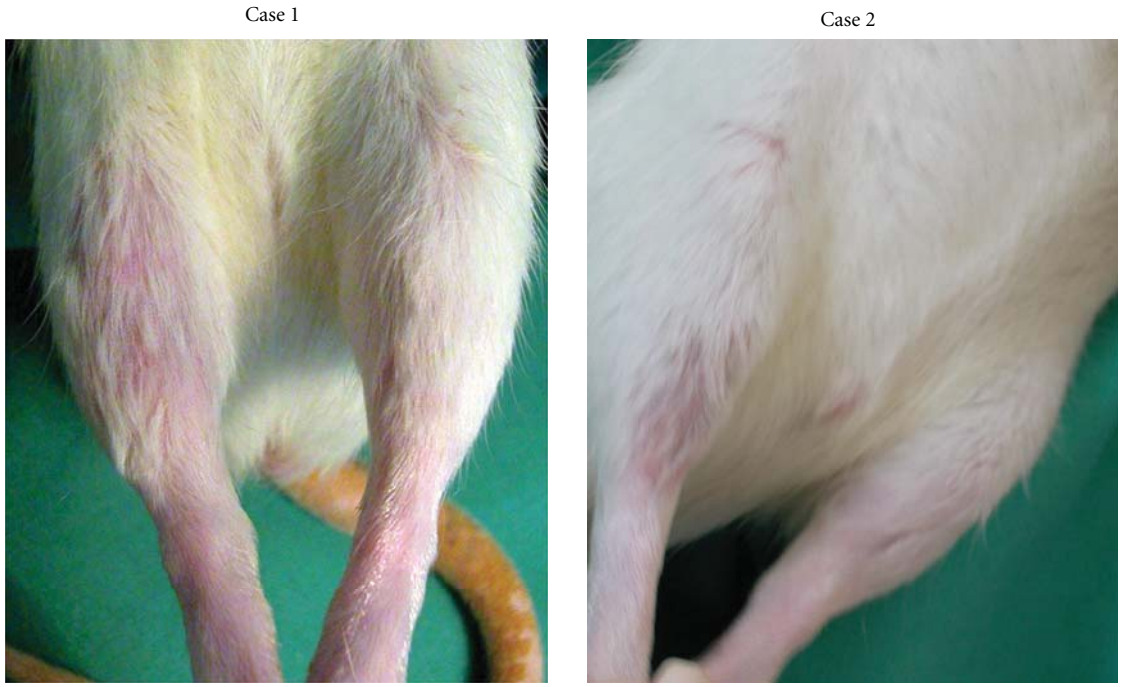
2.3.1. Sequential Planar Scintigraphy. Bone blood flow and metabolism were assessed using ^{99m}Tc-HDP. After the intravenous injection of 9 mCi of ^{99m}Tc-HDP and under general anesthesia, the acquisition was recorded using a single-head gamma camera (Sopha DSX, SMV-GE) equipped with a 1.5 mm pinhole collimator (165 mm focal length, 44 mm radius of rotation) and with the following parameters: 256×256 matrix, 1.14 zoom, and 140 (±20%) keV energy window. Two acquisitions were performed: a dynamic HDP uptake (blood flow) consisted of images obtained at 1 second intervals for 60 seconds reflecting vascularity and a delayed (3 hours after) acquisition of HDP uptake reflecting osteoblastic metabolism [18].

Changes in accumulation of the tracer in irradiated bone and surrounding tissues were evaluated by measuring uptake within regions of interest (ROI) on the computer-processed images software (Dysplay, Console Vision, General Electric). Values were expressed as percentage (%) of total body activity.

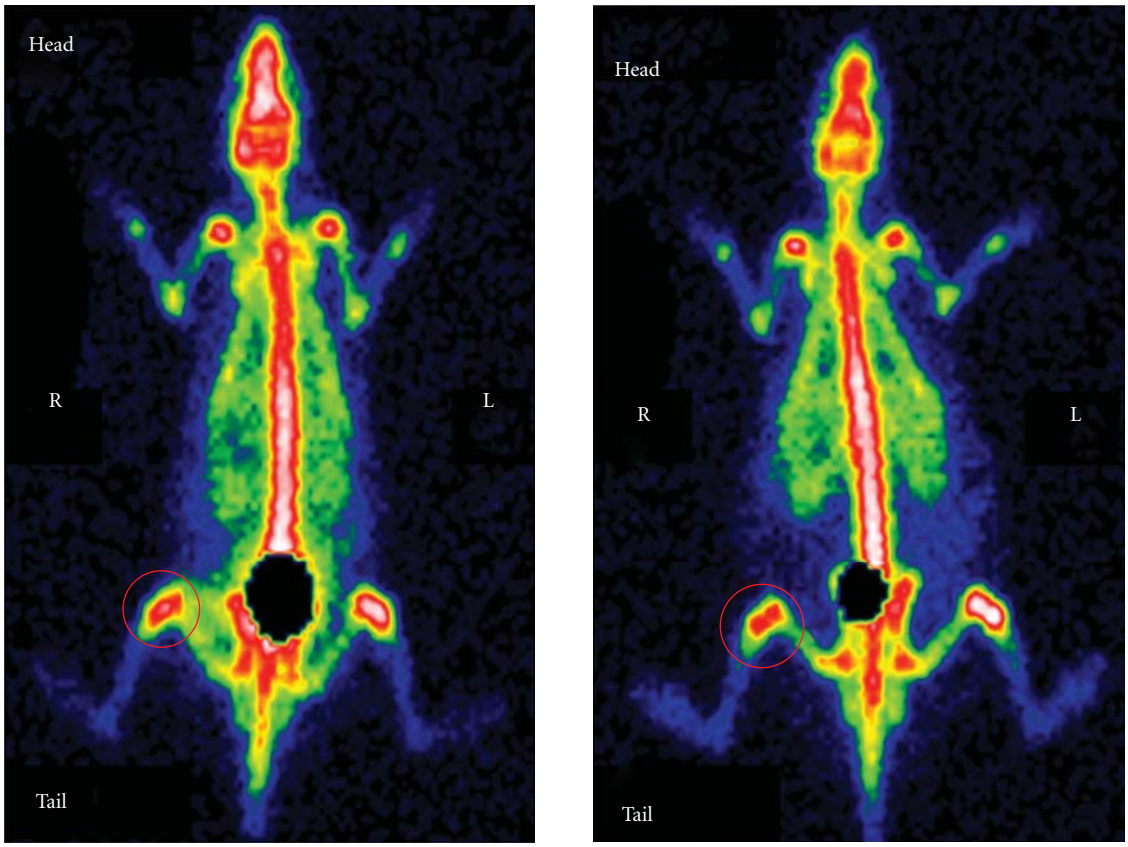
2.3.2. Dual ^{111}In/^{99m}Tc Scintigraphy. Planar scintigraphic images of the body distribution of ^{111}In-labeled BMSCs were provided by the same single-head gamma camera (Sopha DSX, SMV-GE) already described [8, 19]. Two 20% energy windows centered on the 172 KeV and 246 KeV photopeaks of ^{111}In were applied. The initial image was recorded 2 H after cell transplantation during a 15-min period and then at day 2 (48 H) and day 7 (168 H) during time periods of 20 and 40 min, respectively. ^{111}In activity from each image was expressed relative to the total injected activity (total body activity determined at 2 H) and after additional corrections for the physical decay of ^{111}In (2.9 days).

2.3.3. BMSC Isolation, Cell Culture and Flow Cytometry. Autologous bone marrow cells, harvested from the left tibias by punction, were cultured and expanded as previously described in detail elsewhere [19, 20]. Briefly, aspired whole bone marrow cells were suspended in Iscove's modified Dulbecco's culture medium (Life Technologies, Cergy Pontoise, France) containing 10% fetal bovine serum (Life Technologies, Cergy Pontoise, France), 0.1 mmol/L β mercaptoethanol (Sigma, France), 100 U/mL penicillin, and 100 μg/mL streptomycin. The cells were grown in a 5% humidified CO_2 atmosphere at 37°C, and the medium was changed every 2 days.

To ascertain the mesenchymal phenotype of transplanted BMSCs, the expression of CD34, CD44, CD45, and CD90 surface antigens of cells prior to implantation (passage 4) was

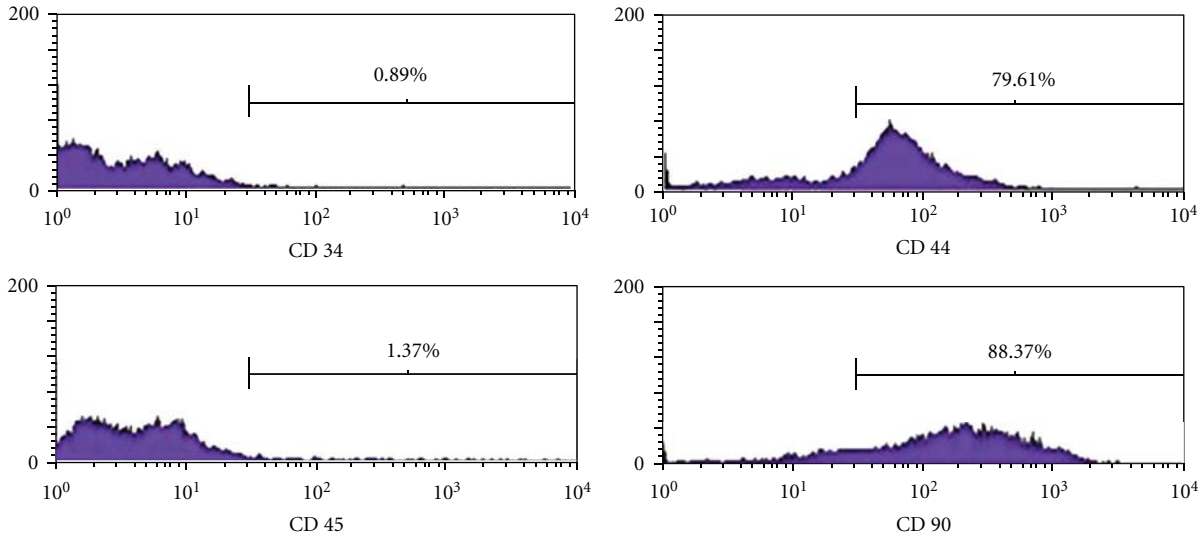


(a) Macroscopic images



(b) Scintigraphic images

Figure 1: Animal model of hindlimb irradiation. (a) Examples of pictures showing alopecia of the hindlimb 3 months after irradiation at a monodose of 30 Gy. (b) Examples of scintigraphic imaging showing the decrease of bone ^{99m}Tc-HDP on the irradiated hindlimb 3 months after irradiation.



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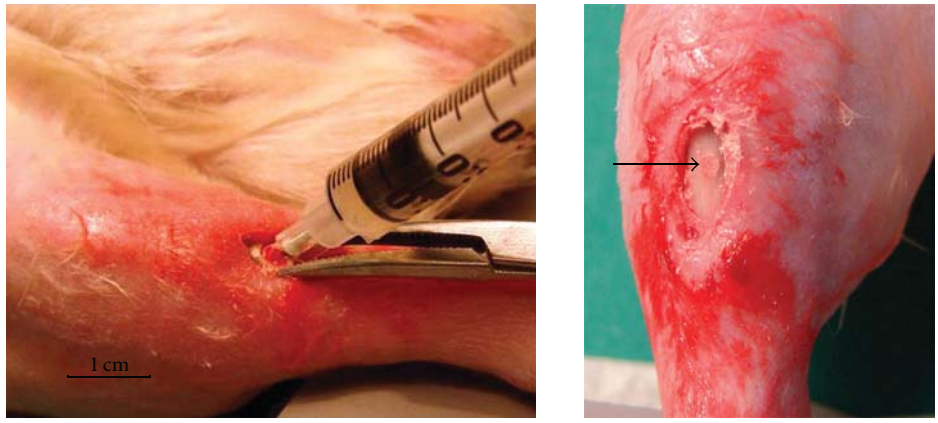

(b)

Figure 2: Mesenchymal quality of the engrafted BMSCs and injection procedure. (a) flow cytometry data depicting several conventional surface antigens of mesenchymal cells (CD34−, CD44+, CD45−, and CD90+) prior to implantation (passage 4). (b) technique of intramedullary injection (left panel) and on the right panel, the arrow indicates the bandage on the surgical site after BMSC engraftment.

Table 1: Pretherapeutic value of ^{99m}Tc-HDP bone uptake of the rat hindlimbs. Results were expressed as percentage of total corporel activity.

		Group 1		Group 2	
		Nonirradiated hindlimb	Irradiated hindlimb	Nonirradiated hindlimb	Irradiated hindlimb
Bone blood flow	Knee	7.9 ± 1.0	8.3 ± 1.2	8.1 ± 1.3	8.3 ± 1.3
	Tibia	3.82 ± 0.6	3.0 ± 0.8*	3.79 ± 1.0	3.2 ± 0.3*
	Foot	2.4 ± 0.7	2.6 ± 0.8	2.7 ± 0.6	2.6 ± 0.7
Bone osteoblastic metabolism	Knee	10.3 ± 3.0	9.9 ± 1.7	11.3 ± 4.9	8.9 ± 1.9
	Tibia	2.2 ± 0.2	2.1 ± 0.1	2.3 ± 0.2	2.0 ± 0.3
	Foot	3.8 ± 0.7	3.3 ± 1.0	3.7 ± 3.0	3.5 ± 1.4

* $P < 0.05$ versus contralateral nonirradiated legs.

analysed using flow cytometry method (FACSCalibur; Becton Dickinson, Meylan, France) and the Cellquest software (Becton Dickinson, Meylan, France) [20].

2.4. Intramedullary Implantation of Radiolabeled BMSCs

2.4.1. Cell Labeling and Cell Transplantation. As already described [7, 8], BMSCs (2×10^6 cells/mL) were trypsinised and incubated at 37°C with 15 MBq of ^{111}In-oxine (Mallinckrodt Medical B.V., Holland) during a 10-min period, the labelling

Table 2: Post-therapeutic value of ^{99m}Tc-HDP bone uptake of the irradiated hindlimbs. Results were expressed as relative to the unirradiated hindlimb.

		Hindlimb	posttherapeutic 48 H	posttherapeutic 168 H	posttherapeutic 2 months
Bone blood flow	Knee	Irradiated untreated	−0.40 ± 1.30	−0.76 ± 1.62	+0.16 ± 0.91
		Irradiated treated	−0.30 ± 0.89	−1.18 ± 0.87	+0.20 ± 0.98
	Tibia	Irradiated untreated	+0.95 ± 1.43	+0.21 ± 1.45	−0.01 ± 0.49
		Irradiated treated	+2.00 ± 0.68*	+0.70 ± 1.03*	+0.40 ± 0.53
	Foot	Irradiated untreated	+1.05 ± 1.12	+0.26 ± 1.57	+0.46 ± 0.31
		Irradiated treated	+1.64 ± 1.25	+1.02 ± 0.96	+0.56 ± 0.90
Bone osteoblastic metabolism	Knee	Irradiated untreated	−2.12 ± 1.25	−1.06 ± 1.06	−1.21 ± 1.13
		Irradiated treated	−1.68 ± 1.43	−1.57 ± 1.44	−1.16 ± 0.98
	Tibia	Irradiated untreated	+0.28 ± 0.85	−0.04 ± 0.67	−0.01 ± 0.66
		Irradiated treated	+0.77 ± 0.56*	+0.47 ± 0.58*	+0.07 ± 0.56
	Foot	Irradiated untreated	+0.36 ± 0.89	+0.01 ± 1.19	+0.07 ± 0.59
		Irradiated treated	+1.07 ± 1.21	+0.58 ± 0.62	+0.43 ± 0.96

* $P < 0.05$ versus contralateral nonirradiated legs.

process being stopped by 5-min centrifugation at 950 g. This 10-min incubation period was previously found to result in both a sufficiently high labeling efficiency (69%) and absence of significant deterioration of cell viability (96%) [8].

After stab incision, a 1 mm diameter drill hole was performed perpendicularly to the orientation of the tibial cortical bone. The ^{111}In-labelled cells were conditioned in a 1 mL syringe (2×10^6 cells in 50 μL) and were injected through the mini-invasive perforation into the bone marrow of the left tibia. To prevent leakage of transplanted cells in the surrounding tissues a biocompatible bandage (IRM Dentsply 78467 Konstanz Germany) was positioned over the drilling site.

2.4.2. Statistics. The statistical analysis was carried out with the Statistical Package SPSS version 14.0 (SPSS, Inc., Chicago, Ill, USA). We used Mann-Whitney tests for the unpaired comparisons and Wilcoxon tests for the paired comparisons in each group. For each test, a P value < 0.05 was considered to be indicative of a significant difference.

3. Results

3.1. Animal Model of Hindlimb Irradiation and Pretherapeutic Data. No animal died throughout the study's period. The 30-Gy irradiation induced 3-4 weeks later a persisting alopecia in the irradiated hindlimb (Figure 1(a)) without affecting however the daily locomotor activities of those animals.

At 2-mo scintigraphic imaging, radiation-induced bone defects appear as areas of attenuation of signal intensity covering the irradiated lower limb, with pronounced effect in the tibia (see Figure 1(b), e.g.,). The pretherapeutic data of the group control and the cell-treated group were resumed in the Table 1. In both groups, compared with the total body activity, irradiation of the hindlimb produced similar alteration in tibial values of bone perfusion blood flow (early uptake of ^{99m}Tc-HDP) and bone osteoblastic metabolism (late uptake of ^{99m}Tc-HDP). For example, bone perfusion blood flow was 3.2 ± 0.8% at the irradiated tibia compared to 3.8 ± 1.0% found in the healthy one ($P < 0.05$). A slight decrease in bone metabolism of circa 10% was found in irradiated tibias, but values did not reach statistical significance (2.0 ± 0.3% versus 2.3 ± 0.6% found in healthy counterparts).

3.2. Cell Identification, Short-Term In vivoTracking, and Post-therapeutic Data

3.2.1. Cell Identification Prior to the Cell Grafting. Flow cyto-metry analyses (Figure 2(a)) showed that the engrafted BMSCs of passage 4 expressed strong expression of CD44 and CD90 surface antigens (>80%). Thus, these cells were negative for CD45 and CD34 (percentage of positive cells were 2.41 ± 2.47% for CD45 and 1.99 ± 2.72% for CD34). These data were consistent with our previous studies [20] and in accordance with criteria defined by the International Society for Cellular Therapy (ISCT) [21].

3.2.2. Effect of BMSCs on Bone Blood Flow and Bone Metabolism in Irradiated Hindlimb. ^{99m}Tc-HDP scintigraphic examinations performed after intramedullary implantation of BMSCs have documented, especially in the tibial area, a significant rise in both bone blood flow and bone metabolism during the posttherapeutic first week (Table 2 and Figure 5). At 48 hours, the bone blood flow found in cell-treated tibias was 4.7 ± 0.7% corresponding to an enhancement of 62% compared to basal pretherapeutic values ($P < 0.01$). Similarly, the bone metabolism was 35% higher than that measured before treatment, values were 2.7 ± 0.5% ($P < 0.01$ versus pretherapeutic data). These effects persisted at 7 days, bone blood flow was 4.5 ± 1.0% ($P < 0.01$ versus pretherapeutic data), and bone metabolism was 2.6 ± 0.6% ($P < 0.05$ versus pretherapeutic data). At 2-mo followup, these uptake values were found to be down to 3.1 ± 1.4% for the bone blood flow and 1.7 ± 0.3% for the bone metabolism.

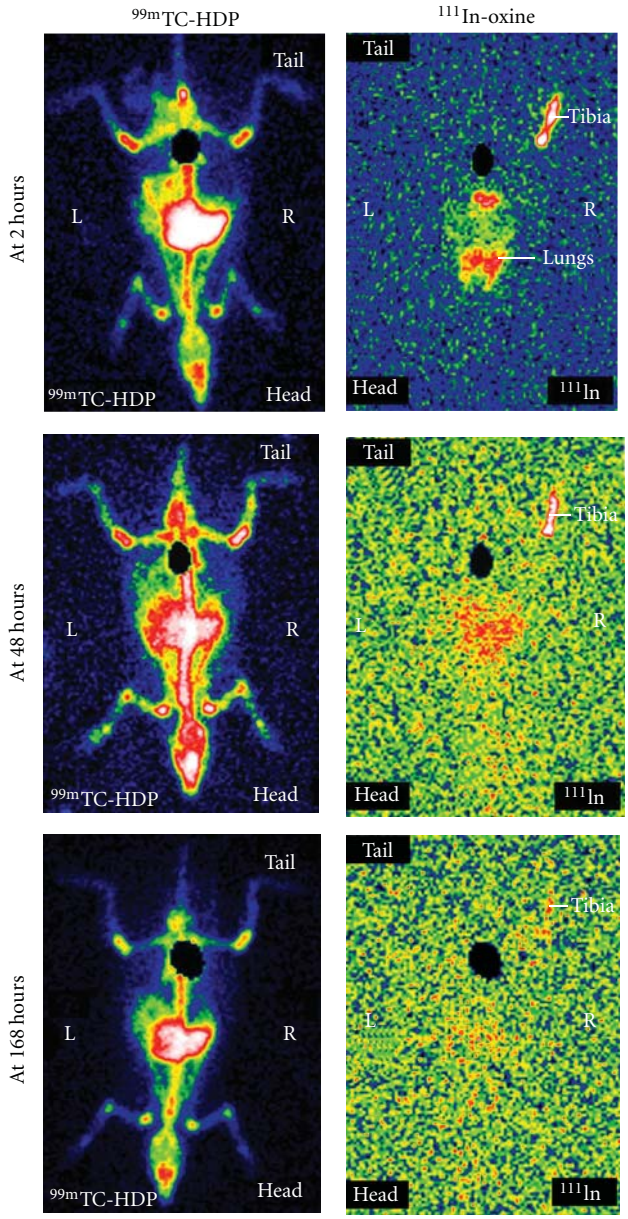

Figure 3: Example of scintigrams from ^{111}In/^{99m}Tc dual-SPECT showing the short-term retention of ^{111}In-oxine-labeled BMSCs following intramedullary injection.

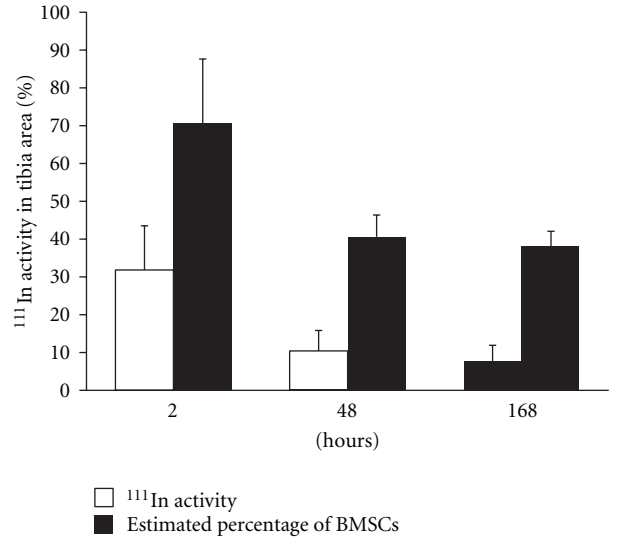

Figure 4: *In vivo* evolution of ^{111}In activity found in the tibia during the 7-day followup and estimation of the percentage of BMSCs retained within the injection site. Calculation of BMSCs retention in the tibia was defined as the ration of the mean radioactivity in the tibia to the mean radioactivity that remained in BMSCs at each time point.

4. Discussion

Damage of normal tissue secondary to radiotherapy is still a major problem in cancer treatment. Stem cell therapy seems to be a new treatment option in radio-induced tissue abnormalities [22–24], providing a mean to reduce related side effects and to improve the quality of life of patients. In this study, we investigated the feasibility of BMSCs when injected intramedullary in an experimental rat model of radio-induced degeneration recently described by our group [17].

In the present study, ^{111}In-oxine labelling of BMSCs was successfully used to follow bone retention and body distribution of BMSCS when injected intramedullary within irradiated bone. ^{111}In-labelled cells have been widely used in humans in localizing areas of inflammation by imaging the leukocyte distribution [25]. Furthermore, ^{111}In-labelling techniques have been applied in various experimental settings in animal to analyse the migration of dendritic cells [26], the biodistribution of transplanted hepatocytes [27], and of injected MSCs in animals model of heart or lung disease [7, 28]. As previously described [7], the technique used here reached a high efficiency (69%) with a low toxicity (viability > 95%). In addition, it has been previously demonstrated that the leakage of ^{111}In from labelled cells resulted in a high ^{111}In uptake in the liver and spleen and usually had hepatobiliary and renal excretion pathways [7, 29]. This is in accordance with our observations, and no ^{111}In radioactivity was found in bones outside the area of injection. Approximately 70% of grafted cells could be estimated to be retained within bone damaged area 2 hours after transplantation. The "disappearance" of radiolabeled grafted cell may be explained by the method used for BMSCs injection which could be associated with a leakage of BMSCs from the injection site before bandage and residual BMSCs in the injection syringes. These data are fully consistent with those of the study of Tran et al. [7], where approximately 60% of ^{111}In labeled BMSCs were still present 2 hours after direct transplantation in a necrotic rat myocardium and were retained within the heart throughout the 7 days of follow-up. In the present study, after 48 hours, BMSCs number decreased to approximately 40% and remained un-changed until the 7th day. The mechanism responsible for cell loss during the first two days remains to be explored. These results highlighted that at short term, the engrafted BMSCs

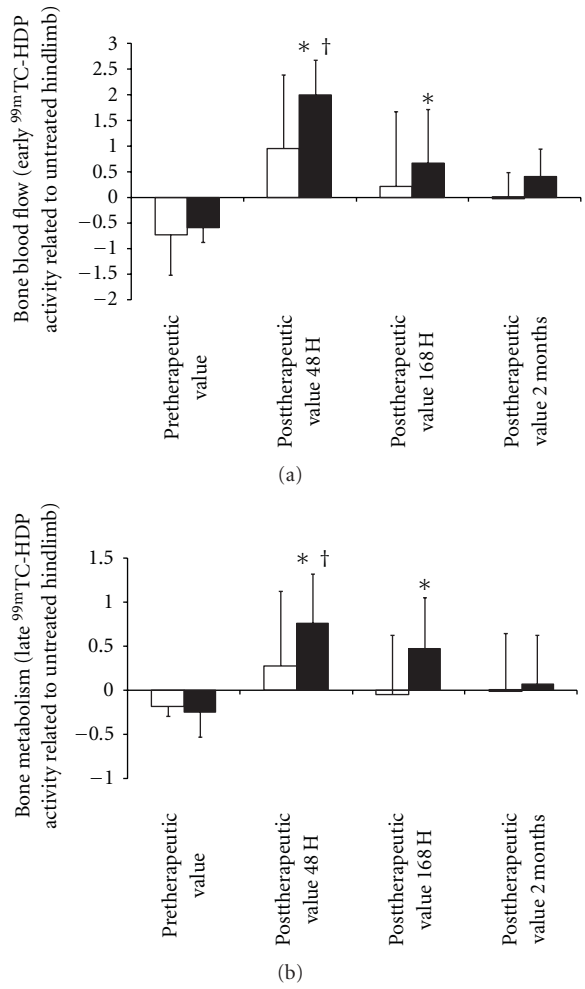

Figure 5: Two-month evolution of bone flood flow (a) and bone osteoblastic metabolism (b) in irradiated tibias treated with BMSCs (dark columns) and control (white columns), values being expressed as relative to baseline (% of uptake variations with regard to untreated hindlimb values). $^{*}P < 0.05$ versus pretherapeutic data, $^{\dagger}P < 0.05$ versus control group.

remain localized within the area of injection into irradiated bone.

Many studies of cell therapy using mesenchymal stem cells [14, 16], used the systemic injection as modality of administration. In comparison, using local injection, cells engraftment seems to be better. For example, in François et al.'s study [14], rats were transplanted with a dose of 5×10^6 BMSCs 24 hours after irradiation of the hindlimb at a single dose of 26.5 Gy. Fifteen days later, the implantation rates of BMSCs in bone and bone marrow were, respectively, approximately 12.5% and less than 0.25%. The major limitation of this approach is constituted by the very low number of BMSCs that home to the site of injury [30]. A possible reason for the inefficient engraftment and homing could be the entrapment of BMSCs in the lungs [31]. Moreover, vascular ischemia and fibrosis, characteristic injury of irradiated tissue [1, 32], may prevent homing and engraftment of BMSCs.

After cell therapy, the bone blood flow and bone metabolism evolved similarly, and a significant increase of these values was observed during the seven days following the BMSCs engraftment. The influence of the surgical procedure used in the present study would require further investigation, especially regarding the role of the inflammation cells response and the local recruitment of mesenchymal stem cells [33] that should have been induced by the wound healing after drilling the cortical bone. However, the benefit obtained seems to be transient since 2 months after cell therapy, blood flow time and bone uptake of ^{99m}Tc-HDP did not differ significantly from data measured in ungrafted animals irradiated at 30 Gy. This result slightly differs from those achieved in our previous study [34], in which autologous fat was used as source of mesenchymal stem cell and grafted within irradiated area, inducing clinical benefit that appeared to be linked to the improvement of vascular network and disappearance of necrotic area. Additional results demonstrating the potency of BMSCs therapy in irradiated tissues were recently reported [35] describing a case of regenerative reconstruction of a terminal stage of osteoradionecrosis with BMSCs and progenitor cells. Another explanation that stands for the short effect of engrafted BMSCs might be related to the native hypoxic microenvironment of the medullar area target of the bone. The BMSCs used here were expanded according to most of the conventional cell culture procedures, that is, in normoxic condition (21% O_2). Although they have mesenchymal characteristics, recent works from our group [36] and others [37] have suggested that BMSC, when cultured under 5% O_2 rather than under 21% O_2, had better growth advantage in long-term cell expansion. Thus, the hypoxic BMSC expressed more adhesion and extracellular matrix molecules and displayed more plasticity features. It is then possible that different *in vitro* conditions during the cell selection and expansion might lower their ability to repair when reimplanted in native environment. This hypothesis needs further experimental evidences.

5. Conclusion

In conclusion, the present study shows the feasibility of the intramedullary implantation of BMSCs in the attempt to rehabilitate the irradiated bone. Our data suggested that BMSCs appear to remain around the injection site, without evident body redistribution, for at least a 7-day period along with a transient benefice on bone blood flow and bone metabolism. Further experiments are required to evaluate their long-term beneficial effect.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Research Article

Investigation of Hepatoprotective Activity of Induced Pluripotent Stem Cells in the Mouse Model of Liver Injury

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To date liver transplantation is the only effective treatment for end-stage liver diseases. Considering the potential of pluripotency and differentiation into tridermal lineages, induced pluripotent stem cells (iPSCs) may serve as an alternative of cell-based therapy. Herein, we investigated the effect of iPSC transplantation on thioacetamide- (TAA-) induced acute/fulminant hepatic failure (AHF) in mice. Firstly, we demonstrated that iPSCs had the capacity to differentiate into hepatocyte-like cells (iPSC-Heps) that expressed various hepatic markers, including albumin, α-fetoprotein, and hepatocyte nuclear factor-3β, and exhibited biological functions. Intravenous transplantation of iPSCs effectively reduced the hepatic necrotic area, improved liver functions and motor activity, and rescued TAA-treated mice from lethal AHF. 1, 1′-dioctadecyl-3,3,3′, 3′-tetramethylindocarbocyanine perchlorate cell labeling revealed that iPSCs potentially mobilized to the damaged liver area. Taken together, iPSCs can effectively rescue experimental AHF and represent a potentially favorable cell source of cell-based therapy.

1. Introduction

Acute or fulminant hepatic failure (AHF) is a severe liver injury accompanied by hepatic encephalopathy which causes multiorgan failure with a high mortality rate. The use of chemical reagents, such as thioacetamide (TAA) [1–4], acetaminophen [5], or galactosamine [6, 7], may reproduce a number of important clinical characteristics of AHF, such as hypoglycemia, encephalopathy, and increased blood levels of hepatic enzymes. Consequently, the experimental models of AHF induced by these chemicals were widely used for the investigation of the pathophyisology and therapeutic strategies of AHF.

Liver transplantation has been shown to be an effective treatment for this liver failure. However, the drawback of the procedure is the shortage of donor organs combined with needing the immunosuppressant treatment [8]. Therefore, transplantation of hepatocytes or hepatocyte-like cells may provide great promise because cellular therapy is the relative simple and less invasive procedure. Recently, the use of embryonic stem cells (ESCs) has attracted attention for cellular therapy because of their capability to proliferation

indefinitely and their potential to differentiate into all types of cells including hepatocytes [9–12]. Heo et al.'s study demonstrated that ESCs gave rise to functional hepatocytes that effectively integrated into and replaced injured parenchyma without formation of teratoma in the mouse model of liver injury [13]. There is evidence suggested that bone marrow (BM) is another source of hepatic progenitors [14, 15]. However, BM cells also contribute functionally and significantly to liver fibrosis [16]. We should be vigilant for the possibility of organ fibrosis induced by BM cell-based therapy.

Induced pluripotent stem cells (iPSCs) are novel stem cell populations induced from mouse and human adult somatic cells through reprogramming by transduction of defined transcription factors [17, 18]. Recent studies further suggested that iPSCs were indistinguishable from ESCs in morphology, proliferative abilities, surface antigens, gene expressions, epigenetic status of pluripotent cell-specific genes, and telomerase activity [19]. Previous studies have shown that both human and mouse iPSCs can efficiently differentiate into functional hepatocytes in vitro [20, 21], which may be helpful in studying liver development and regenerative medicine. In the present study, we first differentiated iPSCs into iPSC-hepatocyte-like cells (iPCS-Heps) using a stepwise protocol and monitored the expression of hepatic markers on iPCS-Heps. Next, we transplanted iPSCs into TAA-treated mice and found that iPSCs were incorporated into livers and significantly improved the hepatic functions, motor activity, and mortality rate of mice. This cellular therapy opened an era for cell-based transplantation by overcoming the ethical controversy over ESCs.

2. Materials and Methods

2.1. In Vitro Hepatic Differentiation. The first clone of murine iPSCs Re7 were generated from mouse embryonic fibroblasts (MEF) derived from 13.5-day-old embryos of C57/B6 mice from our lab, and the secondary clone of murine iPSCs were kindly provided by Dr. Shinya Yamanaka. The iPSCs were cultured as previously described [17, 18]. iPSCs were seeded at 2×10^4 cells/cm^2 maintained in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) containing 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin in gelatin-coated plates, prior to induction by a 2-step procedure. iPSCs were differentiated by using step-1 differentiating medium, consisting of DMED supplemented with 20 ng/mL HGF (Peprotech), 10 ng/mL bFGF (Peprotech), and 0.61 g/L nicotinamide. After 7 days, step-1 differentiating medium was changed to step-2 maturation medium containing DMEM supplemented with 0.1 μM Nicotinamide, dexamethasone (Dex; Sigma), and 1% insulin-transferrin-selenium (ITS; Sigma). Medium changes were performed twice weekly. iPSCs treated with medium supplemented with no growth factors were used as the negative control. For embryoid body (EB) formation, iPSCs were dissociated into a single cell suspension by 0.25% trypsin-EDTA and plated onto nonadherent culture dishes in DMEM with 15% FBS, 100 mM MEM nonessential amino acids, 0.55 mM 2-mercaptoethanol, and antibiotics at a density of 2×10^6 cells/100 mm plate. After 4 days in floating culture, EBs were transferred onto gelatin-coated plates and maintained in the same medium for 24 hours. EBs were then assigned for in vitro hepatocyte differentiation by using a two-step procedure as previously described, with some modifications [22].

2.2. RT-PCR. Total RNA was isolated from iPSCs and differentiating iPSCs using TRIzol Reagent (Invitrogen). The messenger RNA of hepatic specific markers including hepatocyte nuclear factor-3β (HNF-3β), α-fetoprotein (AFP), albumin (ALB), Transthyretin (TTR), α-antitrypsin (AAT), and tyrosine-aminotransferase (TAT) was reverse transcribed to complementary DNA (cDNA) using a reverse transcription system (Promege). cDNA was amplified using Taq polymerase (Takara, Japan) at 94°C for 5 minutes, 35 cycles of 94°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and extension at 72°C for 10 minutes. The primers used are shown in Table 1. The relative expression of each gene was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

2.3. Periodic Acid-Schiff (PAS) Stain for Glycogen. Cells were fixed in 4% paraformaldehyde and then permeabilized with 0.1% Triton X-100 for 10 minutes. Samples were then oxidized in 1% periodic acid for 5 minutes, rinsed 3 times in deionized (d)H_2O, treated with Schiff's reagent for 15 minutes, and rinsed in dH_2O for 5 to 10 minutes. Samples were counterstained with Mayer's hematoxylin for 1 minute and then rinsed in (d)H_2O. With 12 repetitive experiments, the samples were observed under light microscope.

2.4. Cellular Uptake Assay of Low-Density Lipoprotein (LDL). The uptake capability of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate conjugated to acetylated-LDL (DiI-Ac-LDL; AbD Serotec) of iPSCs and differentiated cells was determined by fluorescent microscopy in 12 repetitive experiments. Cells were incubated with 20 μg/mL DiI-AC-LDL at 37°C for 24 hours. Incorporation of DiI-Ac-LDL into cells was visualized by fluorescence microscopy.

2.5. Immunostaining Analysis. Cells were fixed in 4% paraformaldehyde and then permeabilized in PBS containing 1% bovine serum albumin and 0.3% Triton X-100 before incubating with primary antibodies in PBS/BSA at 4°C overnight. After washing with PBS, cells were incubated with rhodamine-conjugated secondary antibodies of anti-HNF-3β, anti-AFP, and antialbumin at room temperature for 30 minutes. After washing with PBS, the samples were mounting in Prolong Gold with 4,6-diamidino-2-phenylindole (DAPI; Molecular Probes). Fluorescence-labeled cells were observed under a fluorescent microscope.

2.6. Cell-Labeling Protocol. In this study, we transplanted DiI-labeled iPSC to trace the distribution of iPSC. In brief,

Table 1: The sequences for the primers of RT-PCR.

Gene name	Primer sequence	Product length
HNF-3β	Forward: CAGCTACACACACGCCAAAC Backward: GGCACCTTGAGAAAGCAGTC	204 bp
AFP	Forward: TCGTATTCCAACAGGAGG Backward: AGGCTTTTGCTTCACCAG	173 bp
ALB	Forward: GCTACGGCACAGTGCTTG Backward: CAGGATTGCAGACAGATAGTC	260 bp
TTR	Forward: CTCACCACAGATGAGAAG Backward: GGCTGAGTCTCTCAATTC	225 bp
AAT	Forward: AATGGAAGAAGCCATTCGAT Backward: AAGACTGTAGCTGCTGCAGC	484 bp
TAT	Forward: ACCTTCAATCCCATCCGA Backward: TCCCGACTGGATAGGTAG	206 bp
GAPDH	Forward: CTCATGACCACAGTCCATGC Backward: TTCAGCTCTGGGATGACCTT	155 bp

Abbreviations: hepatocyte nuclear factor-3β: HNF-3β; α-fetoprotein: AFP; albumin: ALB; Transthyretin: TTR; α-antitrypsin: AAT; tyrosine-aminotransferase: TAT; glyceraldehyde-3-phosphate dehydrogenase: GAPDH.

1×10^6 mouse iPSCs were suspended in phosphate-buffered saline in the presence of DiI at a final concentration of 1 μg/mL and incubated for 10 min at 37°C followed by 5 min at 4°C and finally washed thrice with PBS.

2.7. Animal Model of Liver Injury. Male BALB/c mice, 7-8 weeks old with weighing 25–30 g, were used for our experiments. Fulminant hepatic failure was induced by intraperitoneal injection of thioacetamide (TAA) (150 mg/kg, Sigma) [23, 24]. Mice were randomly divided into 2 groups: group 1 (phosphate buffer saline (PBS); $n = 36$) and group 2 (2×10^6 iPSCs; $n = 23$) via tail vein injection. To avoid hypoglycemia and electrolyte imbalance [25], subcutaneous injections of a solution containing 10% glucose water mixed with lactate ringer (25 mL/kg) were performed every 12 hours after the injection of TAA. Motor activity measurements and blood sampling were performed 24 hours after the administration of TAA to observe the immediate hepatic damage. In order to observe the prolonged hepatic damage, measurement of motor activity and survival rate and collection of blood samples were performed 72 hours after the administration of TAA in iPSCs- and PBS-treated groups. All mice were caged at 24°C with a 12-h light-dark cycle and allowed free access to water and food. This study was approved by Taipei Veterans General Hospital Animal Committee, and the principles of Laboratory Animal Care were followed.

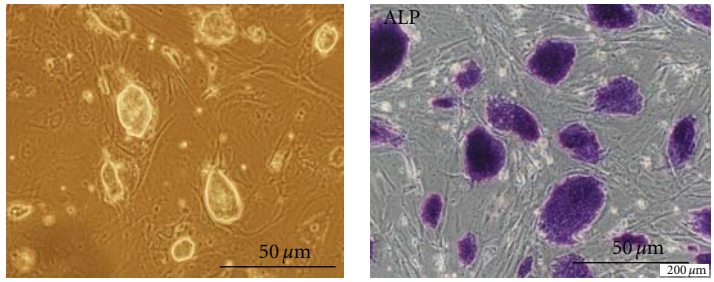
2.8. Measurement of Motor Activity. Motor activities in an open field were determined by using the Opto-Varimex animal activity meter (Columbus Instruments Inc.) [26]. The Opto-Varimex activity sensors utilize high-intensity, modulated infrared light beams to detect animal motion. Animals were housed in transparent cages (17 inches × 17 inches × 8 inches) through which 30 infrared beams pass in the horizontal plane, 15 on each axis. This device differentiates nonambulatory movements (scratching, gnawing) from ambulation on the basis of consecutive interruption of the infrared monitoring beams. An additional row of infrared beams in the horizontal plane (15 on each axis) about 10 cm above the floor was used to count the vertical movements. During the activity measurements, animals have no access to food or chow. All studies were performed under strictly standardized conditions in the dark room for 30 minutes. The counting numbers of the total movements, ambulatory movements, and vertical movements were separately recorded to reflect the motor activities of rats with fulminant hepatic failure. The motor activities were defined as zero in dead mice.

2.9. Liver Functional Tests. Biochemical parameters were measured using standard clinical methods. After anesthesia by ketamine (10 mg/100 g), intracardiac aspiration of blood was performed. A 0.8-0.9 mL of blood sample was collected from the heart into a pyrogen-free syringe containing ~75 units of heparin sodium, then placed in an ice bath and transported immediately to the laboratory. Serum biochemistry tests, including alanine aminotransferase (ALT), aspartate aminotransferase (AST), and total bilirubin, were measured by Vitro DT chemistry system (Johnson & Johnson).

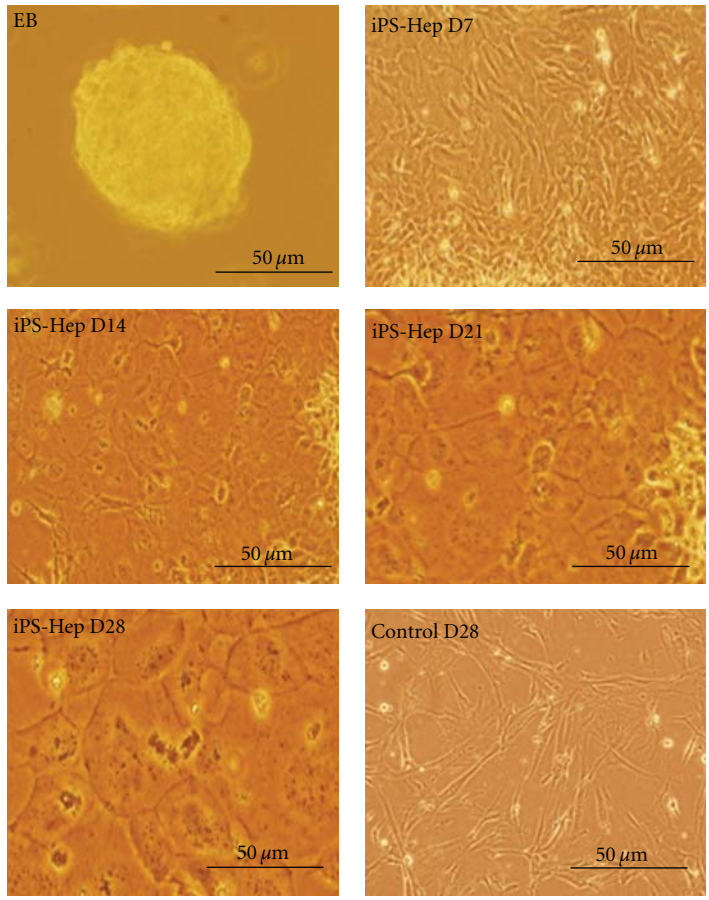
2.10. Statistical Analysis. Results were expressed as mean ± S.D. Statistical analyses were performed by using unpaired Student's t-tests, and the survival rate analyses using log-rank test. Results were considered statistically significant at $P < 0.05$.

3. Results

3.1. In Vitro Differentiation of iPSCs into iPSC-Heps. We introduced retroviruses containing mouse Oct3/4, Sox2, Klf4, and c-Myc into MEF. These iPS clones were positive for



(a)



(b)

Figure 1: In vitro differentiation of iPSCs into iPSC-Heps. iPSCs were seeded at 2×10^4 cells/cm^2, maintained in Dulbecco's modified Eagle's medium. (a) Left: Morphology of iPSC colonies. Right: iPSC colonies were positive for alkaline phosphate stain (purple). (b) The hepatogenic differentiation was induced by a 2-step procedure as described in Section 2. Morphology of undifferentiated and differentiated iPSCs was evaluated at different days after hepatogenic differentiation.

alkaline phosphatase (Figure 1(a)). Under hepatogenic condition, iPSC-derived embryoid bodies (EBs) (Figure 1(b), upper left) were shifted to hepatic differentiation media, and they gradually exhibited broadened and cuboidal morphology with increasing time of induction in vitro and eventually differentiated into iPSC-Heps at D7, D14, D21, and D28 (Figure 1(b)). Immunofluorescence staining with anti-ALB antibody and anti-AFP antibody was observed using the confocal microscope in iPSC-Heps, and showed increased expression of ALB and AFP in iPSC-Heps in the 28th day differentiation (Figure 2). In order to confirm the hepatic characteristics of iPSC-Heps RT-PCR and q-RT-PCR analyses were performed to examine expression of hepatic-specific markers including HNF-3, AFP, ALB, TTR, AAT, and TAT (mean ± SD, $n = 3$). Obviously, after week 2 post differentiation, the expression of hepatic-specific genes was further upregulated in iPSC-Heps compared to un-differentiated iPSCs (Figure 3(a)). Besides, expression of

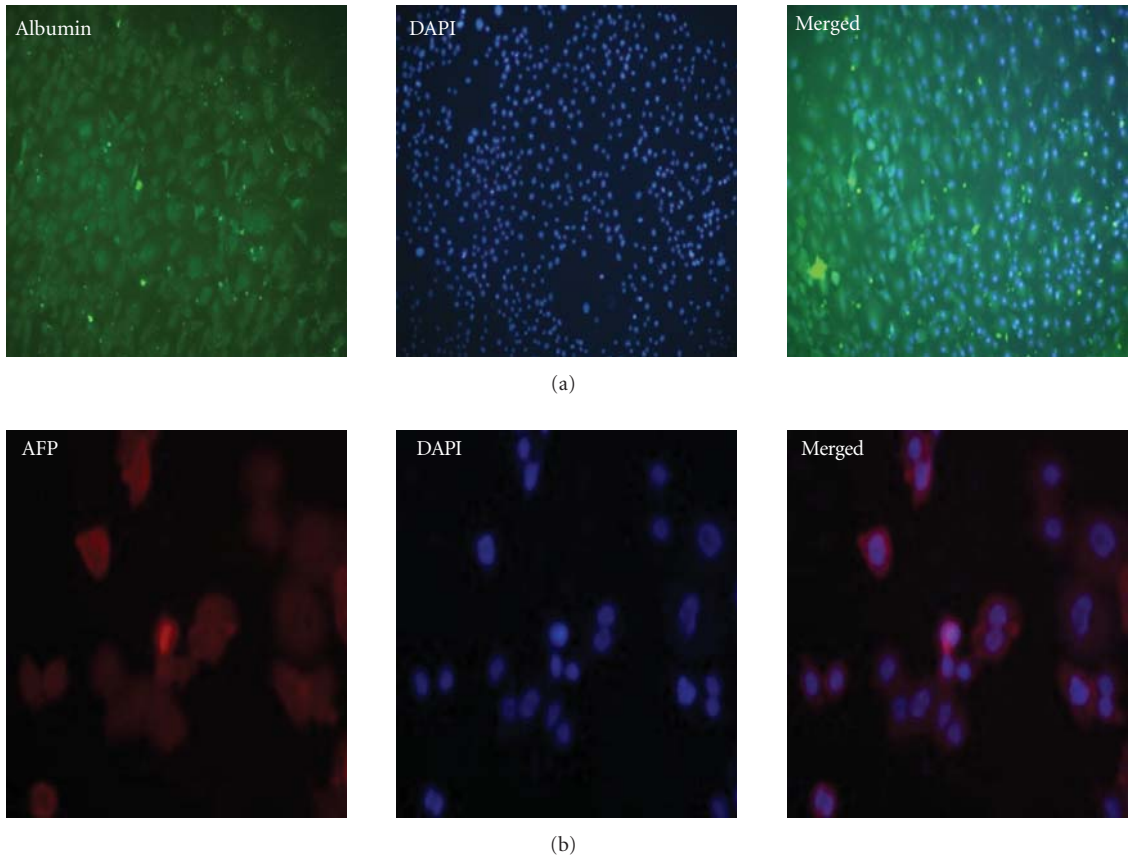


Figure 2: Immunofluorescence staining for several hepatocyte-specific markers in iPSC-Heps. Immunostaining imaging (800x) results showed that several hepatocyte-specific markers detected by using (a) anti-AFP antibody and (b) anti-albumin antibody in iPSC-Heps. Blue signal indicated DAPI-positive cells.

hepatic specific genes was significantly increased at week 2 after differentiation in HNF-3β, TTR, AAT, and TAT, and at week 3 after differentiation in AFP and ALB, but not in undifferentiated iPSCs (Figure 3(b), $^*P < 0.05$ versus iPSC).

3.2. Functional Characterization of iPSC-Heps. We further examined whether the iPSC-Heps with high expression of hepatic-specific genes also possessed biological hepatic functions. The uptake of LDL was measured by incorporation rate of DiI-Ac-LDL and the presence of stored glycogen was determined by PAS staining. Neither cellular uptake of LDL (mean ± SD, $n = 12$) (Figure 4(a)) nor glycogen synthesis (mean ± SD, $n = 12$) (Figure 4(b)) was observed in undifferentiated iPSCs, weeks 1 and 2 after iPSC-Heps differentiation. In contrast, iPSC-Heps showed significantly the ability to take up LDL (Figure 4(a)) and store glycogen (Figure 4(b)) over the three weeks of differentiation ($^*P < 0.05$ versus week 0). Thus, the iPSC-Heps resemble hepatocytes both morphologically and functionally.

3.3. Intravenous Transplantation of iPSCs Exhibited Beneficial Effect on Survival Rate, Hepatic Encephalopathy, and Liver Functions in Mice with AHF. AHF is a severe liver disease accompanied by high mortality and hepatic encephalopathy that causes multiorgan failure. We assessed the therapeutic potential of iPSCs in TAA-induced AHF. We observed that in iPSCs-treated groups, 7/23 (30.4%) mice were died 72 hours post administration of TAA, whereas 26/36 (72.2%) mice were died in control groups. These results showed that the survival rate was significantly improved in mice receiving iPSC transplantation compared to controls (Figure 5(a), $P = 0.0018$ by log-rank test). Furthermore, we examined whether iPSC transplantation could improve hepatic encephalopathy by assessing the motor activity of TAA-treated mice after iPSCs transplantation. Motor activity assay indicated that 72 hours after TAA administration, the total movements (599 ± 110 versus 246 ± 71, $P = 0.006$) and ambulatory movements (364 ± 68 versus 155 ± 45, $P = 0.009$) were significantly increased in iPSCs-treated groups compared to PBS-treated groups (Figure 5(b)). These results demonstrated that iPSC treatment improved TAA-induced hepatic encephalopathy 72 hours after TAA injection.

To evaluate the effects of the 1st clone of iPSC Re7 from our lab on rescuing TAA-induced hepatic failure, we further evaluated whether this cell therapy also rescued liver functions in mice with AHF. There were robust increases in the levels of ALT, AST, and TBIL in PBS recipients 24 hours after TAA administration. These robust increases in hepatic biochemical parameters gradually decreased within 72 hours

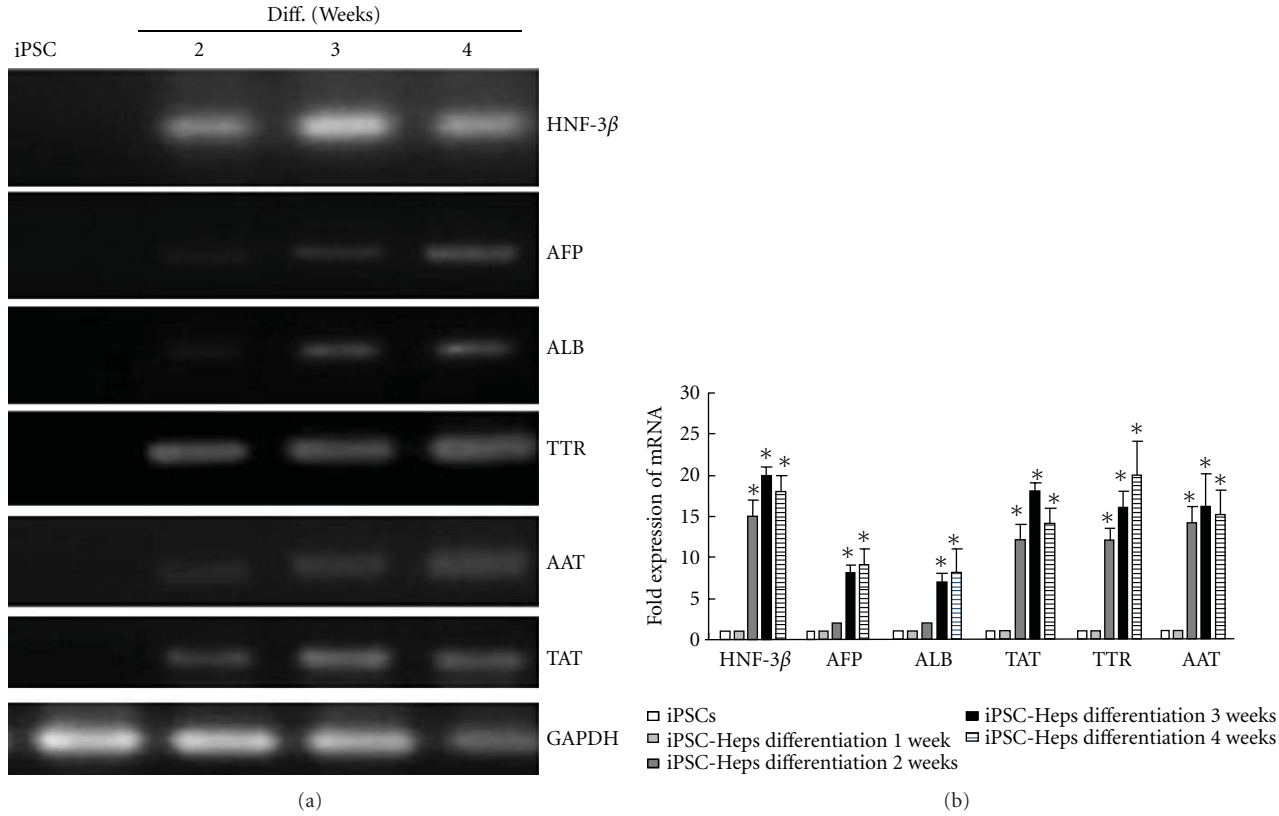


Figure 3: Gene expression of hepatic specific markers for iPSC-Heps. Expression of hepatic specific markers including HNF-3β, AFP, ALB, TTR, AAT and TAT was detected by (a) RT-PCR and (b) q-RT-PCR during the hepatogenic differentiation course of iPSCs. Data shown here are the mean ± SD. $^*P < 0.05$ versus iPSCs.

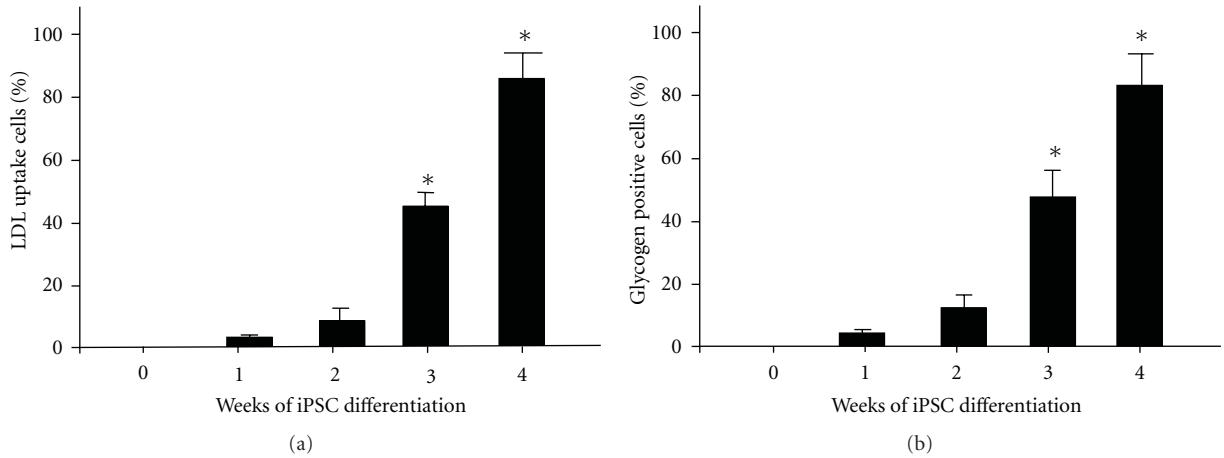


Figure 4: Functional characterization of iPSC-Heps. The uptake of LDL was measured by incorporation rate of DiI-Ac-LDL and the presence of stored glycogen was determined by PAS staining. Both (a) LDL uptake and (b) glycogen synthesis were evaluated over 4 weeks post-differentiation. Data shown here are the mean ± SD. $^*P < 0.05$ versus Week 0.

(Table 2). The iPSC transplantation decreased all of these biochemical parameters at 24 hours and further significantly suppressed them at 72 hours after TAA administration (Table 2, $^*P < 0.05$, iPSC versus PBS). Similar results were identified in the 2nd clone of iPSC from Dr. Shinya Yamanaka (Table 3, $^*P < 0.05$, iPSC versus PBS). These findings demonstrated that intravenously transplanted iPSCs can effectively rescue TAA-induced hepatic failure, restore liver functions, and improve survival in mice with AHF.

3.4. iPSC Transplantation Reduced Hepatic Necrotic Area in Mice with AHF. To explore whether transplantation of iPSCs

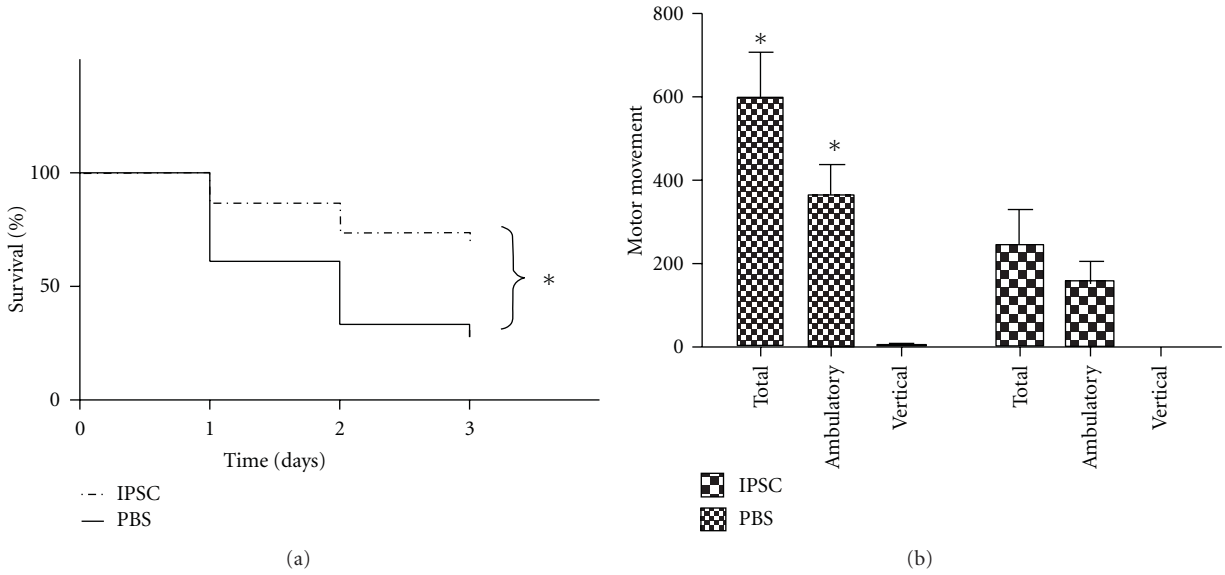


Figure 5: iPSCs increase the survival rate and motor activity of recipient mice. Male BALB/c mice were used for evaluation of the hepatoprotective activity of iPSC in the TAA-treated liver injury model. TAA (150 mg/kg) was given via intra-peritoneum injection and mice were received PBS or iPSCs (2×10^6 cells) via tail vein injection to determinate (a) the survival rate and (b) motor movements. Data shown here are the mean ± SD. $^*P < 0.05$ versus PBS.

Table 2: Effect of intravenous transplantation of PBS or iPSCs clone 1 Re7 from our lab on hepatic biochemical parameters in TAA-treated recipients.

	Alanine aminotransferase (ALT; IU/L)	Aspartate aminotransferase (AST; IU/L)	Total bilirubin (TBIL; mg/dL)
Recipient after 24 hours			
PBS	20702 ± 1631	6956 ± 522	2.0 ± 0.3
iPSCs	5175 ± 509*	1932 ± 290*	1.3 ± 0.2*
Recipient after 72 hours			
PBS	1721 ± 497	1116 ± 286	1.0 ± 0.2
iPSCs	312 ± 62*	248 ± 76*	0.5 ± 0.1*

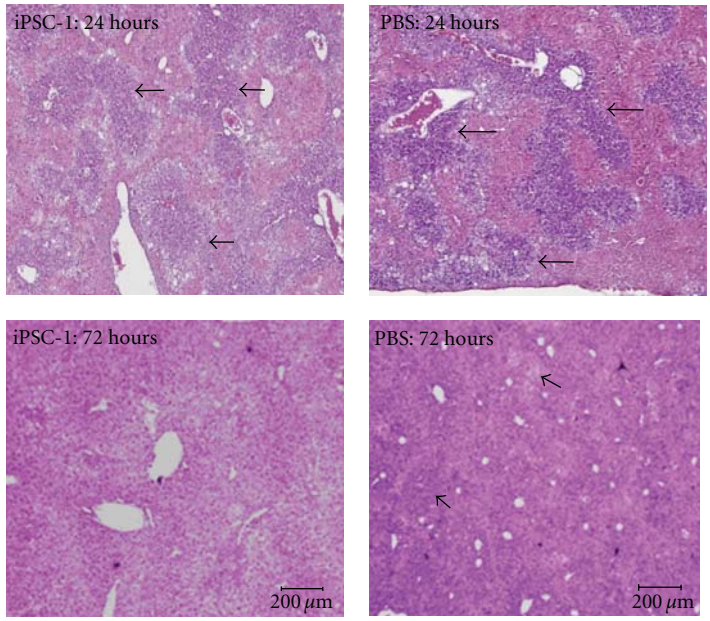
Results were expressed as mean ± SD. $^*P < 0.05$ versus PBS.

repairs hepatic necrosis, we compared H&E-stained liver sections from TAA-treated mice that received infusion of either iPSCs or PBS via tail vein. After TAA treatment, AHF happened immediately with obvious hepatic necrosis area. However, histological examination revealed that the rescuing effect of transplantation of iPSC clone 1 from our lab was initially observable in 24 hours than PBS group, and the numbers of necrotic areas (black arrow) in iPSC transplantation start to decrease significantly (Figures 6(a) and 6(b), $^*P < 0.05$ versus PBS). Furthermore, lymphocyte infiltration (hallow arrow) and necrotic areas (black arrow) were remarkably diminished in 72 hours in iPSC transplantation (Figures 6(a) and 6(b), $^*P < 0.05$ versus PBS) in iPSC clone 1 from our lab. Similar results were identified at iPSC clone 2 from Dr. Shinya Yamanaka in 24 and 72 hours (Figure 6(c), $^*P < 0.05$ versus PBS).

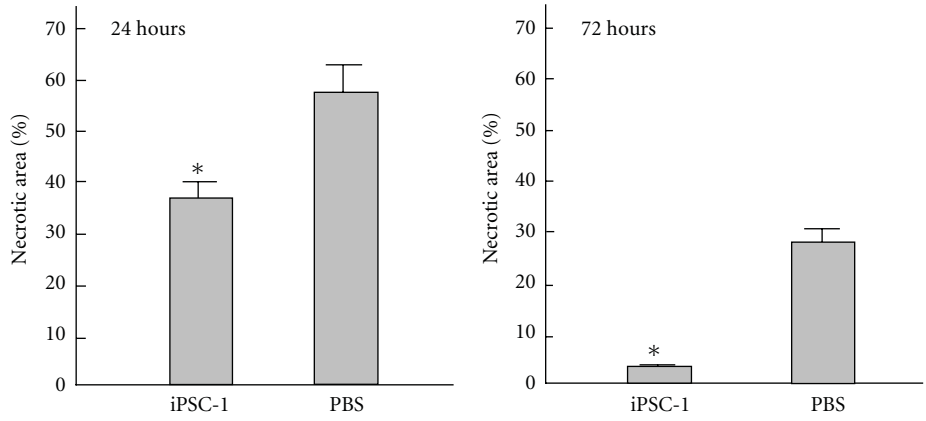
3.5. iPSCs Mobilized to the Damage Area of TAA-Injured Liver. To further explore the fate of intravenously transplanted iPSCs from tail vein, we labeled iPSCs with DiI and explored if the iPSCs could migrate and incorporate into the damaged liver in TAA-treated mice. As detected by immunofluorescence staining, we observed that DiI-labeled iPSCs were found around central veins and were scattered the damaged liver areas (Figure 7(a) light field and 7(b) dark field). This implied that iPSCs can migrate from the peripheral space into areas of damaged liver in TAA-treated mice and may have favorable effects on improving liver functions and motor activity, and rescuing TAA-treated mice from lethal AHF. These hepatoprotective properties may subsequently improve hepatic encephalopathy and impaired motor activity.

4. Discussion

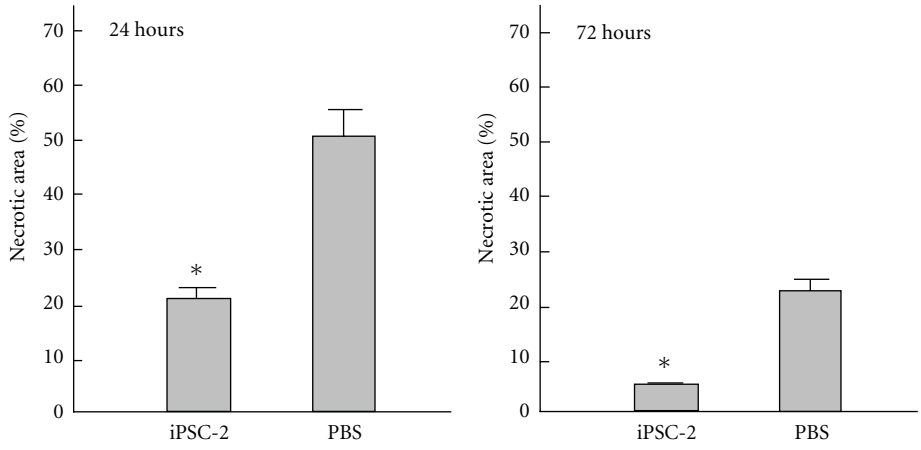
In this present study, we first showed that iPSC-Heps can be generated from iPSCs using the stepwise differentiation protocol. We demonstrated that intravenous transplantation of iPSCs can mobilize to the damaged liver area and extensively reduced the hepatic necrotic area, improved liver functions and motor activity, and rescued TAA-treated mice from lethal in mice with TAA-induced liver failure. Taken



(a)

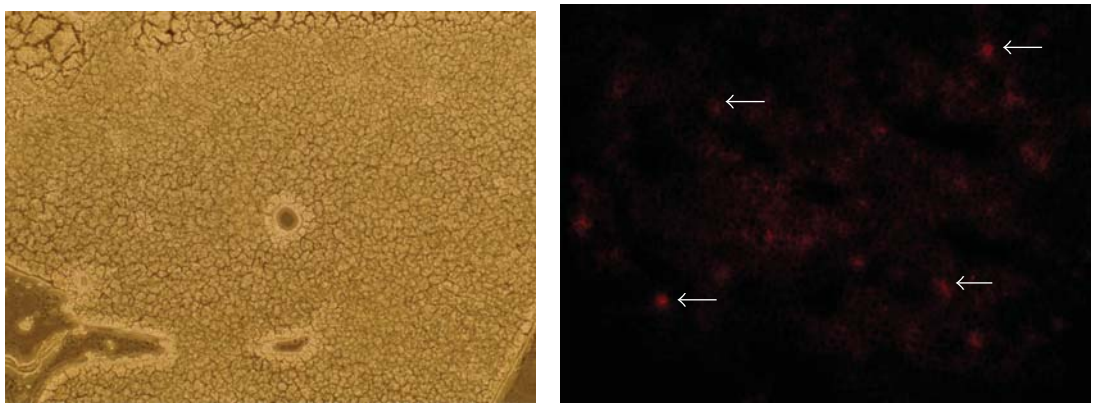


(b)



(c)

Figure 6: Effects of iPS cells on histopathological changes in recipient mice. Results showed the representative H&E stain of TAA-treated liver tissue receiving iPSCs or PBS treatment after 24 hours or 72 hours in iPSC clone 1 from our lab and clone 2 from Shinya Yamanaka. In (a) and (b), the necrotic areas in iPSC-treated group were significantly reduced than PBS-treated group in 24 hours and 72 hours after TAA administration in iPSC clone 1. In (c), similar results were identified in iPSC clone 2. $^*P < 0.05$ versus PBS.



(a) (b)

Figure 7: Mobilization of DiI-labeled iPSCs to the liver of mice with AHF. DiI fluorescent staining was used to trace the migration of the cells in the body. The transplanted DiI-labeled iPSCs was indicated as red spots under dark field microscopy (b) in the damaged liver area in TAA-treated mice. White arrows indicated the DiI-labeled iPSC and results indicated histological examination under (a) light field; (b) dark field.

Table 3: Effect of intravenous transplantation of PBS or iPSCs clone 2 from Dr. Shinya Yamanaka on hepatic biochemical parameters in TAA-treated recipients.

	Alanine aminotransferase (ALT; IU/L)	Aspartate aminotransferase (AST; IU/L)	Total bilirubin (TBIL; mg/dL)
Recipient after 24 hours			
PBS	17768.2 ± 786.9	6875.2 ± 929.1	3.87 ± 0.45
iPSCs	3157.4 ± 336.8*	1512.9 ± 257.1*	1.53 ± 0.46*
Recipient after 72 hours			
PBS	1553.4 ± 67.1	984.7 ± 26.2	1.53 ± 0.18
iPSCs	217.7 ± 7*	175.1 ± 8.4*	0.92 ± 0.13*

Results were expressed as mean ± SD. *$P < 0.05$ versus PBS.

together, our data showed that iPSCs can effectively rescue experimental AHF and may provide an alternative cell source to hepatocyte transplantation for acute/fulminant liver diseases. Nevertheless, the underlying mechanisms of iPSC-mediated therapeutic effect remained largely unknown.

It has been clearly demonstrated that mesenchymal stem cells (MSCs) have a capacity to mobilize and integrate into damaged tissues and provide immunomodulatory effects to undergo tissue repair via paracrine effects [27]. Consequently, MSC transplantation has been regarded as a new clinical approach for tissue regeneration. Intravenous transplantation of MSCs has been shown to mobilize to the damaged liver and improved hepatic functions in mice with AHF [28]. Interestingly, in the present study, the distribution of DiI-labeled iPSCs demonstrated that iPSCs could also mobilize to the damaged area of liver, in a manner similar to that of MSCs. BM-derived MSC is widely used for MSC-based regeneration study, whereas such cell is relatively inaccessible [29, 30], and the cell number and the differentiating potential of BM-derived MSCs were decreased with age [31]. Given that iPSCs were generated from somatic cells and exhibited remarkable therapeutic potential on fulminant hepatic failure, iPSC could be another choice for cell-based therapy against fulminant hepatic failure.

Cross-gender and whole-liver transplantation studies in rodents indicate that BM-derived or extrahepatic stem cells can differentiate into hepatocytes [32]. Previous studies also showed that early hepatic precursors can be generated from ESCs in vitro and these precursors can differentiate into functional hepatocytes that rescued and incorporated into diseased liver parenchyma [13]. Although iPSCs possess pluripotent properties similar to those of ESCs, whether the transplanted iPSCs differentiated into hepatocytes or iPSC-Heps after incorporation into the liver area was unknown. We speculated that there were some possible mechanisms for iPSC-mediated hepatoprotective effect, including (a) restoration of impaired hepatic function by engrafted iPSCs that may undergo differentiation and have mature hepatocyte functions in vivo, (b) stimulating the proliferation of new functional hepatocytes that may restore hepatic function, via a paracrine manner, and (c) providing certain immunomodulatory effects similar to those of MSCs and repair damaged tissue. Nevertheless, further investigations will be required to clarify these speculations.

Previous studies have demonstrated that iPSCs can differentiate into different types of cells including cardiomyocytes, endothelial cells, and neural cells in vitro [33–36].

Given the potential to generate patient-specific cell populations without the need for human embryonic cells, iPSC technology has received great excitement by research and medical communities. However, many questions regarding the actual molecular process of induced reprogramming remain unanswered and need to be addressed. Teratoma formation [37] and genomic instability [38] have been reported to be the major risk in the transplantation of iPSCs generated by four exogenously introduced genes (Oct4/Sox2/Klf4/c-Myc). Notably, there are no observable tumor-like structures in the liver after a follow-up observation for one month after iPSC transplantation in the present study. To fully exclude the risk of teratoma formation and nonviral vector or replaced the pro-oncogens of c-Myc and Klf4 may be a more safe way and should be developed in the future study.

Comparing with MSCs and ESCs, iPSCs are relatively accessible and with less ethical issues and immunorejection. Our results provided evidence that iPSCs can migrate from systemic circulation to the damaged liver in TAA-treated mice and immediately improve liver functions and rescue survival benefit shortly after transplantation. Therefore, this iPSC transplantation may provide an alternative of therapeutic strategy for patients with acute/fulminant hepatic failure.

Conflict of Interest

The authors declare that they do not have anything to disclose regarding funding from industries or conflict of interest with respect to this manuscript.

Acknowledgments

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Research Article

Preclinical Studies with Umbilical Cord Mesenchymal Stromal Cells in Different Animal Models for Muscular Dystrophy

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Umbilical cord mesenchymal stromal cells (MSC) have been widely investigated for cell-based therapy studies as an alternative source to bone marrow transplantation. Umbilical cord tissue is a rich source of MSCs with potential to derivate at least muscle, cartilage, fat, and bone cells *in vitro*. The possibility to replace the defective muscle cells using cell therapy is a promising approach for the treatment of progressive muscular dystrophies (PMDs), independently of the specific gene mutation. Therefore, preclinical studies in different models of muscular dystrophies are of utmost importance. The main objective of the present study is to evaluate if umbilical cord MSCs have the potential to reach and differentiate into muscle cells *in vivo* in two animal models of PMDs. In order to address this question we injected (1) human umbilical cord tissue (hUCT) MSCs into the caudal vein of *SJL* mice; (2) hUCT and canine umbilical cord vein (cUCV) MSCs intra-arterially in GRMD dogs. Our results here reported support the safety of the procedure and indicate that the injected cells could engraft in the host muscle in both animal models but could not differentiate into muscle cells. These observations may provide important information aiming future therapy for muscular dystrophies.

1. Introduction

Mesenchymal stromal cells (MSCs) have been extensively explored over the last years to understand their stem cell properties and clinical application. MSCs were first isolated from bone marrow (BM), but similar populations have been reported afterwards in other tissues, such as adipose tissue, dental pulp, placenta, umbilical cord, and fallopian tube [1–4]. They comprise a population of cells with ability to self-renew and differentiate into specific functional cell types including chondrocytes, osteocytes, adipocytes, and myocytes *in vitro*. However, examining the differentiation potential of MSCs *in vivo* still stands as one of the most important way to address their stemness capacity and direct their use for future cell-based therapies.

Among the genetic diseases of great medical relevance are the progressive muscular dystrophies (PMDs), a clinically and genetically heterogeneous group of disorders for which there is no cure. They are caused by the deficiency or abnormal muscle proteins, resulting in progressive degeneration and loss of skeletal muscle function [5]. Duchenne muscular dystrophy (DMD), which affects 1 in 3500 male births, is the most common and severe form. It is caused by mutations in the dystrophin gene leading to the absence of the muscle dystrophin protein, an essential component of skeletal muscle [6]. The clinical course of DMD is severe and progressive. Affected individuals exhibit muscular weakness by the age of 5 years, lose their independent ambulation around 12 years, and, without special care, they succumb due to respiratory failure or cardiomyopathy in their late teens or early twenties [5].

The murine model for DMD, the mdx mouse, also lacks muscle dystrophin. However, in opposition to affected boys, they have an almost normal phenotype [7, 8]. On the other hand, the golden retriever muscular dystrophy dog (GRMD) has a frameshift point mutation within the splice acceptor

site in intron 6 of the dystrophin gene, which results in the complete absence of the muscular protein [9]. Although the disease course is variable, and neonatal death is frequent, with very rare exceptions [10], GRMD dogs are severely affected and represent the best animal model for human DMD.

Differently from DMD, the limb-girdle muscular dystrophies (LGMDs) constitute a subgroup of 22 different forms identified until now, most of them with autosomal recessive inheritance [11, 12]. They are characterized by the involvement of the pelvic and shoulder girdle musculature. Among the autosomal recessive forms, one of the most prevalent is caused by mutations in the dysferlin gene resulting in two phenotypes: miyoshi myopathy (MM) which affects distal muscles at onset, with preferential early involvement of the gastrocnemius and LGMD2B with a more pronounced limb-girdle involvement [13]. Dysferlin expression is reduced or absent in these patients [14, 15].

A 171-bp in-frame deletion in the murine dysferlin cDNA was identified in a mouse model, the *SJL* mice, with a corresponding reduction in dysferlin levels to 15% of normal. The spontaneous myopathy of the *SJL* mice begins at 4–6 weeks of age and is nearly complete by 8 months of age with a progressive inflammatory change in muscle [16]. The *SJL* mice deletion is in-frame and, therefore, does not cause a total absence of the protein.

Adult skeletal muscle has the potential to regenerate new muscle fibers by activating a population of mononucleated precursors, which otherwise remain in a quiescent and non-proliferative state [17]. However, the continuous and gradual muscle degeneration in progressive muscular dystrophies leads to a depletion of satellite cells, and, consequently, the capability to restore the skeletal muscle is lost [18, 19]. The possibility to repair the defective muscle through cell therapy is a promising approach for the potential treatment of PMD, independently of specific mutations.

We have recently shown that human umbilical cord tissue (hUCT) is a rich source of MSC with ability to differentiate into skeletal muscle cells *in vitro* [3, 20]. We also described that canine MSCs could be isolated from umbilical cord vein (cUCV) and that they represent a good candidate for preclinical studies [21]. Human umbilical cord MSCs are obtained after full-term delivery of the newborn, from a sample that would be inevitably discarded. The process is noninvasive, painless, and without harm for the mother or the infant. These cells also lack the expression of the major histocompatibility complex (MHC) class I and II antigens which render them to be highly tolerated in transplantations [22, 23] and excellent candidates for cell replacement therapy in PMDs.

However, it is not known if umbilical cord MSCs show the same *in vitro* muscle differentiation capacity as *in vivo*. In order to address this question we injected umbilical cord MSCs in two different animal models of PMDs, *SJL* mice, and GRMD dogs, aiming to compare their ability to engraft into the host muscle and express muscular proteins. Although the injected cells could reach the musculature in both animal models, they were unable to differentiate into muscle cells. In GRMD dogs, it is very difficult to evaluate the therapeutic effect of any procedure due to their great clinical variability. However, we observed that *SJL*-injected mice had a functional performance significantly better than the control noninjected animals. These results may have important implications for future therapeutic approaches.

2. Materials and Methods

2.1. Ethics Statement. This study was approved by the human research ethics committee (*Comitê de ética em pesquisa—seres humanos*—CEP) and by the animal research ethics committee (*Comissão de ética no uso de animais em experimentação*—CEUA) of Institute of Bioscience and University Hospital of University of São Paulo. hUCT MSC were collected from donated umbilical cord (UC) units, after all mothers sign the written informed consent, in accordance with the ethical committee of Institute of Bioscience and University Hospital of University of São Paulo (CEP), permit number 040/2005. Animal care and experiments were performed in accordance with the animal research ethics committee (CEUA) of the Biosciences Institute, University of São Paulo, permit number 034/2005.

2.2. Animal Models. *SJL* mice were purchased from the Jackson Laboratory. The GRMD dog colony was established with a female GRMD carrier, Beth, donated by Dr. Joe Kornegay (University of North Carolina). All animals were housed and cared for in the University of Sao Paulo.

GRMD dogs were genotyped, at birth, from blood genomic DNA extracted with the kit GFX Genomic (GE Healthcare). For PCR reaction, the primers GF2 and GR2 and the temperature conditions were used as previously reported [24]. DMD diagnosis was confirmed by the digestion of PCR products with the enzyme Sau96I (New England Biolabs) and by elevated serum creatine kinase (CK) levels.

Dog leukocyte antigen (DLA)-identical littermate donor/recipient pairs were determined based on the identity for highly polymorphic MHC class I (C.2200) and MHC class II (C. 2202) microsatellite markers, formerly described [25].

2.3. Harvesting and Expansion of hUCT and cUCV MSC. Human umbilical cord (UC) units were collected and transferred to the laboratory under sterile conditions. hUCT and cUCV were isolated, characterized, and expanded as described elsewhere [3, 21]. Briefly, UC of full-term deliveries were filled with 0.1% collagenase (Sigma-Aldrich) in phosphate-buffered saline (PBS, Gibco) and incubated at 37°C for 20 minutes. Then, each UC was washed internally with proliferation medium consisting of Dulbecco's modified Eagle's medium-low glucose (DMEM-LG; Gibco) supplemented with 10% of fetal bovine serum (FBS; Gibco). Detached cells were harvested after gentle massage of the umbilical cord and centrifuged at 300 g for 10 minutes. Cells were resuspended in proliferation medium, seeded in 25-cm^2 flasks, and maintained at 37°C in a humidified atmosphere containing 5% CO_2. After 24 hours of incubation, nonadherent cells were removed, and culture medium was replaced every 3 days. Adherent cells were cultured until reaching 90% confluence and passaged using TrypLE (Invitrogen).

2.4. Cell Transplantation

2.4.1. SJL Mice. Two-month-old *SJL* mice were divided into two groups ($n = 7$): experimental (group A) and control (group B). Each animal from group A was injected in the tail vein with 1×10^6 of hUCT MSCs in 0.1mL of Hank's Buffered Salt Solution (HBSS, Gibco). The animals were injected for 6 months, weekly in the first month and then monthly. The control group B were uninjected animals. All results were analyzed blindly. The code for each of the mice groups was disclosed only after the completion of all the studies. Two months after the last cell transplantation, the animals were euthanatized using a CO_2 chamber.

2.4.2. GRMD Dogs. Approximately 1×10^7 cUCV or hUCT cells were injected through the femoral artery of 3 GRMD dogs. Dogs were sedated, and the injections were performed using a 22 gauge intravenous catheter connected to the injection syringe containing the cells resuspended in a final volume of 10 mL of HBSS. Transplantation protocol started when dogs were 51-day old, and each dog received 7 consecutive injections with 30-day interval. All dogs were given standard supportive care and have been followed up during all experimentation. Completion time of the study was determined when the dogs died of natural DMD-related causes.

2.5. Muscle Biopsies. In the *SJL* study, muscle biopsies were collected after animals were euthanized. Samples were taken from distal and proximal muscles localized in the hind leg and foreleg of both experimental and control animals.

In GRMD dogs, biopsies were obtained from *biceps femoralis*. The first procedure (B144) was realized two days after the third injection, when dogs were 144-day old. The second procedure (B312) was done 3 days after the seventh injection, when dogs were 312 days old. During these procedures, the animals were under effect of anesthesia and sedation.

Each biopsy was divided into two pieces. The first piece was reserved for histological analysis and prepared by embedding in optimal cutting temperature compound and stocked in liquid nitrogen. The other fragment was used for molecular analysis and prepared by snap freezing in liquid nitrogen.

2.6. Engraftment Analysis. DNA samples were obtained from muscle biopsies using DNeasy Blood & Tissue Kit (Qiagen). The presence of human DNA in the host was evaluated as described in Pelz et al. [26].

To detect the presence of Y chromosome in the female-injected animals, we evaluated the amplification of the sex-determining region Y (SRY) gene by PCR using the primers and temperature conditions previously described [27]. PCR products were separated by electrophoresis on 6% polyacrylamide gels and stained with ethidium bromide. Nonsaturated digital images were obtained using ImageQuant imaging system (GE HealthCare).

2.7. Protein Analysis. Immunohistochemistry (IHC) and western blot (WB) were performed according to the methodologies previously described [28]. The following primary antibodies were used: antidystrophin NCL-DYS1 and NCL-DYS2 (Novocastra Laboratories); specific antihuman-dystrophin MANDYS106 2C6 and MANDYS108 4D8 (a kind gift from Dr. Glenn Morris and Dr. Nguyen thi Man, from the North East Wales Institute, Wrexham, UK); specific antihuman nuclei MAB1281 (Chemicon).

For IHC, samples were incubated with antimouse IgG-Cy3-conjugated secondary antibody (Chemicon), and, when necessary, slides were counterstained with 4′, 6-diamidino-2phenylindole (DAPI; Sigma). Slides were examined in Axiovert 200 microscope (Carl Zeiss), and images were captured using Axiovision 3.1 software (Carl Zeiss).

For WB, horseradish peroxidase (HRP)-conjugated antimouse secondary antibody (Santa Cruz Biotechnology) was used to detect immunoreactive bands with enhanced chemiluminescence (ECL) plus kit (GE Healthcare).

2.8. Functional Assessment. In order to verify whether injected hUCT MSCs would improve motor ability in *SJL*-injected mice, we performed motor ability tests before and after 2 months of the last injection. Mice were examined, weighed, and submitted to the following tests: (a) the *inclined plane test* evaluated by measuring the maximal angle of a wood board on which the animal was placed until it slipped; (b) *the wire hanging test* to determine the ability of the mouse suspended on a horizontal thread by its forelegs, to reach it with its hind legs and the length of time they were able to stay hanging; (c) *the ambulation test* which was performed to determine the mean length of a step measured in hindfoot ink prints while mice freely run in a corridor (length: 50 cm; width: 8 cm; height of lateral walls: 20 cm) [29].

2.9. Statistical Analysis. Observations were quantified blindly. Numerical data are the mean ± sd (standard deviation). The statistical analysis of the equivalence between the injected and uninjected mice was achieved by the one-tailed Student's t-test, at the significance level of $P = 0.05$, and the results were expressed by the percentage variation between their performance before and after hUCT MSC transplantation.

3. Results

3.1. DMD Typing, DLA Matching of Littermates and Transplantation Setup. For this study, we had 3 affected GRMD dogs available from same litter: 1 affected male, L3M6; two affected females, L3F1 and L3F2. However, only the dogs L3M6 and L3F1 had DLA-histocompatible pairs from the male littermates L3M7 and L3M5, respectively. The donor-recipient pairs with DLA identity were chosen where the recipients possessed the dystrophin mutation, and the donors were wild-type littermates. Thus we injected cUCV cells from DLA-compatible donors into the dogs L3M6 and L3F1. Since L3F2 did not have any DLA-compatible donor, we injected this dog with male hUCT MSCs. Both cell type

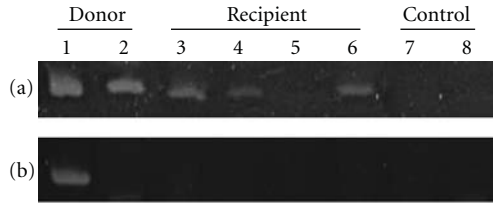

Figure 1: Engraftment of male human and canine umbilical cord MSCs into muscle of female GRMD dogs. Polymerase chain reaction analysis for (a) sex-determining region Y (SRY) sequence and (b) canine-specific dystrophin sequence. Muscles samples shown are the following: (1) male cUCV MSC from L3M5; (2) male hUCT MSC; (3, 4) B144 and B312 from affected female L3F1; (5, 6) B144 and B312 from affected female L3F2; (7) female canine control DNA; (8) female human control DNA.

injected were previously characterized by immunophenotyping and differentiation potential [3, 21].

3.2. Capacity of Umbilical Cord MSCs to Reach and Engraft at the Host Muscle of Transplanted Animals. In order to verify if human and canine umbilical cord MSCs were able to reach and colonize the host muscle, we analyzed the biopsies of transplanted female dogs, L3F1 and L3F2, which received male cUCV and hUCT cells respectively. By PCR analysis, we detected the presence of the Y-chromosome marker SRY in muscle biopsies of both affected females, indicating the presence of the injected cells in the musculature of these animals (Figure 1). In addition, scattered human cells were confirmed in the affected female L3F2 by immunohistochemistry (IHC) using specific antibody for human nuclei (Figure 2).

We also found similar results in the *SJL* mice model injected with hUCT MSCs into the caudal vein [30]. PCR analysis detected human DNA in the foreleg and hind leg muscles of all seven injected mice (data not shown).

3.3. Muscle Proteins in Transplanted Animal Models. To explore the myogenic differentiation followed by the engraftment of umbilical cord MSCs, we analyzed the expression of dystrophin in the host muscle of transplanted GRMD dogs. Through western blot (WB) analysis (Figure 3(a)), no dystrophin was found in the muscles of injected animals indicating that the engrafted cells were unable to produce muscular proteins. In addition, we did not observe the expression of human dystrophin, by IHC analysis (Figure 4) or RT-PCR analysis (data not shown), in the muscle biopsies of the affected female L3F2 that received hUCT MSCs.

Three months after we finalized the injections in GRMD animals, the dog L3M6 died of natural GRMD-related causes. Aiming to investigate if the injected cells into the femoral artery were able to spread all over his body, reach different muscular groups, and restore the dystrophin expression, we collected eight different muscle samples at his necropsy. However, no dystrophin expression was found by WB analysis in any analyzed tissue (Figure 3(b)).

Similarly from what we observed in GRMD dogs, hUCT MSCs were able to engraft in the host muscle of injected *SJL* mice but were not able to differentiate into muscle cells and express human dystrophin (data not shown) [30].

3.4. Functional Assessment. Clinical assessment in GRMD dogs is very difficult due to the great variability in their clinical course [10]. From the 3 transplanted animals, one affected male dog (L3M6) and one affected female dog (L3F2) died 3 months after the last cell injection at 414 days of age and at 3 years and 5 months of age, respectively. However, the female affected dog L3F1 is alive at the age of 4 years.

In *SJL* mice, we performed three standardized motor ability tests and compared their performance before and after cell transplantation [30]. Our results showed a statistically significant difference between the two groups. While uninjected animals worsen significantly their performance, in the injected group, the disease remained stable (35.14 + 9.55% versus 13.47 ± 10%; $P = 0.0014$, Student's t-test, $n = 7$).

4. Discussion

Repairing skeletal muscle damage is a challenge for cell-based therapies, given the unique architecture of the tissue, which comprises around 640 types of skeletal muscles that make up about 40 percent of the body's weight in a normal individual. Thus, the successful use of stem cell for clinical application in PMDs will depend on finding an easily obtainable source that could be expanded in quantities suitable to reach the entire musculature, engraft, and restore the defective protein. Although high levels of engraftment are very difficult to be achieved, it has been reported that levels of 20–30% are able to ameliorate dystrophic pathologic lesions [31, 32].

In the past decades, human umbilical cord has been used as an alternative source to bone marrow for cell-based therapies because of its hematopoietic and mesenchymal cell components. We recently showed that hUCT is a richer source of MSCs in comparison to human umbilical cord blood (hUCB) [3, 20]. In addition, we demonstrated that MSC from hUCT and hUCB have different gene expression profiles [33]. Since umbilical cord is easily obtained and a rich source of MSCs, we investigated their ability to originate muscle cells *in vivo* and restore the expression of defective muscular proteins in different animal models of PMDs.

Jazedje et al. [34] and Gang et al. [35] demonstrated that both hematopoietic and mesenchymal stromal cells, respectively, from umbilical cord blood were able to differentiate into skeletal muscle *in vitro*. In addition, Secco et al. [3] reported the myogenic potential, *in vitro,* of MSCs from human umbilical cord tissue. Although different cell populations from umbilical cord show apparently a similar ability to differentiate into muscle cells at least *in vitro*, preclinical studies are of utmost importance to verify if this also happens *in vivo*.

Kong et al. [36] injected human umbilical cord blood cells intravenously into *SJL* mice. These authors reported that a small number of cells engrafted in the recipient muscle and were capable of myogenic differentiation. More recently, Kang et al. [37] reported a boy that was cured of chronic granulomatous disease (CGD) after being transplanted with

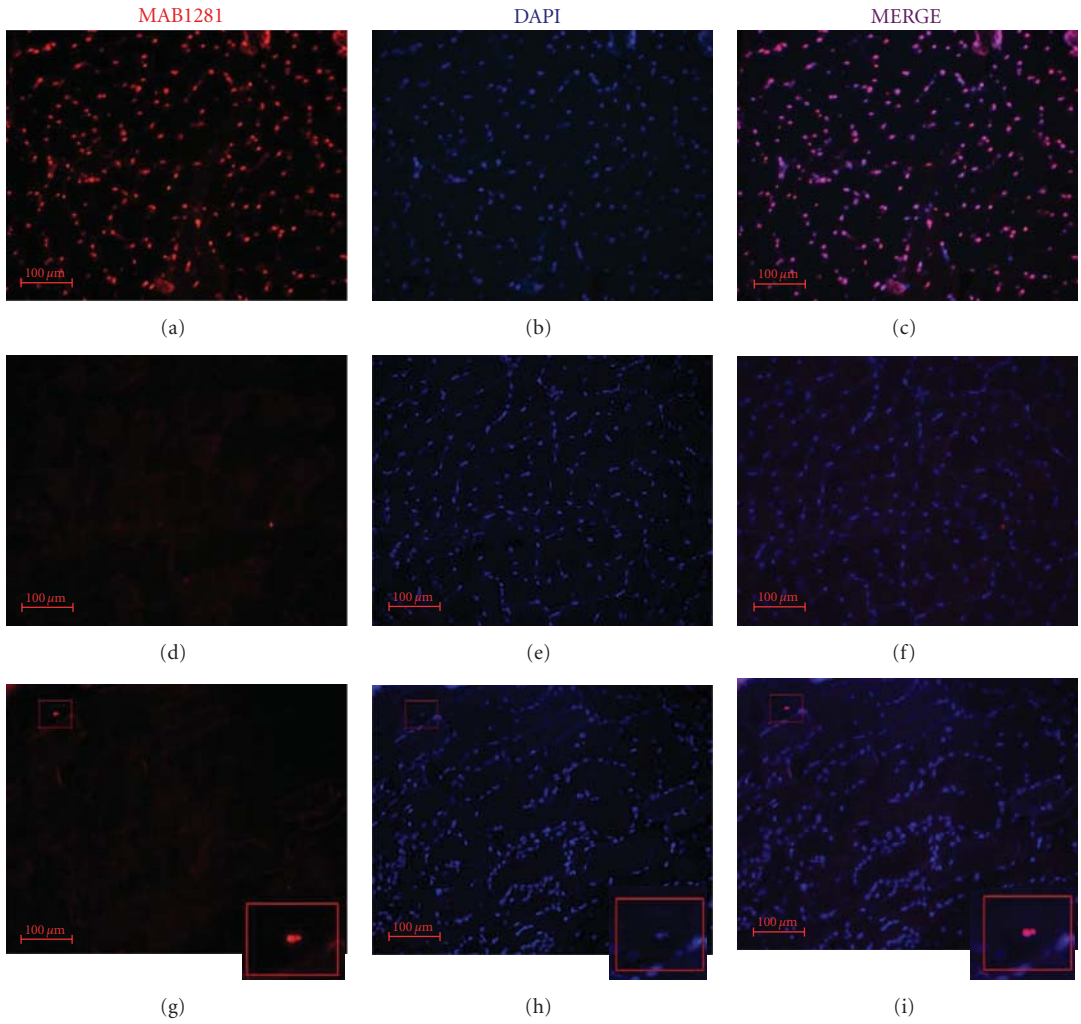

Figure 2: Presence of human nuclei at recipient dog muscle after umbilical cord MSCs transplantation. Scattered human cells into *biceps femoralis* (B312) of affected female L3F2 identified by the antihuman nuclei antibody MAB1281. Preparations were counterstained with 4′, 6-diamidino-2phenylindole (DAPI). (a–c) human muscle; (d-e) nontransplanted canine muscle; (g-h) B312 from L3F2. Insets in (g-h) show details of human nucleus. Images were acquired with the same exposure time and magnification of 200x.

allogeneic umbilical cord blood cells. Unfortunately, two years latter, he was diagnosed with DMD, and analysis of his muscle biopsy demonstrated no expression of donor dystrophin.

In the present study, we were interested to investigate the potential of MSCs from umbilical cord tissue for *in vivo* muscle regeneration. In our first trial, we did nine injections of one million cells into the caudal vein of *SJL* mice, the murine model of limb-girdle muscular dystrophy 2B. DNA analysis in transplanted animals showed that the hUCT MSCs were able to reach the host muscle through systemic delivery. However, we did not find human dystrophin through WB in the same muscle samples where the human DNA was present. In addition, the functional ability tests did not show any clinical improvement. These results were expected since the human umbilical cord MSCs were not able to originate human muscle proteins. However, surprisingly, the performance of noninjected animals was significantly worse than the "treated" animals [30]. The results reported here were done with the same methodologies used in our previously report where we injected human adipose multipotent mesenchymal stromal cells (hASCs) [38]. Differently from hUCT MSCs, hASCs injected in *SJL* mice resulted in *in vivo* expression of human muscle proteins and functional amelioration. These results suggest that although MSCs from different sources show apparently similar properties *in vitro,* they may be more or less efficient to differentiate into specific cell lineages *in vivo* according to the niche where they come from.

In the second trial, we used the golden retriever muscular dystrophy (GRMD) dogs, the canine model of Duchenne Muscular Dystrophy, aiming to evaluate the ability of MSCs from umbilical cord to regenerate the dystrophic muscle in a large animal model using a protocol already described [39]. As reported by Sampaolesi et al. [39], intra-arterial delivery of wild-type dog mesoangioblasts resulted in an extensive

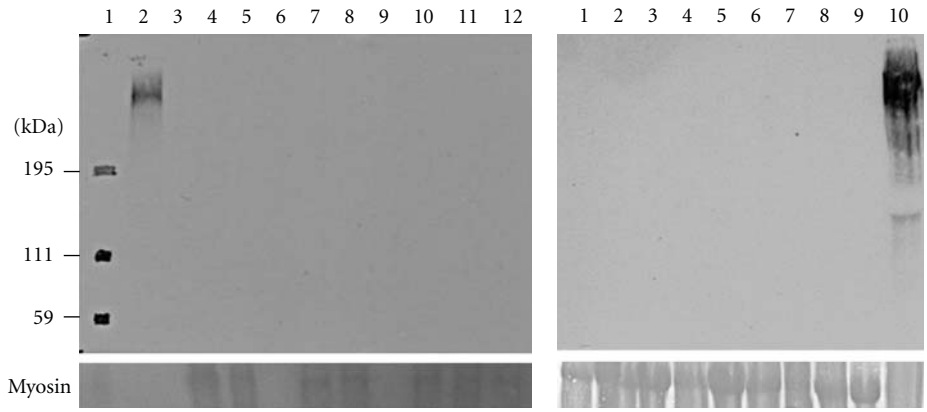

Figure 3: Dystrophin expression analysis. Western blot using antidystrophin rod-domain DYS1 antibody. Samples shown are the following: (a) (1) kaleidoscope protein standard; (2) wild-type canine muscle; (3, 6, 9) blank; (4, 5) B144 and B312 from affected male L3M6; (7, 8) B144 and B312 from affected female L3F1; (10, 11) B144 and (12) B312 from affected female L3F2. (b) (1) Biceps femoralis; (2) biceps brachialis; (3) triceps brachialis; (4) quadriceps femoralis; (5) tibialis cranialis; (6) diaphragm; (7) sartorius; (8) gastrocnemius; all from affected male L3M6 at necropsy. (9) GRMD muscle; (10) wild-type canine muscle. Myosin content in the Ponceau S prestained blot was used to assess the amount of loaded proteins.

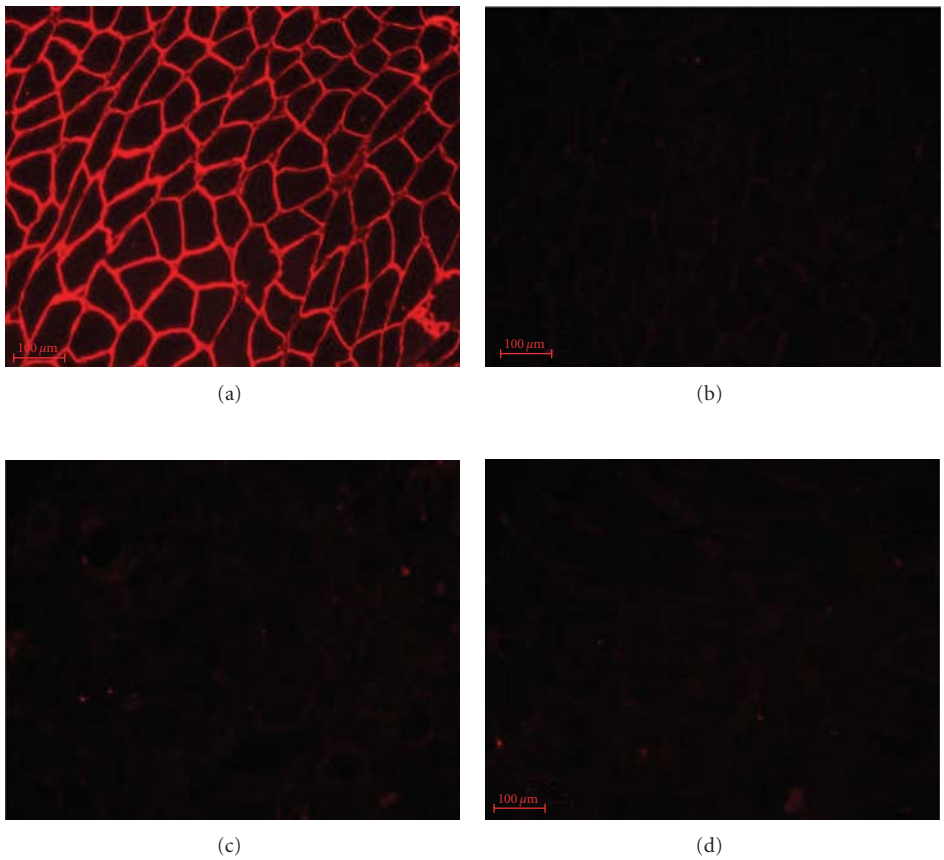

Figure 4: Human dystrophin expression analysis. Immunofluorescence using specific antihuman dystrophin antibody, Mandys106/2C6. (a) human normal muscle; (b) canine wild-type muscle; (c) B144 and (d) B312 from affected female L3F2. Images were acquired with the same exposure time and magnification of 200x.

recovery of dystrophin expression in transplanted animals. In the present study, we injected seven consecutives injections of one billion cells into the femoral artery of GRMD dogs. Using the Y chromosome as a track marker, we could show the successful engraftment of male cells into the *biceps femoralis* muscle of female affected dogs that received both canine and human umbilical cord MSCs. Furthermore, human cell engraftment into the canine muscle was also confirmed using the antihuman nuclei antibody. Similarly to what we observed in the mouse model, human and canine umbilical cord MSCs were able to reach the musculature in injected affected dogs, but no dystrophin expression were detected in those animals after transplantation.

Although no relevant number of GRMD dogs were evaluated in preclinical cell transplantation assays by us and others [39–41], due to the difficulty and high cost of such studies, its very important to test the safety and efficiency of different cell sources in a large animal model of PMD before starting any attempt of clinical trials. In addition, since the disease course in GRMD dogs is extremely variable, it is very difficult to analyze any amelioration or better performance due to any preclinical study [10]. Although one of the injected dogs did not survive long after the last injection and one affected female dog died at age of 3 years and 5 months, we still have in our kennel in Sao Paulo one injected female at age of 4 years that is being followed up.

In this study, we showed, in both animal models, that even without differentiating in muscle cells, systemic injections of umbilical cord MSCs are apparently safe and may possibly have a positive effect when interacting with the host muscle. Therapeutic effects of MSCs are believed to occur not only by direct differentiation into injured tissues but also by productions of paracrine factors that inhibit apoptosis, stimulate endogenous cell proliferation, and/or activate tissue resident stem cells in the site of injury. As reported by Prockop [42], MSCs secrete, in response to injury, large quantities of bioactive molecules, such as cytokines, antioxidants, proangiogenic, and trophic factors. Also, there are growing evidences that umbilical cord MSCs possess important immunomodulatory properties that may enable them to survive in an allogeneic or xenogeneic environment [43]. First, UCT MSC have low immunogenicity and suppress the proliferation of activated splenocytes and T cells. Second, UCT MSC do not express human leukocyte antigen (HLA)-DR and costimulatory molecules CD40, CD80, and CD86 that are required for T-cell activation. Third, UCT MSC synthesize HLA-G6, an immunosuppressive isoform of HLAs [44–46]. Finally, UCT MSC can be tolerated in animal models. These cells are not rejected when transplanted into SCID mice or even as xenografts in immune-competent rats [47–50]. As suggested by Chen et al., prostaglandin E_2 is the principal mediator of this potent immunomodulatory property of umbilical cord MSC [51].

In short, here we analyzed, for the first time, the ability of mesenchymal stem cells obtained from human and canine umbilical cord tissue to engraft into recipient dystrophic muscle after systemic delivery, express muscle proteins in the dystrophic host, and the safety of the procedure. Our results showed that, in both murine and canine models of PMD, umbilical cord MSCs were able to reach the host musculature but were not able to complete full differentiation in skeletal muscle cells.

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