Stem Cells International

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Editorial Board

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Editorial

Multipotent to Pluripotent Properties of Adult Stem Cells

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Stem cells have captured the attention of both researchers and the public alike because of the promise of tissue regeneration, drug screening, and organogenesis. Stem cells are broadly classified as embryonic or adult with differentiation capacities ranging from pluripotent and multipotent to unipotent. In general, pluripotent stem cells are ascribed to cells derived from the inner cell mass of blastocyst stage embryos or to those generated experimentally using reprogramming factors. Adult stem or progenitor cells are generally tissue-restricted but reside in most organs. While the use of pluripotent stem cells has been limited to a few clinical trials due to safety concerns, adult stem cells have shown evidence of safety and are broadly employed in both clinical trials (http://www.clinicaltrial.gov/) and clinical practice. Transplantation of adult bone marrow hematopoietic stem cells, for example, represents a standard method of clinical care for autoimmune diseases and hematological disorders. While hematopoietic stem cells cannot be expanded *in vitro*, other stem cells such as mesenchymal stem cells (MSCs) can be easily expanded from autologous and allogeneic sources for clinical testing. Moreover, adult-derived testicular spermatogonial stem cells (SSCs) have been reported to be the only cells in the body that can be dedifferentiated/reprogrammed to a pluripotent state *in vitro* and grown into ES-like colonies.

It has been advocated that adult stem cells have remarkable plasticity, transdetermination, and transdifferentiation ability. These properties have raised hope that adult stem cells could become a universal source of stem cells for tissue/organ repair in lieu of embryonic stem cells, which can easily form tumors. MSCs, for example, can be isolated from various tissues like bone marrow, gut, lungs, liver, blood, adipose tissue, umbilical cord Wharton's jelly, dental pulp, amniotic fluid, and so forth and have the ability to form bone, cartilage, adipose tissue, skeletal muscle, liver, neurons, skin, pancreatic islets, endothelial cells, intestine, renal, epithelial, and germ cells. More importantly, MSCs can be given across allogeneic barriers. Nevertheless, many trials with MSCs have shown only marginal benefits and some have proposed that the effects may be more paracrine in nature than being regenerative.

This special issue, comprised of 10 articles, focuses on adult stem cells that examine the following claims: Why are MSCs ubiquitous? Are they indeed stem cells or just stromal cells which constitute the somatic niche for tissue specific stem cells in various body organs? Have we looked carefully at MSCs growing in a culture dish? Are ES-like colonies observed only from testicular biopsy but also from ovarian and endometrial biopsies? The article by D. Bhartiya addresses these issues and reviews the available literature on the presence of novel pluripotent very small ES-like stem cells (VSELs) as a subgroup among MSCs. It also highlights that Oct-4A transcripts need to be studied to conclude pluripotent state rather than Oct-4. An article by Dr. P. Rameshwar's group studied the safety issue of MSCs since these stem cells have been shown to support tumor growth. Other papers have discussed various sources of adult stem cells including dental pulp stem cells (S. Arrifin et al.), satellite stem cells in skeletal muscles (S. Fujimaki et al.), and bone marrow stem cells (I. Catacchio et al.) and their multipotent properties and transdifferentiation potential. Further characterization of these stem cells and their functional potential is essential. The role of cannabinoid receptor type I in differentiation and

survival of MSCs is discussed by A. Gowran et al. M. Akita et al. show that CD133 positive cells have the capacity to form endothelial capillary tubes in 3D culture. Cultures of cardiac explants result in the formation of cardiospheres which contain both stem and progenitor cells implicated in cardiac regeneration (L. Barile et al.). Y. Togo et al. have reviewed the literature on the use of MSCs as a nonviral gene carrier for gene transfer *in vitro*. Finally, N. Sakayori et al. discuss how neural stem/progenitor cells function is regulated by lipids.

The diverse areas of the manuscripts in this issue underscore the wide field of adult stem cell biology. The papers indicate that more in-depth research and functional studies are required to take adult stem cells safely to the clinic for regenerative medicine.

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Review Article

A Review of Stem Cell Translation and Potential Confounds by Cancer Stem Cells

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Mesenchymal stem cells (MSCs) are multipotent cells found in both fetal and adult tissues. MSCs show promise for cellular therapy for several disorders such as those associated with inflammation. In adults, MSCs primarily reside in the bone marrow (BM) and adipose tissues. In BM, MSCs are found at low frequency around blood vessels and trabecula. MSCs are attractive candidates for regenerative medicine given their ease in harvesting and expansion and their unique ability to bypass the immune system in an allogeneic host. Additionally, MSCs exert pathotropism by their ability to migrate to diseased regions. Despite the "attractive" properties of MSCs, their translation to patients requires indepth research. "Off-the-shelf" MSCs are proposed for use in an allogeneic host. Thus, the transplanted MSCs, when placed in a foreign host, could receive cue from the microenvironment for cellular transformation. An important problem with the use of MSCs involves their ability to facilitate the support of breast and other cancers as carcinoma-associated fibroblasts. MSCs could show distinct effect on each subset of cancer cells. This could lead to untoward effect during MSC therapy since the MSCs would be able to interact with undiagnosed cancer cells, which might be in a dormant state. Based on these arguments, further preclinical research is needed to ensure patient safety with MSC therapy. Here, we discuss the basic biology of MSCs, discuss current applications, and provide evidence why it is important to understand MSC biology in the context of diseased microenvironment for safe application.

1. Introduction

Stem cell therapy is not a new field but should be considered as an expanded field to successful bone marrow transplantation for several disorders such as autoimmune diseases and hematological malignancies. Decades of clinical application to reconstitute the hematopoietic system have led to improved methods to increase the age for transplants, resulting in benefit to an aging population [1, 2]. The long history of a focus on hematopoietic stem cells resulted in scientists overlooking other organs with tissue-specific stem cells. This past decade corrected this oversight, resulting in an "explosion" in the number of papers, journals, and scientific meetings on stem cells. The new focus correlated with an increase in registered stem cell clinical trials (clinicaltrials.gov). Those involved in the educational system across the globe are aware that stem cells are moving rapidly to the clinic while the education of future scientists and practicing physicians lags. This review discusses whether clinical trials with stem cells need a pause while scientists and a team of supporting experts become involved in robust investigational studies. We argue that such delay will ensure that stem cell delivery is done safely.

The field of stem cell provided invaluable information in cancer biology, including insights into cancer stem cells. As scientists begin to understand the latter type of stem cells,

one has to ponder if undiagnosed cancer and cancer stem cells would hinder the translation of stem cell to patients. While the information on cancer stem cells is likely to lead to novel approaches to target otherwise evasive cancer cells, their "silence" or dormant phenotype existence has to be a major consideration for the safe treatment with stem cells.

Mesenchymal stem cells (MSCs) continue to show promise in cell therapy [3]. Although there are several reasons to explain why MSCs reached the clinic, a major advantage is based on the science. There is no question that embryonic stem cells (ESCs) can form any cell type. However, ESCs easily respond to *ex vivo* conditions to differentiate into different cell types. ESC "instability" poses challenges with regard to the cells' efficiency to generate a homogeneous population of a desired cell type. More importantly, ESCs can quickly form tumors when placed in an animal [4]. An attractive feature of MSCs is their ability to be used as "off-the-shelf" source for cell therapy [5], making them readily available. However, the advantages currently considered with MSCs do not give these stem cells a "green light" for absolute safety. A major issue that will be discussed in this review is the role of MSCs in cancer. Another issue with MSCs involves the culture conditions to obtain a heterogeneous population.

Despite many reports that MSCs are heterogeneous, it is difficult to determine if this occurs endogenously or if the heterogeneity is an artifact of the culture methods. This difference is an important question that needs to be addressed. Stem cell biologists will need to collaborate with biomaterial companies since they are likely to have existing "libraries" of different surfaces. Robust testing of different surfaces would determine if the type of culture method limits our ability to obtain a pure population of MSCs. However, one must be mindful that there might be an advantage to a heterogeneous population of MSCs. There is a possibility that transplanting heterogeneous MSCs in patients could be advantageous since the different cell subsets might interact to achieve a more effective response such as tissue repair. At this time, there are no "solid" experimental studies to validate the advantage of using a heterogeneous population of MSCs although this question is among many unanswered but fundamental "black boxes" in the field of stem cell biology. These questions seem to arise daily and answers are needed for effective translation to patients. One cannot help but note that the contents within requests for proposals for stem cells by funding agencies, such as the national institutes of health, do not emphasize safety issues. At this time, one wonders if the issue of safety will come to the forefront after deleterious outcomes. If so, this could slow if not end the field of translation in stem cell biology.

2. Mesenchymal Stem Cells (MSCs)

MSCs are in trials for different disorders (clinicaltrials.gov). In parallel, there is intense research to understand how stem cells can be translated for different disorders. MSCs can migrate towards a region of tissue injury, partly due to the expressed chemokine receptors responding to high levels of chemokines at the site of tissue damage [6].

A stem cell can differentiate into multiple lineages and undergo self-renewal. Stem cells play an important role in developmental processes, tissue repair, and protection. In recent years, the use of stem cell for several diseases such as neural and cardiac disorders has become a common theme with great promise as the future of medicine.

MSCs are adult stem cells found in human first-trimester fetal blood, liver, bone marrow, umbilical cord blood, peripheral blood, fetal membrane, placenta, adipose tissues, amniotic fluid, and multiple organs [7–11]. In adults, however, the major organs of MSCs are bone marrow and, if the individual is obese, adipose tissue. Unlike other stem cells, MSCs are easy to isolate and expand *in vitro*, whereas ESCs can form teratoma, along with the ethical issues linked to its derivation from the inner cell mass of human blastocyst [12]. Induced pluripotent stem cells (iPSCs) are derived from somatic cells through the expression of multiple genes. The iPS cells share behavior similar to ESC such as teratoma formation [13].

MSCs are spindle-shaped fibroblastoid cells. Phenotypically, MSCs express CD44, CD73, CD146, CD166, CD90, CD29, CD105/SH2/CD1-5, vimentin and endoglin, SSEA-1, and SSEA-4 [14, 15]. MSCs do not express markers associated with hematopoietic cells such as CD45 and CD34. MSCs can generate osteocyte, chondrocyte, adipocyte, myocardiocytes, neurons, hepatic cells, and bone marrow stromal cells [16–18]. We define transdifferentiation as the ability of stem cells to jump germ layer. Others argue against the transdifferentiation because it is believed that MSCs are derived from neuroectodermal cells.

MSCs interact with both innate and adaptive immune cells to exert dual immune responses, stimulation, and suppression. The type of immune response depended on the tissue microenvironment [19]. In the adult bone marrow, MSCs can be found around the blood vessel forming an interface between the periphery and the cavity [19, 20]. This location strongly suggests that MSCs could be immunologically involved in bone marrow homeostasis [21]. There are several reports stating a switch in the immune property of MSCs, depending on the level of proinflammatory cytokines. As an example, at low level, IFN-γ allows MSCs to be antigen presenting cells by inducing the expression of MHC-II whereas at high levels of IFN-γ, the MSCs switch functions to be immune suppressor [22]. MSCs can engulf foreign particles such as bacteria and, through MHC-II expression, activate T cells [23]. During the activation of T cells, IFN-γ levels are increased. This causes MHC-II expression to be decreased on MSCs and concomitant increase in the program cell death ligand 1 (B7-H1) to suppress the immune response [24].

IFN-γ also interact with other cytokines such as TNF-α to inhibit T-cell activation and to enhance the cytotoxic effect of natural killer (NK) cells and the proliferation and maturation of dendritic cells (DC) [25–27]. Although the exact pathways by which MSCs suppress the immune system remain an active area of investigations, the reports showed MSCs releasing cytokines such as IL-6 and IL-10 to inhibit T-cell receptor-dependent and receptor-independent proliferation of T cells. In line with the suppressive role of MSCs, these stem cells can induce and expand regulatory T cells (T_{regs}), which

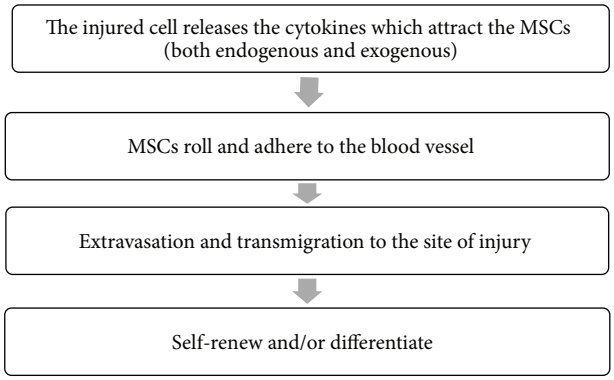

Figure 1: A general scheme is presented to provide an overview on the migration of MSCs to an area of tissue injury. Top row shows the release of cytokines at the region of the tissue to attract the MSCs (rows 2 and 3). Upon reaching the tissue, the MSCs can self-renew and suppress the inflammation or can differentiate to replace the damaged tissue.

are $CD4^+/CD25^+$. T_{regs} can act as negative regulators of inflammatory processes such as autoimmune diseases. Also, in the presence of breast cancer, MSCs can induce T_{regs} through the production of TGF-β [28–30].

In an experimental model of lupus, MSCs inhibit the proliferation and differentiation of B cells [31]. This occurred partly through IFN-γ, which activated the programmed death ligand pathway (PDL-1). The translation of the *in vitro* findings did not show a significant difference in proteinuria but showed a decrease in the deposition of glomerular immune complex. MSCs can also decrease B-cell function through the downregulation of chemokine receptors [32].

The involvement of MSCs with the innate immune system is linked to the expression of Toll-like receptor (TLR), 1–8 [33]. TLR can influence the expression of several cytokines, such as IL-6, IL-8, and IL-10 [19, 33, 34]. TLRs are single membrane noncatalytic proteins, which are important to the innate immunity [35]. TLR can activate NFκB for the regulation of inflammatory cytokines [34].

Figure 1 demonstrates the basic principle of MSCs being attracted to the sites of tissue injury for tissue replacement or remaining as stem cells where they self-renew and prevent further damage. Tumor cells produce cytokines and can be considered an area of tissue damage. Thus, it is not a surprise that MSCs can also home to regions of tumors. The ability of MSCs to migrate to tumors can be explored by engineering the cells to express antitumor cytokines such as IL-12 [36]. Similarly, MSCs are being developed to transport drugs, including those within nanoparticles for brain tumors [37, 38].

3. MSCs-Transplantation

Clinical trials with stem cells, including those with MSCs, are registered in a national database (clinicaltrials.gov). Animal models of spinal cord injury, bone fracture, autoimmune disorder, rheumatoid arthritis, and hematopoietic defects indicated a clinical application for MSCs [6, 39–43]. The transplantation of MSCs could be from allogeneic or autologous sources. It appears that autologous transplant might pose a risk because when expanded MSCs are reintroduced into its host, the MSCs might be perceived differently from the endogenous (unexpanded) MSCs [22]. Due to the immune suppressor properties of MSCs, these cells are widely used to minimize graft versus host disease (GvHD).

The intent of transplanting MSCs for GvHD is to eventually replace or reduce the level of steroids to prevent the untoward effect associated with steroids [36]. The biggest issue with allogeneic transplantation to replace the hematopoietic system is the mismatch at the major histocompatibility complex (MHC) between the donor and recipient. As third party cells, MSCs can exert immune suppression known as veto function [44]. This property forms the basis for MSCs as third party cells to subjects receiving allogeneic bone marrow cells in transplantation. Based on the ability of MSCs to suppress allorejection, the method can be similarly applied for organ transplant. An application to suppress organ rejection will require experimental studies in large animals, which are costly but in the long-term will benefit patients and also reduce healthcare cost.

IL-10 is important during allogeneic transplantation because it inhibits IFN-γ production and suppresses the antigen-presenting cells, indicating that the IL-10 would prevent MSCs converting to immune-enhancing cells [23, 45]. IL-10 primed MSCs resulted in a lower mortality rate than untreated MSCs and showed significant reduction of reduced GvHD [46]. The application of MSCs for acute GvHD underscores the promise of allogeneic MSCs for stem cell therapy. Similarly, clinical trials using allogeneic MSC for acute myocardial infarct improved the patients' condition, although the mechanisms by which this occurs have not been described [5, 36]. MSCs are tested in ongoing clinical studies for neurological disorders such as Parkinson's disease and multiple sclerosis [36].

4. Regenerative Medicine: Other Applications

Thus far, this review mostly discussed the safety of MSCs as therapeutic use for immune suppression. However, MSCs can be used in regenerative medicine to repair damaged tissues [47]. This field represents another arm of stem cell treatment treatment in which the cells are not given to "self" but to another individual representing a different allogeneic host. Although still ongoing, this type of treatment is valuable to tissue repair to preserve organs before the damage requires transplantation. In addition to tissue regeneration, stem cells, in particular ESCs, can also be used to screen drugs.

ESCs can be induced to form any cell type such as cardiac cells. Thus, a single clone of ESC can be used to test different cell types through a rapid screening process. This will be an efficient method to prevent expensive studies and to enhance the process of getting new drugs to the clinic [48]. Although limited, ESC-derived cells are in the clinic to treat macular degeneration, heart failure, neurodegenerative disorders, and diabetes (clinicaltrials.gov). ESCs have been shown to differentiate into neural cells such as dopamine and serotonin neurons [49]. While these neurons could be

used as a treatment for degenerative diseases or to repair stroke damage, there are lingering concerns with the use of ESC-derived cells. Besides the ethical reasons, these ESC-derived cells have the potential to either differentiate or dedifferentiate into the cell type that they were originally programmed to create [50].

Transplantation of hematopoietic stem cells (HSC) has been tried since 1959 to repopulate the hematopoietic system. The method has since been applied to humans for cancer, primary immunodeficiency, and other heritable and acquired diseases [51]. More recently, hematopoietic transplantation is combined with MSCs as stem cell immunotherapeutics to prevent acute GvHD [52]. Hematological malignancies such as myelomas seem to be better targeted with autologous stem cell transplant. A clinical trial with MSCs for GvHD did not show significant progress indicating additional research to effectively bring MSCs as adjuvant to transplantation [21].

The treatment of cardiac damage was tried with transplanting bone marrow mononuclear cells, which includes a mixed population of HSCs, MSCs, progenitor cells, and other hematopoietic cells [53]. Cardiac repair trials with these bone marrow cells include Bone Marrow Transfer to Enhance ST-Elevation Infarct Regeneration (BOOST) and the Transplantation of Progenitor Cells and Regeneration Enhancement to Acute Myocardial Infarction (TOPCARE-AMI). These studies resulted in improvement of the left ventricular efficiency [54–56].

To reiterate, MSCs can differentiate into different cell types such as osteocytes, chondrocytes, fibroblast, adipocytes, myocardiocytes, neurons, and hepatic cells [16–18, 57]. The differentiated cells would benefit from the field of tissue engineering for tissue repair. MSCs can also secrete factors to regulate the microenvironment to aid the tissue repair process [57]. The discussion on MSCs as immune regulators and the influence of this property to tissue repair underscores the "attractiveness" of these cells for translation to patients. Indeed, there are listed clinical trials that use MSCs for the treatment of diabetes, cirrhosis of the liver, ulcerative colitis, and spinal cord injuries (clinicaltrials.gov). In addition to bone marrow derived cells, MSCs also show promise for cardiac repair [58, 59].

5. Cancer Stem Cell

The cancer stem cell hypothesis proposes a small subpopulation of cancer cells that contribute to tumor formation. These cells, known as cancer stem cells (CSCs), have the ability to evade therapeutic treatments thus allowing for recurrence of cancer and metastasis [60]. Current therapies often attack the bulk of tumor cells leaving the CSCs. The surviving CSCs can continue to form new tumors or can adapt dormancy. CSCs can be caused by genetic mutations as well as other molecular changes associated with oncogenesis [61]. The method by which the CSCs survive as dormant cells and remain undetected and evade treatment is unknown. However, the experimental evidence strongly suggested that the host microenvironment and intercellular communication between the CSCs and microenvironmental cells can cause the CSCs to remain as dormant cells or cause tumors through the generation of proliferating cells and metastasis to other sites [61]. This section is included in this review because it underscores the problem that MSC treatment will encounter when they are transplanted into a subject with dormant CSCs. This issue is particularly important because MSCs can support tumor growth and also protect from the immune system. This confound is discussed below.

6. Breast Cancer Cells: MSC Interaction

This section further expands on CSCs of the breast since the method by which these cells adapt dormancy has been well studied. It is the goal of this review that, as MSCs progress in the clinic, the safety issue described throughout would extrapolate on the information on breast cancer for other cancers. The phenotype of breast CSCs have been well described although this is still a work in progress [62]. Breast cancer has a predilection to home and integrate to the bone marrow where they retain dormancy [63]. Once in the bone marrow, the cancer cells establish quiescence by intercellular communication with resident stromal cells [62]. Cells within the bone marrow microenvironment can also support reverse dormancy for the eventual progression and metastasis, which could partly explain resurgence [64]. MSCs, which constitute the stromal compartment of the bone marrow, can influence the migration of the cancer cells in and out of the bone marrow [64]. MSCs support the growth, invasiveness and metastatic potential of breast cancer [65–67].

7. Potential Confounds of MSC Treatment

There are several reports on the involvement of MSCs to support and protect solid tumors [68–70]. A major consideration when treating a patient with MSCs is undiagnosed tumor. For example, in the case of breast cancer, the experimental studies as well as the clinical evidence indicated that breast cancer cells can survive in a state of mitotic arrest for long periods as dormant cells [44, 62, 71–75]. In many cases of cancer resurgence, the bone marrow has been identified as the source of tertiary metastasis indicating the survival of initiating cancer cells in bone marrow [71–74]. This indicates that the bone marrow could be home to dormant breast cancer cells and that the original disseminated tumor cells can survive for >10 yrs [75, 76].

The heterogeneity of breast cancer cells is being developed as a hierarchy and this organizational structure is based on the relative maturation of the different subsets [62]. MSCs can induce T-cell responses such as regulatory T cells (T_{regs}) to protect the cancer cells from immune cytotoxicity. The future of MSC therapy will depend on how T cells respond to the different subsets of breast cancer cells. This is important with regard to safety when MSCs are used for treatment because while they protect the tumor from the immune system through T_{regs}, they can also support the growth of tumor cells [28, 77]. Breast cancer cells interact with MSCs through membrane-bound stromal cell-derived factor 1α (CXCL12) and its receptor, CXCR4 [78, 79]. It is unclear if all subsets interact equally. This is important in going forward to understand if stem cells, such as MSCs, should be delivered

in conjunction with other agents to counteract the MSCs supporting dormant tumor cells. This brings up another point on selecting who should oversee the treatment with MSCs. In most countries, the physicians are specialized and become experts in his or her field. The field of stem cell challenges the isolationist approach. The building of teams of different subspecialty to include translational scientists would be most efficient to bring stem cells to the clinic.

As discussed above, MSCs can support tumors through increased growth and/or protect the tumor by suppressing the immune response [28, 78, 80–87]. Thus, it is important to study the immune response between MSCs and each subset of breast cancer cells. Ideally, it would be advisable to eradicate cancer and then deliver stem cells. However, at this time, there is no method to eradicate cancer, despite intense studies on the interaction between distinct cancer cell subsets and the microenvironment [88]. At this time, stem cell therapy will need to consider how the stem cells might affect the recipient who is a cancer survivor. One would assume that the surviving cancers are phenotypically stem cells. *In vivo* studies are lacking to understand how cancer stem cells interact with MSCs. Until these studies are conducted, the existence of cancer stem cells remains a liability issue for stem cell therapy. The authors of this paper do not believe that this is an easy issue for those involved in bringing stem cells to patients, but it is a fundamental problem that requires research to safely bring stem cells to patients.

Another safety issue that has little attention is the crosstalk between stem cells and molecules within the microenvironment where stem cells home and integrate. Stem cells, through specific receptors, can initiate a crosstalk with the milieu within an area of tissue injury. The site of tissue damage is likely to produce inflammatory mediators that can interact with specific receptors on the stem cells. The stem cells, in turn, would respond and produce soluble factors to activate the cells within the microenvironment [89]. Thus, it is important to understand how stem cells will respond within an area of tissue injury and whether this could be a question of safety before stem cells are given to patients.

8. Summary and Conclusion

This paper provides evidence to support future application of MSCs for regenerative medicine and anti-inflammatory processes. The applications for MSCs are discussed with the information that these cells could cause untoward effects. Thus, we noted a need for additional research to ensure patient safety. In particular, we cited the importance of studies to dissect the interaction between the transplanted MSCs and the tissue microenvironment. More importantly, the treatment of MSCs needs to be safe. We therefore discussed the interaction between MSCs and different subsets of breast cancer cells, in particular the cancer stem cells. MSCs can interact with cancer stem cells and support their growth. Going forward, MSC treatment will need to consider that the host may have undiagnosed cancer that could be influenced by the transplanted MSCs. We propose that parallel research studies are needed on cancer stem cells and MSCs. In summary, we propose that robust studies are needed to examine MSC biology in different diseases prior to clinical application since this will improve patient safety and increase the efficacy of stem cell treatment.

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Research Article

Differentiation of Dental Pulp Stem Cells into Neuron-Like Cells in Serum-Free Medium

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Dental pulp tissue contains dental pulp stem cells (DPSCs). Dental pulp cells (also known as dental pulp-derived mesenchymal stem cells) are capable of differentiating into multilineage cells including neuron-like cells. The aim of this study was to examine the capability of DPSCs to differentiate into neuron-like cells without using any reagents or growth factors. DPSCs were isolated from teeth extracted from 6- to 8-week-old mice and maintained in complete medium. The cells from the fourth passage were induced to differentiate by culturing in medium without serum or growth factors. RT-PCR molecular analysis showed characteristics of $Cd146^+$, $Cd166^+$, and $Cd31^-$ in DPSCs, indicating that these cells are mesenchymal stem cells rather than hematopoietic stem cells. After 5 days of neuronal differentiation, the cells showed neuron-like morphological changes and expressed MAP2 protein. The activation of *Nestin* was observed at low level prior to differentiation and increased after 5 days of culture in differentiation medium, whereas *Tub3* was activated only after 5 days of neuronal differentiation. The proliferation of the differentiated cells decreased in comparison to that of the control cells. Dental pulp stem cells are induced to differentiate into neuron-like cells when cultured in serum- and growth factor-free medium.

1. Introduction

Dental pulp tissue contains many types of cells including committed cells (e.g., endothelial cells) and uncommitted cells (i.e., DPSCs). DPSCs are of mesenchymal stem cells (MSCs) [1]. In mice, the majority of MSCs were isolated from bone marrow [2] and peripheral blood [3, 4]. These MSCs can be characterized by the expression of specific gene markers such as *CD44*, *CD73*, *CD90*, *CD105*, *CD117*, and *CD166* [5, 6].

DPSCs are capable of differentiating into multilineage cells [7–9] including neuron-like cells [10]. Neuron-like cells differentiated from MSCs derived from bone marrow cells [11–13] and brain [14]. However, MSCs derived from dental pulp, that is, DPSCs, are also capable of differentiating into neuron-like cells [10]. The characteristics of MSCs from bone marrow are similar to those cells derived from dental pulp [11]. Both types of MSCs express *Cd44*, *Cd106*, *Cd146*, and *Cd166* [15–17].

Many factors are involved in neuronal differentiation including nestin [18], tubulin3 (Tub3) [19], and MAP2 [20]. Nestin is involved in the radial growth of axons during neuronal differentiation in vertebrate cells [19, 21]. Therefore, Nestin is known as a neural marker and its presence can be considered as a criterion for the ability to differentiate

Table 1: Primers involved in RT-PCR experiments.

Gene/accession no.	Primers	Sequences	Expected product size (bp)	Annealing temperature (°C)
Cd146 (NM_023061)	Forward	5′-GGACCTTGAGTTTGAGTGG-3′	479	60
	Reverse	5′-CAGTGGTTTGGCTGGAGT-3′		
Cd166 (NM_009655)	Forward	5′-AACATGGCGGCTTCAACG-3′	630	61
	Reverse	5′-GACGACACCAGCAACGAG-3′		
Nestin (NM_016701)	Forward	5′-CGCTCGGGAGAGTCGCTT-3′	215	64
	Reverse	5′-CCAGTTGCTGCCCACCTTC-3′		
Gapdh (NM_008084)	Forward	5′-CAACGGCACAGTCAAGG-3′	717	62
	Reverse	5′-AAGGTGGAAGAGTGGGAG-3′		
Tub3 (NM_023279)	Forward	5′-ACGCATCTCGGAGCAGTT-3′	125	61
	Reverse	5′-CGGACACCAGGTCATTCA-3′		
Cd31 (NM_001032378.1)	Forward	5′-GGTCTTGTCGCAGTATCAG-3′	355	58
	Reverse	5′-ATGGCAATTATCCGCTCT-3′		

into neurons [18, 22]. However, Nestin has shown to be expressed by other cell types such as hair follicle stem cells [23], pericytes [24], endothelial cells [25], myofibroblasts, and pancreatic fibroblasts [26]. Therefore, analysis on expression of other specific neuron markers such as Tub3 [27, 28] and MAP2 [29, 30] has been done concurrently for neuronal confirmation. Tub3 and MAP2 play a role in the stability of axons and neuronal cell bodies [20, 31]. Certain growth factors, such as epidermal growth factor, basic fibroblast growth factor, and retinoic acid, were used for neuronal induction [32–35]. Dimethyl sulfoxide (DMSO) was also used to induce transformation of MSCs into neuron-like phenotypes *in vitro* [12, 13]. The objective of the present study was to examine the directed differentiation of DPSCs into neuron-like cells in the absence of chemical induction.

2. Materials and Methods

2.1. Isolation of Dental Pulp Cells. Incisor teeth were extracted from 6- to 8-week-old mice under sterile conditions and placed in medium containing 1X PBS (Sigma, USA). The extracted dental pulp was washed with 1X PBS containing 1% (v/v) penicillin-streptomycin (Invitrogen, USA).

Dental pulp tissue was incubated for 1 hour in 4-unit collagenase type I at 37°C, followed by several rounds of enzymatic disaggregation. The cells were centrifuged at 1200 g for 10 minutes at 25°C and cultured in complete medium consisting of α-MEM (Invitrogen, USA) supplemented with 20% (v/v) FBS (Biowest, USA) and 1% (v/v) penicillin-streptomycin. The cells 1×10^5 cells/mL obtained were put in a T25 flask containing complete medium and cultured in an incubator with 5% CO_2 atmosphere and 95% humidity at 37°C.

After 24 hours, the suspended cells were removed from the medium, and the flask was washed with 1X PBS solution. The cells were grown in complete medium until 80% confluency. A solution of 0.25% (v/v) trypsin-EDTA (Sigma, USA) was used to detach the dental pulp cells from the flask surface for subculturing in another flask at 1×10^5 cells/mL. For cryopreservation and storage, cells at the fourth passage were placed in cryovials containing freezing medium consisting of α-MEM, 10% (v/v) DMSO (Sigma, USA), and 50% (v/v) FBS and stored in liquid nitrogen. In this study, the cells used were at the fourth passage.

2.2. Differentiation of Dental Pulp Stem Cells into Neuronal Cells. Approximately 1×10^5 cells were transferred into 24-well plates containing complete medium and were allowed to grow for 24 hours until adherence. The medium was discarded, and the cells were washed with PBS. The cells were then cultured in serum- and growth factor free-medium consisting of α-MEM and 1% (v/v) penicillin-streptomycin for 5 days. The medium was changed every 2-3 days during this 5-day period. As a control, the same number of cells was cultured in complete medium (consists of α-MEM, 1% (v/v) penicillin-streptomycin, and 15% (v/v) fetal bovine serum) for 5 days. Cell morphology was monitored on days 2 and 5 of neuronal differentiation using an Olympus phase-contrast microscope.

2.3. Molecular Analysis Using RT-PCR. Control and differentiated cells were detached using 0.25% (v/v) trypsin-EDTA and centrifuged at 1400 g for 10 minutes. Total RNA was extracted using TRI-reagent (Sigma, USA) according to the manufacturer's protocol. The purity of the total RNA was assessed spectrophotometrically at 260 and 280 nm, with an $A_{260} : A_{280}$ ratio of 1.8–2.0 considered acceptable. Approximately, 300 ng of total RNA sample was used for each RT-PCR reaction with the Access Quick RT-PCR system kit (Promega, USA). The primers were designed using the Primer Premier 5.0 software program based on sequences obtained from NCBI. Information on the primers is summarized in Table 1.

Primary cDNA synthesis was performed using AMV reverse transcriptase for 45 minutes at 45°C followed by deactivation for 2 minutes at 94°C. The amplification consisted of denaturation for 30 seconds at 94°C, annealing for 60 seconds, and extension for 60 seconds at 68°C, performed for 40 cycles. A final extension step was performed for 7 minutes at 68°C. The amplified products were separated using

1% (w/v) agarose gel electrophoresis, stained, and analysed. The amplified products were subjected to DNA sequencing and verified using the BLASTN program from NCBI. As for expression level analysis, a total of 1 μg of total RNA was used for each amplification and intensity was determined using online ImageJ 1.47 program (http://rsbweb.nih.gov/ij/).

2.4. Immunocytochemistry of MAP2. The induced neuronal cells were washed with 1X PBS and fixed at 4°C with 4% (v/v) paraformaldehyde for 2 hours. The cells were washed 3 times with 0.05% PBS-Tween 20 followed by 2% PBS-Triton-X for 10 minutes. Then, the cells were washed again 3 times with 0.05% PBS-Tween 20. The cells were incubated at 37°C with 10% goat serum diluted in 0.05% (v/v) PBS-Tween 20 plus 0.01 mg/mL BSA for 30 minutes prior to incubation of primary antibody. This solution was removed and primary antibody at 1 : 200 diluted with 0.05% (v/v) PBS-Tween 20 plus 3% (v/v) goat serum and 1 mg/mL BSA were added for 24 hours at 4°C. Then, the cells were washed 3 times with 0.05% (v/v) PBS-Tween 20 followed by incubation of secondary antibody anti-mouse IgG-FITC for 30 minutes at room temperature diluted at 1 : 50 with Tween 20 plus 3% (v/v) goat serum and 1 mg/mL BSA. Finally, the cells were washed 3 times with 0.05% (v/v) PBS-Tween 20 prior to analysis using fluorescence microscope. Negative control used was undifferentiated cells without neuronal induction, that is, cultured in complete medium.

2.5. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT) Assay. Approximately 1×10^4 cells were seeded in 96-well plates and incubated for 24 hours at 37°C. Two groups of cells were cultured: one in complete medium (control) and the other in serum- and growth factor-free medium. After, 24-hour incubation, approximately 20 μL MTT (5 mg/mL) was added to each sample and the samples were incubated for another 4h at 37°C. The mixture was slowly removed, 200 μL of DMSO was added to each well, and the wells were measured by an ELISA Plate Reader at 570 nm. Each experiment was repeated in triplicate. The cells were subjected to MTT assay on the first, third, and fifth days of neuronal induction. The number of viable cells for each analysis was determined using a standard graph created prior to the experiment.

2.6. Statistical Analysis. Data from the differentiated and control groups were compared using paired t-test in SPSS program version 16.0.2. Differences with a P value < 0.05 were considered statistically significant. Data obtained were presented as average (mean ± SD; standard deviation) from three independent experiments ($n = 3$).

3. Results

3.1. Identification of Mesenchymal Stem Cells in Dental Pulp Tissue. The identity of dissociated cells isolated from dental pulp tissue using collagenase was confirmed by their capacity to form adherent colonies consisting of sphere-like clusters of cells (Figure 1). Averages of 6.8×10^4 cells/cm^2 were found capable to obtain colonies after 24 hours cultured in the complete medium. Then, the suspended cells were discarded and only adherent cells were expanded in the medium. The suspended cells may have been cells that were unable to survive in the medium. The colonies began to change their shape during the second passage. The cells assumed a fibroblast-like morphology with a long, thin body during the fourth passage and became confluent after 2 to 3 days of *in vitro* culture in complete medium.

Molecular analysis was performed to validate the types of cells in the fourth passage. The total RNA was extracted from the fourth passage of dental pulp cells and was subjected to RT-PCR analysis (Figure 2). This analysis showed *Cd146* and *Cd166* amplicons in these cells, whereas activation of *Cd31* was not observed. The amplicon of *Gapdh* was found in dental pulp cells both before and after differentiation. Analysis of expression level for *Cd146* and *Cd166* was shown to produce 118.0 ± 16.8% and 77.5 ± 14.3%, respectively, when compared to *Gapdh* (100%) which was used to normalize the cellular mRNA level.

3.2. Morphological Changes into Neuron-Like Cells and Expression of MAP2 Protein. After 5 days of culture, most of the cells showed a morphological change to a long, thin body shape (Figure 3). The cytoplasm was contracted toward the nucleus and assumed a multipolar shape. The cells displayed small, spherical, and contracted bodies and a conical cytoplasm with branches resembling the neuronal perikaryon, axon, and dendrite. The perikaryon, dendrite, and axon of a neuronal cell are indicated, respectively, by a white arrow, an open arrowhead, and a black arrow. Approximately 60%–70% of the cell population differentiate into neuron-like cells, an indication that differentiation occurred due to the absence of serum and growth factors but not because of spontaneous differentiation. DPSCs expressed the neuron-specific protein marker MAP2 after 5 days of culture in serum- and growth factor-free medium (Figure 3). However MAP2 was not detected in DPSCs or undifferentiated cells without neuronal induction, that is, cultured in complete medium (negative control) (Figure 3).

3.3. Activation of Neuronal Markers. The cells showed expression of *Cd146*$^+$, *Cd166*$^+$, and *Cd31*$^-$ before differentiation. RT-PCR analysis showed the presence of a *Nestin* amplicon (~215 bp) in the cells, both before and after differentiation. However, the intensity of *Nestin* activation was significantly higher after differentiation compared with before differentiation (Figure 4(a)). An amplicon of *Tub3* (~125 bp) was observed after day 5 of neuronal differentiation (Figure 4(b)). *Tub3* was shown to be activated after 5 days of neuronal differentiation. *Gapdh* was used as a positive control both before and after differentiation. A *Gapdh* amplicon (~717 bp) was found in cells both before and after differentiation, indicating that *Gapdh* remains activated in both types of cells (Figure 4(c)).

3.4. Proliferation of Dental Pulp Stem Cells during Differentiation. Cell viability studies were performed to assess

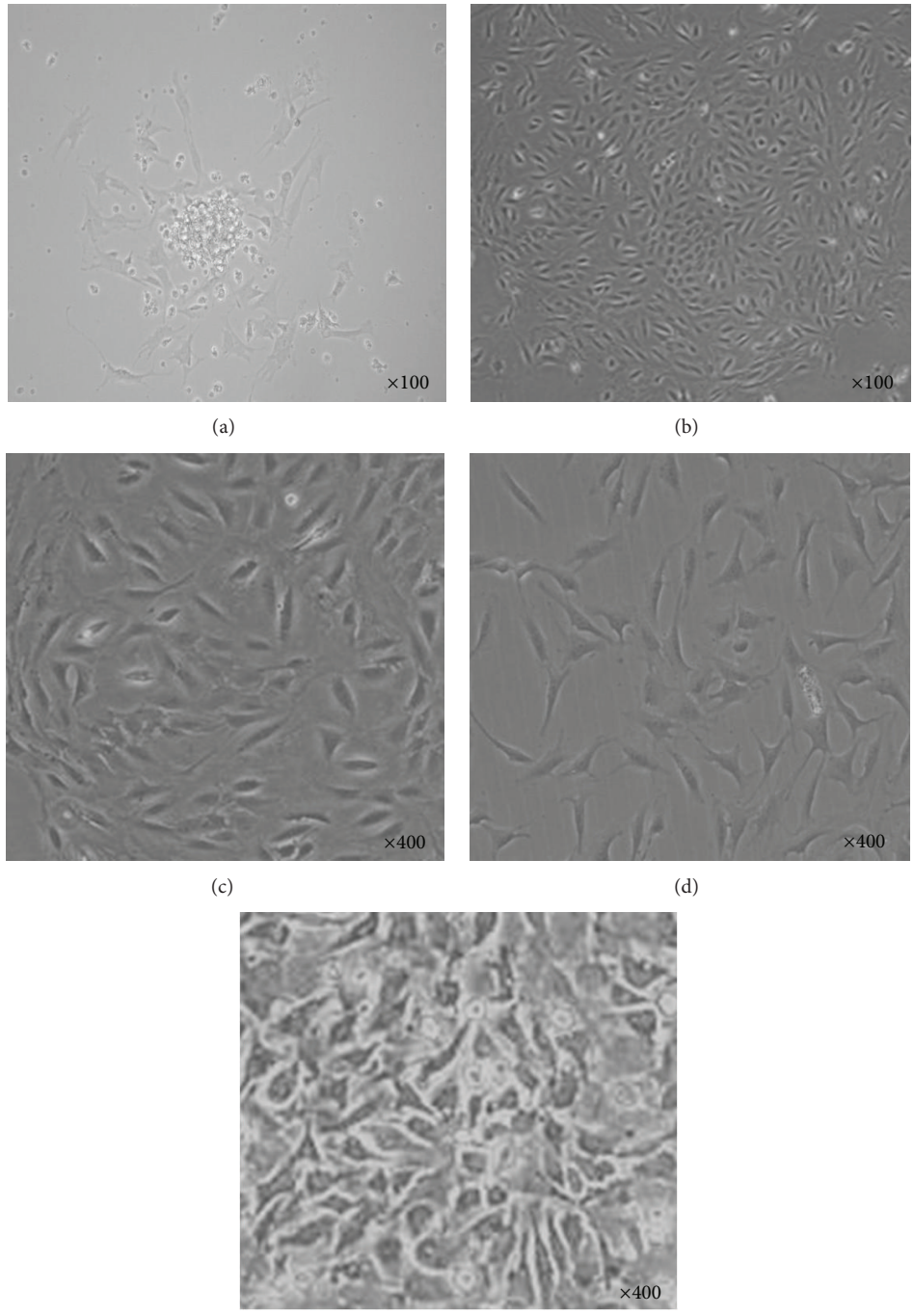

Figure 1: Characteristics of isolated and *in vitro* mouse dental pulp stem cells. Colonies derived from dental pulp at the first passage (a) and after 24 hours of culture (b). Colonies began to show changes in shape after the second passage (c), a fibroblastic cell shape after the fourth passage (d), and confluence after 2-3 days of culture in complete medium (e).

the proliferation capacity of both differentiated and undifferentiated cells during neuronal differentiation. The numbers of control and differentiated cells were significantly ($P < 0.05$) increased upon differentiation compared with day 0 of culture (Figure 5). However, the proliferation capacity of the cells began to change gradually after 24 hours of culture until day 5 of neuronal differentiation, with the number of control cells remaining higher. Both types of cells maintained their growth rate during the initial 24 hours, of culture. However, the differentiated cells showed a reduction of growth rate after 24 hours whereas the undifferentiated (control) cells maintained their growth rate, resulting in an increased number of viable

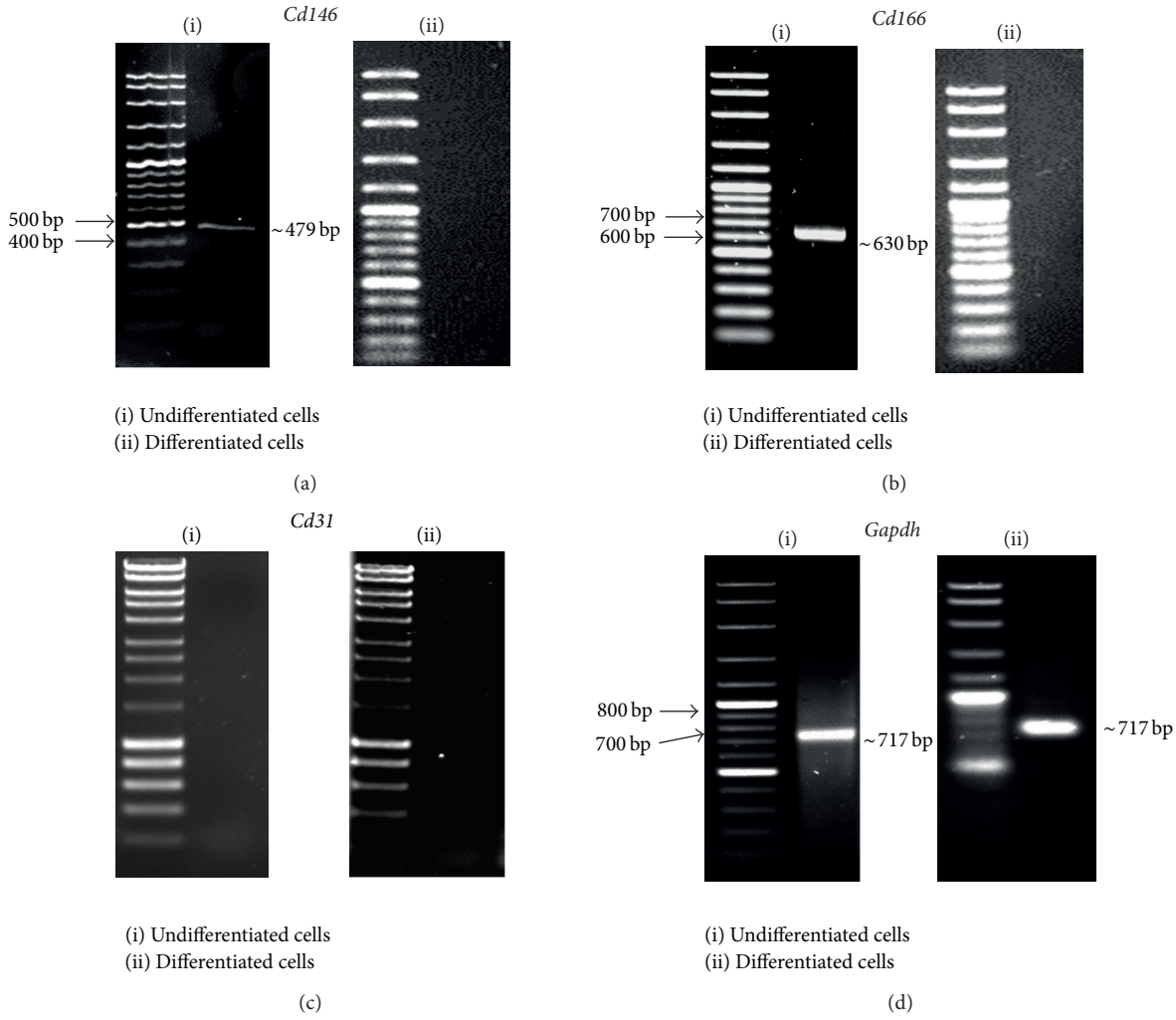

Figure 2: Activation of mesenchymal stem cell markers. The activation of *Cd146* (~479 bp) (a) and *Cd166* (~630 bp) (b) was observed only in cells before differentiation, indicating that the cells were mesenchymal stem cells. *Cd31* was inactivated before and after neuronal differentiation (c). *Gapdh* (~717 bp), a housekeeping gene, was expressed before and after differentiation (d). Panel (i) is representing undifferentiated cells, that is, cells before neuronal differentiation, while panel (ii) is representing differentiated cells, that is, cells after neuronal differentiation.

cells. Statistical analysis showed a significant difference ($P < 0.05$) in cell number between the two types of cells at days 3 and 5 of culture (Figure 5).

4. Discussion

The cells formed a fibroblast-like morphology during the fourth passage. Molecular analysis showing *Cd146* and *Cd166* amplicon but not *Cd31* validated that the fibroblast-like cells were MSCs rather than hematopoietic stem cells. *Cd146* and *Cd166* are mesenchymal stem cell markers [36, 37]. *Cd146* is an early mesenchymal stem cell marker expressed within dental pulp tissues [36]. *Cd166* is a cell adhesion molecule that plays important roles in tight cell-to-cell interaction and in the regulation of MSCs differentiation [38]. While *Cd166* is expressed in a wide variety of tissues, it is usually restricted to subsets of cells involved in processes of dynamic growth and/or migration, including neural development and immune response [39]. The amplicon of *Gapdh* found in DPSC both before and after differentiation indicated that *Gapdh* is expressed in both types of cells.

To confirm the differentiation of DPSCs to a neuronal phenotype and to demonstrate that this differentiation was not an artefact, three analyses were performed: morphological changes, expression of MAP2, and activation of neuronal markers. After 2 days of culture, most of the cells resembled multipolar neuron. However, some fibroblast-like cells with spread-out morphology were still observed in the population. We suggest that DPSCs changed gradually and differentiated into neuron-like cells after 5 days when cultured in serum- and growth factor-free medium. DPSCs from various tissues differentiated into neuronal cells by displaying neuron morphology [40, 41], similar to our observations. Thus,

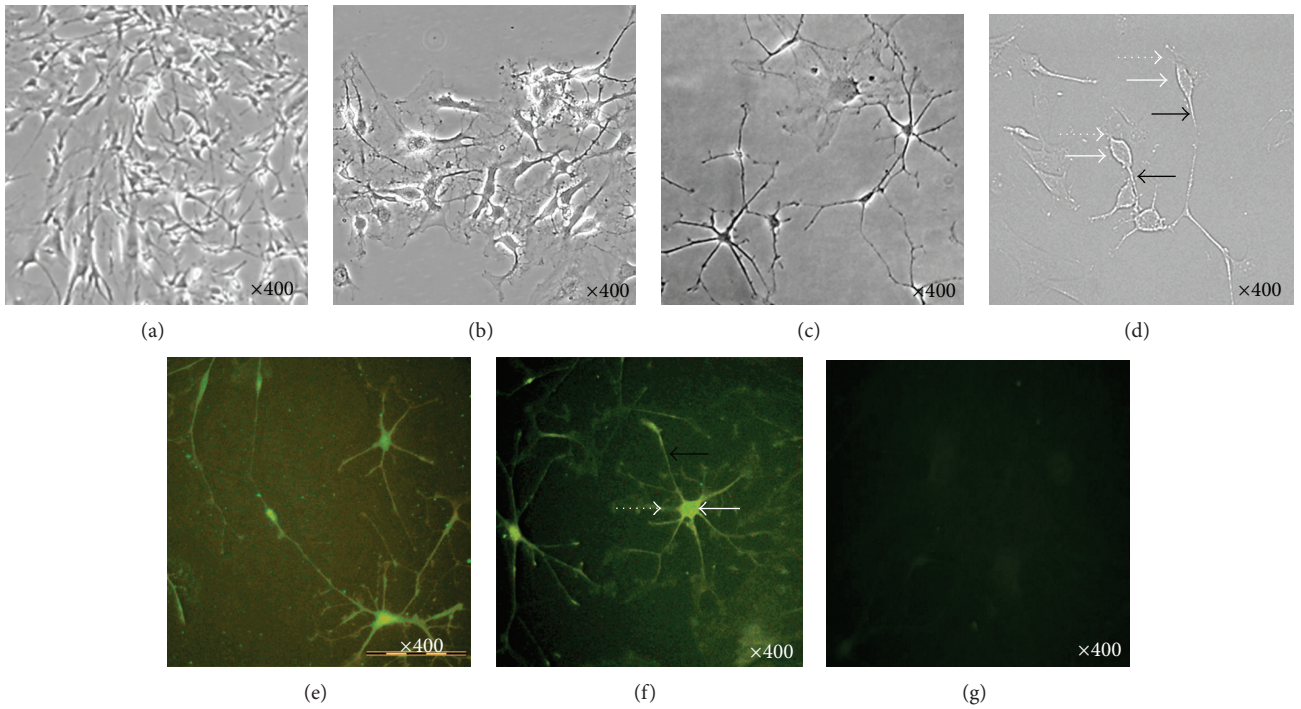

Figure 3: Characteristics of differentiated cells. Neuron-like cells appeared among the dental pulp stem cells ((a), (b)) 5 days after neuronal induction (c). The perikaryon, dendrites, and axons of neurons are indicated, respectively, by white arrows, open arrowheads, and black arrows (d). Immunofluorescence staining for neuron markers MAP2 was performed after 5 days of neuronal induction ((e), (f)). MAP2 marker was not detected in DPSCs without neuronal induction, that is, negative control (g).

morphological analysis indicated that DPSCs isolated from dental pulp tissue differentiated into neurons.

RT-PCR analysis showed the presence of a *Nestin* amplicon. *Nestin* is a neuron marker in adult rat and human brains [42]. Both *Nestin* and *Tub3* were also used as markers to investigate neuronal differentiation in the hippocampus of mice after DPSC implantation [43]. The expression of both *Nestin* and *Tub3* after differentiation indicated that the DPSCs differentiated into neuronal cells. Nestin is one of the intermediate filaments found in the cytoskeleton of vertebrate cells [19, 44]. *Nestin* expression was used to track the proliferation, migration, and differentiation of neuronal stem cells. *Tub3* is expressed in all eukaryotic cells. It contributes to microtubule stability in neuronal cell and plays a role in axonal transport. In the present study, *Tub3* was activated after 5 days of neuronal differentiation. *Gapdh* was used as a positive control both before and after differentiation. *Gapdh* is a housekeeping gene which has always been activated by all mammalian cells whether by undifferentiated or differentiated cells [45]. It was used to determine the RNA quality of isolated DPSCs and to normalize the levels of mRNAs. A *Gapdh* amplicon was found in cells both before and after differentiation, indicating that *Gapdh* remains activated in both types of cells.

Nestin activation was increased during neuronal differentiation. *Nestin* is known to be expressed within fibrous dental pulp tissue, however, its expression continued to be detected by the majority of DPSCs following neuronal induction; that is, expression of *Nestin* was increased when cells differentiated into neuron [28]. MAP2 and *Tub3* are expressed only after neuronal differentiation and are therefore utilized as markers of mature neuronal cells during the final stages of growth [46–48]. MAP2-positive neuronal cells have been shown to be adult neuronal cells produced by neuronal induction. Varied expression of *Nestin* in several cell types such as hair follicle stem cells, pericytes, endothelial cells, myofibroblasts, and pancreatic fibroblasts makes *Nestin* not specific to neuron [23–26]. However, combination of *Nestin* expression together with the expression of other neural markers, that is, *Tub3* and MAP2, can support neuronal differentiation. In addition, characterization of the neuron-like cell by morphological analysis also showed a positive result.

While growth factors such as FGF and retinoic acid have been employed in previous studies, neuronal induction was induced in the present study solely by excluding serum and growth factors from the complete medium. Our observations on *Tub3* and *Nestin* activation, neuron-like cells morphology, and MAP2 expression provide evidence for the directed differentiation of mouse dental pulp stem cells into neuron-like cells in serum- and growth factor-free medium.

The proliferation of the differentiated cells continued although their growth rate was lower than that of the control group. Theoretically, proliferation and differentiation of cells cannot occur simultaneously. The signalling cues that coordinate these two processes are largely unknown. However, cell differentiation and proliferation are regulated simultaneously

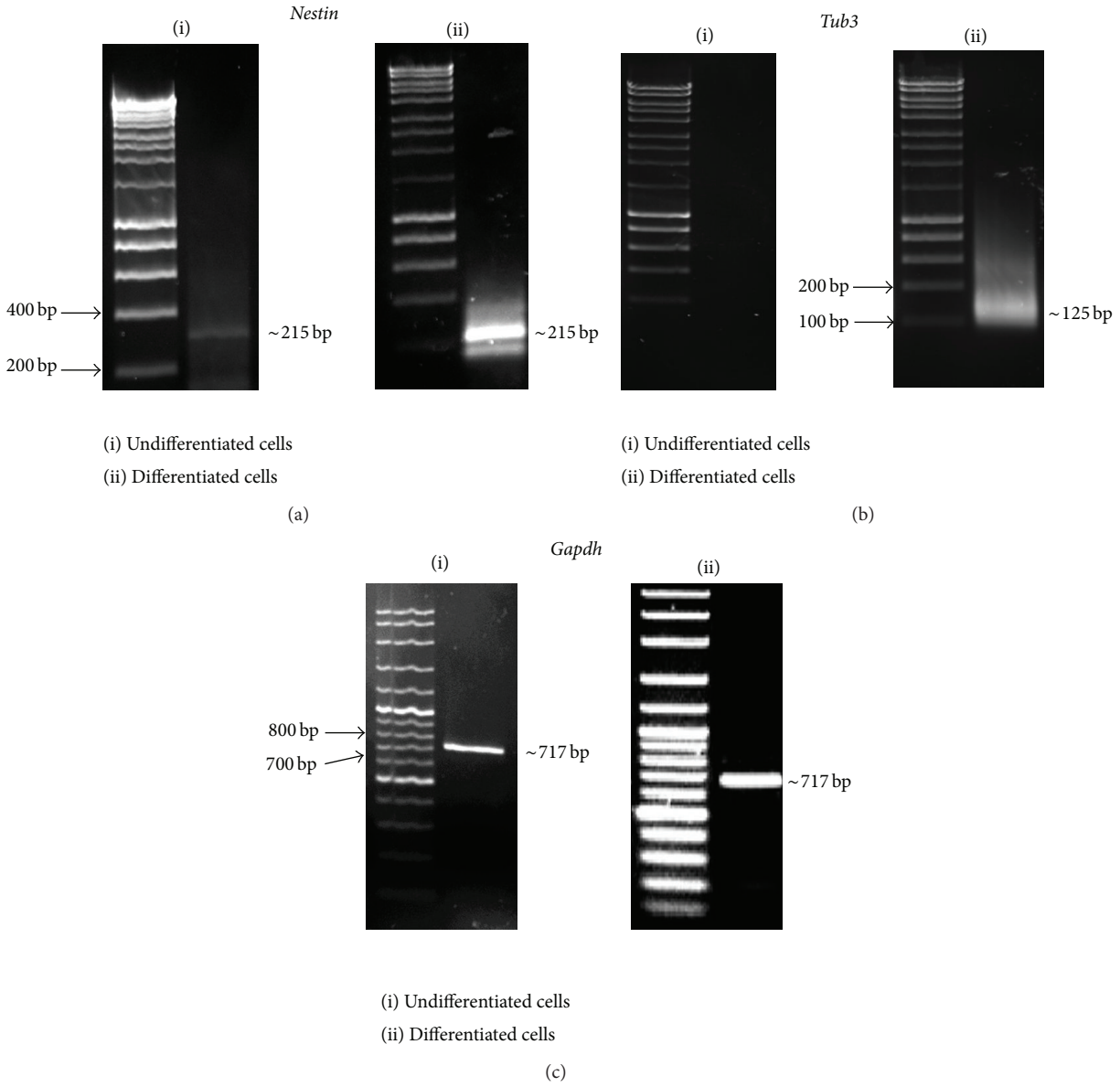

Figure 4: Activation of specific neuronal markers.The activation of *Nestin* (~215 bp) (a) and *Tub3* (~125 bp) (b) indicated that the cells had differentiated into neurons. *Gapdh* (~717 bp), a housekeeping gene, was activated before and after differentiation (c). Panel (i) is representing undifferentiated cells, that is, cells before neuronal differentiation, while panel (ii) is representing differentiated cells, that is, cells after neuronal differentiation.

but independently, that cells often start differentiating long before they stop dividing, and that the initiation of differentiation is not restricted to any particular segment of the cell cycle [49, 50]. In the present study, differentiated cells began to divide slowly after 24 hours of culture and continued until the end of the differentiation period, suggesting that the cells undergo differentiation immediately after neuronal differentiation has been induced. Although the number of cells increased upon the induction of differentiation, the cells subsequently showed a growth rate reduction and focused on the differentiation process.

Capabilities of adult stem cells to differentiate into cells from different germ layers or cell lineages are known as transdifferentiation. Stem cells from bone marrow which originated from mesoderm were shown to be able to differentiate into liver, lung, gastrointestinal tract, and skin cells which derived from endoderm and mesoderm [51]. Peripheral blood stem cells (hematopoietic stem cells) also were found able to differentiate into mature cells which were not originated from hematopoietic cells such as liver, skin epithelial, and gastrointestinal tract cells [52]. Changes that occur to cell microenvironments such as addition of certain growth or differentiation factors during *in vitro* cell culture were found to be able to induce transdifferentiation [53]. Deletion of serum also contributed to this transdifferentiation since addition of serum in the medium allowed growth

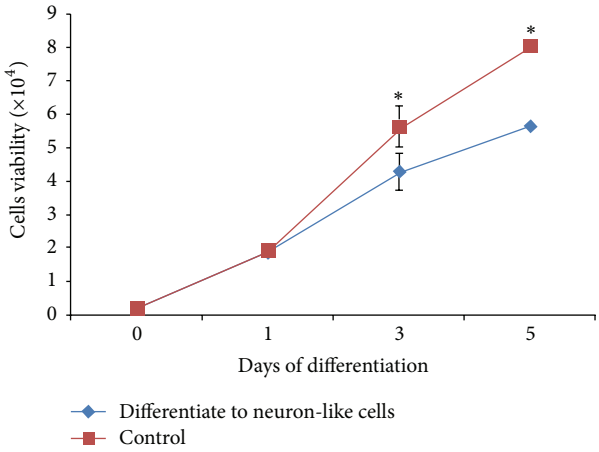

Figure 5: Viability of cells during directed differentiation. The viability of control (undifferentiated cells cultured in complete medium) versus differentiated cells cultured in serum- and growth factor-free medium. The numbers of both control and differentiated cells were significantly increased upon differentiation compared with day 0 of culture. The results are summarized as the mean ± SD. Statistical significance was determined using SPSS program version 16.0.2. *Statistical analysis showed significant differences ($P < 0.05$) of viable cells at days 3 and 5 of culture as compared to control.

and maintenance of cells and prevented embryonic stem cells differentiation into neuronal cells [54]. In this study, the absence of serum and growth factors during culture may lead to DPSCs transdifferentiation into neuron-like cells.

5. Conclusions

Dental pulp stem cells are induced to differentiate into neuronal cells when cultured in serum- and growth factor-free medium.

Authors' Contribution

Shahrul Hisham Zainal Ariffin is head of this project. He designed the experiment and characterized the cell lines. Shabnam Kermani performed the experiments. Intan Zarina Zainol Abidin analyzed the data and corrected and rewrote the paper. Rohaya Megat Abdul Wahab designed the experiment and analyzed the data. Zulham Yamamoto wrote the paper. Sahidan Senafi and Zaidah Zainal Ariffin analyzed the data and corrected the paper. Mohamad Abdul Razak designed the experiment and the research approaches. All authors have read and approved the paper.

Acknowledgments

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Review Article

Impact of Lipid Nutrition on Neural Stem/Progenitor Cells

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The neural system originates from neural stem/progenitor cells (NSPCs). Embryonic NSPCs first proliferate to increase their numbers and then produce neurons and glial cells that compose the complex neural circuits in the brain. New neurons are continually produced even after birth from adult NSPCs in the inner wall of the lateral ventricle and in the hippocampal dentate gyrus. These adult-born neurons are involved in various brain functions, including olfaction-related functions, learning and memory, pattern separation, and mood control. NSPCs are regulated by various intrinsic and extrinsic factors. Diet is one of such important extrinsic factors. Of dietary nutrients, lipids are important because they constitute the cell membrane, are a source of energy, and function as signaling molecules. Metabolites of some lipids can be strong lipid mediators that also regulate various biological activities. Recent findings have revealed that lipids are important regulators of both embryonic and adult NSPCs. We and other groups have shown that lipid signals including fat, fatty acids, their metabolites and intracellular carriers, cholesterol, and vitamins affect proliferation and differentiation of embryonic and adult NSPCs. A better understanding of the NSPCs regulation by lipids may provide important insight into the neural development and brain function.

1. Introduction

Neural stem/progenitor cells (NSPCs) are a specific population of cells in nervous system that has self-renew capacity and multipotency. In early brain development, NSPCs divide symmetrically and increase their number to produce sufficient NSPC pool. Subsequently, an NSPC divides asymmetrically to produce one NSPC and one differentiated cell in an orderly fashion [1]. NSPCs at the early developmental stage generate a large amount of neurons, whereas those at the late developmental stage generate mainly glial cells [2]. New neurons migrate out and form synapses with other neurons, establishing neuronal networks, which are supported by glial cells including astrocytes and oligodendrocytes. The fact that all neurons and glial cells consisting the adult nervous system originate from NSPCs shows no doubt about the importance of these NSPCs in brain development.

Neurogenesis was traditionally considered to finish just after birth, although the possibility of neurogenesis in the adult rat brain is suggested already in 1960s [3, 4]. After a few decades of doubt against adult neurogenesis in mammals, Reynolds and Weiss found that cells dissociated from adult mouse brains proliferate to form spherical balls in culture [5]. These spherical balls are called "neurospheres" and are positive for nestin, a marker for NSPCs [6]. Neurospheres can differentiate into neurons, astrocytes, and oligodendrocytes following the withdrawal of growth factors from the culture medium. Clonal culture eventually confirmed self-renewal capacity and multipotency of these spheres [7]. When cells from a tissue form spheres *in vitro*, the original tissue is retrospectively considered to contain NSPCs. This selective culture of NSPCs for forming neurospheres is widely used in NSPC studies. Regarding *in vivo* studies, NSPCs are found as proliferating cells in the inner wall, subventricular zone (SVZ), of the lateral ventricle [8, 9]. It is further proven that cells in the SVZ that have features similar to astrocytes are NSPCs [10]. Regarding hippocampal neurogenesis, proliferating cells [11] and immature neurons [12] exist in the subgranular zone (SGZ) of the adult dentate gyrus. Late 90s is an epoch-making period that multiple papers are published from various laboratories showing the existence of actively proliferating NSPCs

in the SGZ in rats [13] and mice [14], of adult-born neurons in the monkey brain [15] and in the postmortem human brain [16]. From these lines of evidence, it is now widely believed that NSPCs exist not only in the embryonic brain but also at least in two areas of the adult brain: the SVZ of the lateral ventricle and the SGZ of the dentate gyrus in the hippocampus. The NSPCs in the adult brain continuously produce new neurons that have important roles in rodent behaviors (see below), suggesting their significance in brain function.

Lipids are an important nutritional composition because they have high calorific value, compose biological structures, and produce biologically active substances. Lipid is an ambiguous term, and there is no definition widely accepted. It is frequently defined as naturally occurring compounds that are insoluble in water but soluble in nonpolar solvents. However, such a definition is somewhat misleading because many substances that are regarded as lipids are soluble in both water and nonpolar solvents. Lipids are often categorized into simple lipid, compound (or complex) lipid, and derived lipid [17], although another categorization is also well accepted [18]. Simple lipid is an ester of fatty acids and alcohol, for example, fat and wax. Fat is stored in adipocytes and is believed to be used as energy source for all organs except for nervous system. Compound lipid is a lipid with more groups, including phosphoric acid or carbohydrate, for example, phospholipid and glycolipid, respectively. These compound lipids are components of cell membrane. Simple lipids and compound lipids are metabolized or hydrolyzed into derived lipids, for example, fatty acids, steroids, and fat-soluble vitamins. These derived lipids have strong bioactivity and regulate various biological functions.

Fatty acids are one of the derived lipids and behave as signaling molecules, precursors to families of lipid mediators, and components of both simple and compound lipids. In the brain, fatty acids are the major structural components; it is estimated that half of the neuronal membrane is composed of fatty acids [19–21]. Among fatty acids, long-chain polyunsaturated fatty acids (PUFAs), which have more than 16 carbon atoms and more than one *cis* double bond, have been implicated as critical nutritional factors for proper neural development and function [22–24]. Because the physiological properties of PUFAs largely depend on the position of the first double bond from the terminal methyl group of the carbon chain, PUFAs are categorized into *n*-3, *n*-6, and *n*-9 PUFAs by its position. They have the first double bond existing as the third, sixth, and ninth carbon-carbon bond from the methyl group, respectively. Most of lipids are synthesized *de novo* in mammals, while these *n*-3 PUFAs and *n*-6 PUFAs are not synthesized and must be obtained from diets [25]. Thus, *n*-3 PUFAs, including α-linolenic acid (ALA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA), together with *n*-6 PUFAs, including linoleic acid (LA) and arachidonic acid (ARA), are referred to as essential fatty acids. In a more rigorous definition, essential fatty acids are ALA and LA. This is because DHA and ARA can be synthesized from ALA through EPA and from LA, respectively (Figure 1). However, we should keep in mind that the synthesis of DHA from its original precursor, ALA, is very scarce in human [26]. The major end product of *n*-3 pathway is DHA, whereas that of the *n*-6 pathway in mammals is ARA. Actually, PUFAs in membrane phospholipids are mainly composed of DHA and ARA [25]. These fatty acids have indispensable roles in various biological functions.

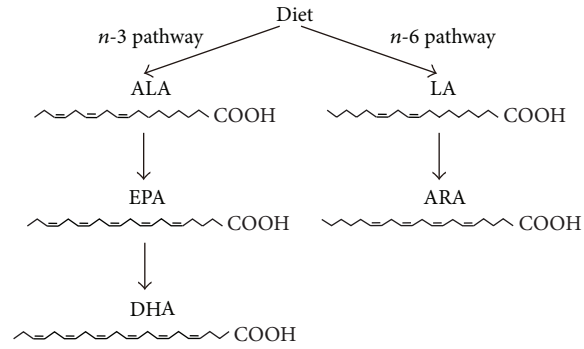

Figure 1: Synthesis of *n*-3 and *n*-6 PUFAs. DHA and ARA can be synthesized from ALA and LA, respectively.

Brain contains a large amount of lipids because neurons have very complicated dendrites and long axons that are ensheathed by the cell membrane of oligodendrocytes. There are many pieces of literature showing that lipids play pivotal roles in neural development and brain function because lipid is included in diet and affects as an extrinsic factor [27]. In this review, we focus on the effects of lipid nutrition in embryonic and adult NSPCs, mainly in rodents.

2. NSPCs and Their Function

2.1. Embryonic NSPCs in the Telencephalon. All neurons except for granule cells in the dentate gyrus and interneurons in the olfactory bulb (see below) are produced from embryonic NSPCs. All regions of the embryonic brain have NSPCs, and each character is slightly different. Here, we focus on embryonic NSPCs in the mouse telencephalon.

During early neural development, NSPCs emerge in the neural tissue at embryonic day (E) 8 in the mouse (or E10 in the rat). At this stage, NSPCs proliferate to expand the pool of NSPCs. Approximately at E10.5, NSPCs that reside in the inner wall of the neural tube start to produce cortical neurons. This region where NSPCs reside is termed the ventricular zone (VZ), and these NPSCs are called radial glial (RG) cells because their processes locate radially within the cortical primordium, and these cells exhibit astroglial properties [28]. RG cells also produce basal progenitor cells that further proliferate in the subventricular zone (SVZ) neighboring the VZ [29–31]. Neurons are produced by direct neurogenesis from RG cells and by indirect neurogenesis from basal progenitor cells [32]. Recently, another subtype of progenitor cells has been reported in the embryonic cortex. These progenitor cells are called outer radial glial (oRG) cells. oRG cells are generated directly from RG cells, form the outer subventricular zone (OSVZ), and produce neurons directly [33, 34]. oRG cells are implicated as an important source for cortical evolution because a recent study suggests that the development of

oRG cells may be the cellular mechanism underlying expansion in primate corticogenesis [35]. The initial neurons produced from RG cells form the preplate, which is subsequently divided into the subplate and the marginal zone. The marginal zone will form layer 1 of the neocortex. From E11.5 to 17.5, RG cells, basal progenitor cells, and oRG cells produce projection neurons of the different neocortical layers in a strictly controlled temporal order, from layer 6 to layer 2/3 (Figure 2) [36, 37], although a recent report has shown that neuronal progenitor cells that will differentiate into upper layer neurons (layers 2–4) are already produced even in early neural development [38]. These neurons develop the cortical plate, which will give rise to the major layers (layers 2–6) of the gray matter of the neocortex, sandwiched by the subplate and the marginal zone. The production of cortical projection neurons is completed by the end of the embryonic period.

During late neural development, astrocytes are differentiated from NSPCs, and their production has its peak just after birth. Thus, there is a transition from neurogenic to gliogenic in the character of NSPCs. This transition is well studied *in vitro* because cultured NSPCs recapitulate the transition. NSPCs cultured for a short period differentiate into neurons, whereas those cultured for a long period produce more glial cells [2]. Moreover, the neurogenic-to-gliogenic fate switch of NSPCs can be observed in culture in clones of single NSPCs [2]. Several molecular mechanisms for the initiation/inhibition of astrocyte differentiation from NSPCs have been proposed [39–42].

Oligodendrocytes in the cortex are produced in three waves: the first and second waves occur in the embryonic ventral telencephalon, and the third wave occurs among postnatal cortical progenitors [43]. During embryonic development, oligodendrocyte precursor cells (OPCs) are thought to be generated from NSPCs located in the ventral telencephalon [43–45]. OPCs then migrate tangentially into the developing cortex [46, 47]. In addition to the production of embryonic OPCs, a postnatal wave of OPCs has been reported in the cortical SVZ [48, 49]. It is thought that a large portion of oligodendrocytes in the adult cortex originates from these OPCs [50]. OPCs differentiate into oligodendrocytes that form the myelin sheath surrounding neuronal axons. In some regions of the healthy adult brain, approximately 60% of OPCs continue to proliferate to generate oligodendrocytes [51]. Therefore, oligodendrogenesis is important throughout life.

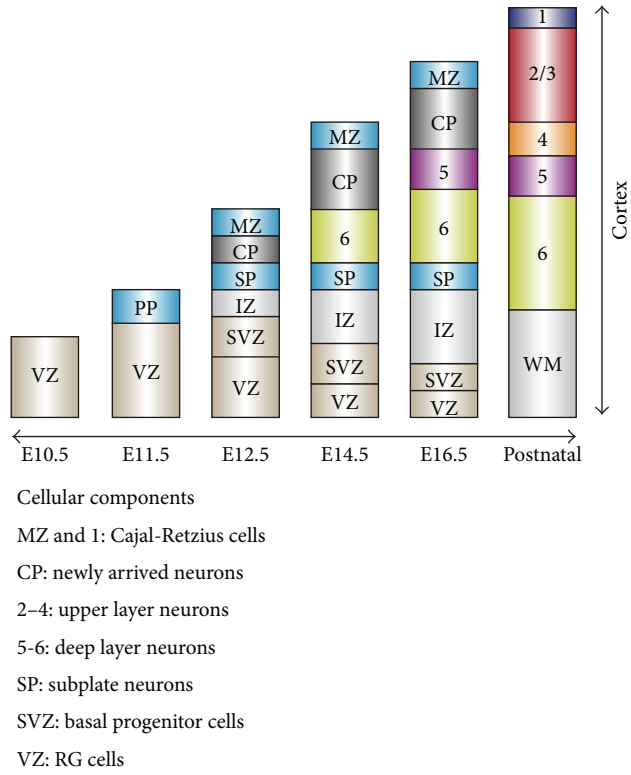

Figure 2: Neocortical development in the mouse. Preplate (PP) is composed of the earliest born neurons, which are differentiated from NSPCs in the VZ. PP is split into the subplate (SP) and marginal zone (MZ). NSPCs in the VZ also produce basal progenitor cells, resulting in the formation of the SVZ. The SP and MZ will form a part of layer 6 and the whole of layer 1 of the neocortex, respectively. Later, newborn neurons that form the cortical plate (CP) will form the multilayered neocortex in the postnatal brain, between layer 1 and layer 6. RG cells: radial glial cells, IZ: intermediate zone, and MW: white matter.

2.2. Adult NSPCs in the SVZ of the Lateral Ventricle. Several cell types are involved in adult neurogenesis in the SVZ (Figure 3). A lineage tracing study and fate-mapping study have revealed that GFAP-expressing cells that have morphological features similar to RG cells serve as quiescent neural stem cells [10, 52]. GFAP-expressing radial glia-like cells are referred to as type B cells [10]. These cells extend their small apical process that retains primary cilium to the ventricle. In addition, their basal processes reach blood vessels and form endfeet [53, 54], suggesting that type B cells are directly regulated by both cerebrospinal fluid and bloodstream. Type B cells give rise to actively proliferating progenitors, referred to as type C cells [10, 55]. Immature neuroblasts called type A cells are generated from type C cells and migrate a long way to the olfactory bulb (OB) through the rostral migratory stream (RMS) [8, 56]. Once type A cells reach the core of the olfactory bulb, they separate from the RMS and migrate radially toward the surface of the OB. Most of the type A cells become GABAergic granule neurons, but a minority of them become GABAergic/dopaminergic periglomerular neurons [57, 58].

Although the potential function of adult-born neurons in the OB is still under investigation, cumulative evidence has indicated their important roles in the OB functions. Half of the adult-born neurons in the OB are incorporated into the preexisting neural circuitry [59], and genetic ablation of newly generated cells in the SVZ resulted in a significant reduction in the number of mature granule neurons in the OB [60]. Many experiments have addressed the functional importance of adult-born neurons in olfactory-related behaviors. Although adult-born neurons in the OB are not required for the discrimination between similar chemical odors and response to innate aversive odor such as fox scent [60–62], they are required for olfactory-fear conditioning [62], olfactory perceptual learning [63], and long-term olfactory memory [64]. A recent study has demonstrated that adult-born

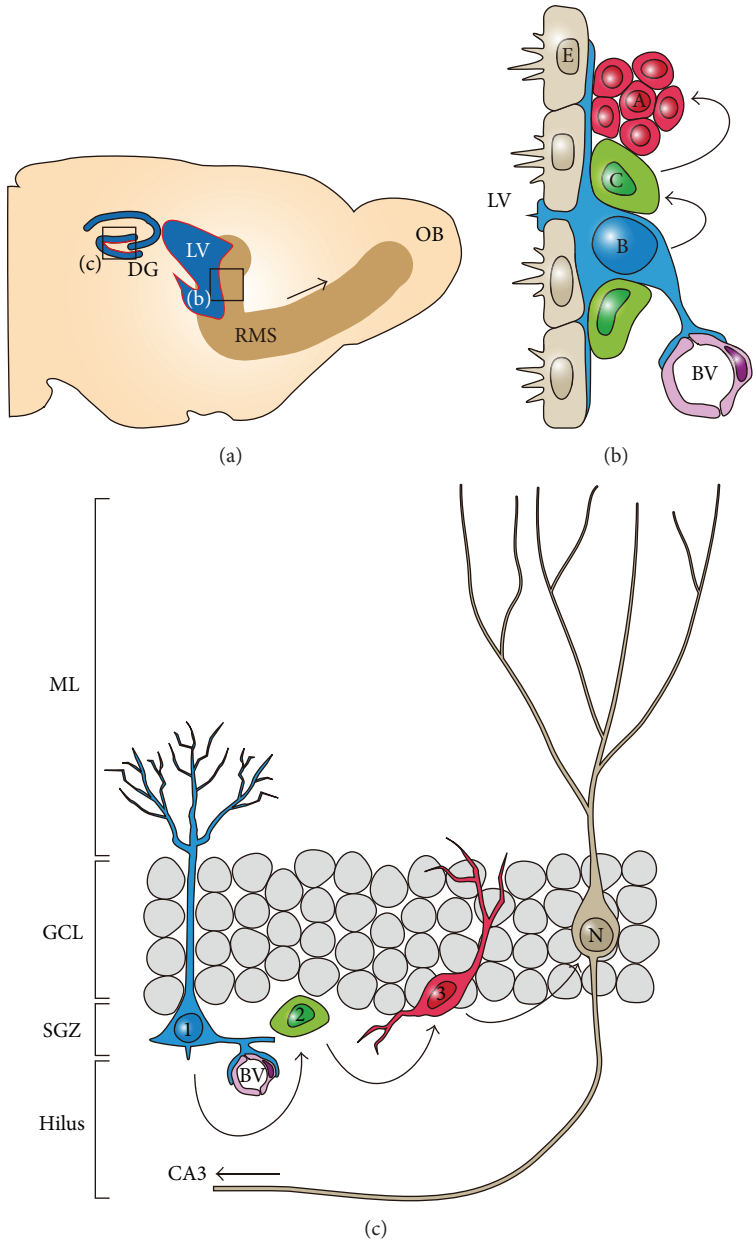

Figure 3: Adult neurogenesis in the rodent brain. (a) A sagittal view of the adult rodent brain. Red areas in the LV and DG indicate the SVZ and the SGZ, respectively, where active adult neurogenesis occurs. Arrow shows the direction of RMS through which immature neuroblasts born in the SVZ migrate to the OB. (b) A schematic image of adult neurogenic niche and sequential progression of adult neurogenesis in the SVZ. E: ependymal cell, A: type A cell, B: type B cell, C: type C cell, and BV: blood vessel. (c) A schematic image of adult neurogenic niche and sequential progression of adult neurogenesis in the SGZ. 1: type 1 cell, 2: type 2 cell, 3: type 3 cell, and N: mature neuron.

neurons affect the response of mice to innate aversive odor when associated with reward [65]. Thus, adult-born neurons in the OB are important in olfaction-dependent behaviors via long-term structural integration.

2.3. Adult NSPCs in the SGZ of the Dentate Gyrus in the Hippocampus. The production and maturation of new granule neurons in the SGZ occur in a sequential manner as well as those in the SVZ (Figure 3). In the SGZ, GFAP-expressing radial glia-like cells are referred to as type 1 cells [66]. Type 1 cells extend their long apical process into the molecular layer (ML) of the dentate gyrus [52, 67], and their basal processes contact with blood vessels in the same fashion as type B cells in the SVZ [68]. Type 1 cells make a transition to fast proliferating intermediate progenitors called type 2 cells, which in turn generate neuroblasts (type 3 cells) [69]. Type 3 cells become immature neurons and migrate a short way into the inner granule cell layer (GCL), where they differentiate into granule neurons. Within two weeks, newborn granule neurons extend their dendrites toward ML and project their axon (mossy fiber) to CA3 pyramidal neurons through the hilus of dentate gyrus [70]. Compared to mature neurons, newborn

granule neurons have hyperexcitability and enhanced synaptic plasticity during a certain period of time, contributing to shaping the existing circuit in response to external stimuli [71–73]. Newborn granule neurons gradually mature and work within preexisting neural circuit.

Adult neurogenesis in the SGZ has been implicated in several hippocampus-dependent behaviors. It has important roles in hippocampus-dependent learning and memory. Morris water maze task that is considered to activate the hippocampal neural circuit enhances the survival of newborn neuron in the dentate gyrus [74]. Irradiation or genetic manipulation of newborn neurons has also shown that adult neurogenesis in the SGZ is required for short- or long-term spatial memory [60, 75–77]. In addition to learning and memory, adult neurogenesis in the SGZ is also required for the formation of contextual fear memory and transition of such a kind of memory from the hippocampus to higher brain regions [78, 79]. Furthermore, other groups demonstrated that adult newborn neurons in dentate gyrus also contribute to the pattern separation by ablation with irradiation of adult neurogenesis in the SGZ [80] and by selectively inhibiting the synaptic transmission of old granule cells in dentate gyrus [81]. Pattern separation is a process to discriminate similar but distinct matters, and it is thought that the dentate gyrus and CA3 of the hippocampus play an important role in this process [82, 83]. Adult neurogenesis in the SGZ has also been implicated in mood control. Patients with major depressive disorder exhibit a reduced hippocampal volume, suggesting that decreased neurogenesis is one of the contributing factors [84]. In fact, antidepressant treatment in rodents and nonhuman primates increases neurogenesis in the dentate gyrus, and ablation of newborn neurons by irradiation attenuates the efficacy of antidepressant such as imipramine and fluoxetine on behavior [85–87]. Although there is criticism regarding the association between depression and neurogenesis (reviewed by Petrik et al. [88]), a recent study indicated that newborn neurons in the dentate gyrus are required for buffering the stress response through hypothalamo-pituitary-adrenal axis (HPA-axis) [89]. It seems that neurogenesis in the adolescent stage may contribute to the establishment of sensorimotor gating in the rat [90] and in the mouse (our unpublished results). Thus, various hippocampal functions are indeed at least in part related to newborn neurons in the SGZ.

Adult neurogenesis in the SGZ is well understood because it is regulated by lots of physiological stimulations. Physical exercise such as voluntary running enhances cell proliferation in the SGZ, and enriched environment promotes the survival of newborn neurons [14, 91]. On the other hand, various stress paradigms, for example, subordination, resident intruder, restraint, and isolation stresses, decrease cell proliferation in the SGZ [15, 92–94]. Aging also decreases in cell proliferation and neuronal differentiation in the SGZ [13]. A recent study has shown that age-associated decline of neurogenesis in the SGZ is attributable to depletion of neural stem cells followed by their differentiation into astrocyte [95]. Further study will reveal flexible characters of adult NSPCs in the SGZ and provide us with an insight into the regulation of stem cell activity in the adult tissue.

3. Modulation of NSPCs by Lipid Nutrition

NSPCs are regulated by various intrinsic and extrinsic factors. Intrinsic factors including genetic networks are difficult to manipulate. However, diet is one of the important extrinsic factors that can be easily manipulated. Here, we review nutritional effects of lipids on NSPCs.

3.1. Fat. Fat is a major dietary source for lipids and has significant involvements in NSPCs. Dietary fat is called triglyceride because it is a triester of glycerol and fatty acids. In triglyceride form, fat cannot be absorbed by the intestines. Pancreatic lipase hydrolyses the ester bond and releases fatty acids from glycerol. These derivatives can be absorbed and used by various organs. It is reported that obesity-inducing high-fat diet (HFD), when administered to mother mice, impaired the proliferation of early postnatal NSPCs but not of embryonic and young-adult NSPCs, in the hippocampus of their offspring [96]. Interestingly, another report has shown that HFD caused impairment of the proliferation of adult NSPCs in the SGZ without causing the apparent obesity [97]. These studies suggest the possibility that excess intake of fat is detrimental in NSPCs.

3.2. n-3 and n-6 PUFAs. Various *in vitro* studies have shown that *n*-3 and *n*-6 PUFAs are involved in the regulation of NSPCs. Previously, we have shown that DHA and ARA affect proliferation and differentiation of embryonic NSPCs [98]. We assayed embryonic NSPCs by neurosphere culture in DHA/ARA-free medium with/without DHA or ARA. For neurogenic NSPCs, DHA and ARA promoted the maintenance of NSPCs, but no detectable effects on differentiation were observed. For gliogenic NSPCs, DHA promoted the maintenance and neuronal differentiation of gliogenic NSPCs. Conversely, ARA did not promote the maintenance of NSPCs but promoted differentiation into astrocytes. We also confirmed that higher concentration of DHA had more toxic effects on the survival of NSPCs compared with that of ARA. This makes sense because DHA has more double bonds than ARA, and lipid peroxidation is a form of oxidative stress, which is toxic to cells and dampens cell survival [99]. These results show that DHA and ARA directly regulate embryonic NSPCs and that the effects of DHA and ARA on embryonic NSPCs depend on the stage of development. Other groups have also shown that DHA promotes the proliferation and neuronal differentiation of cultured NSPCs generated from embryonic stem (ES) cells [100] and that DHA induces the neuronal differentiation of cultured embryonic NSPCs [101–103]. Kan et al. found that both DHA and ARA are necessary for the neuronal differentiation from mesenchymal stem cells [104], suggesting that ARA may also be necessary for neuronal differentiation under some conditions.

The precursors of DHA and ARA, that is, ALA and LA, also affect NSPCs *in vitro*. We have previously shown that ALA and LA promote the maintenance of embryonic NSPCs [105]. On the other hand, it is also reported that conjugated linoleic acid (CLA), a positional and geometrical isomer of LA, promotes the neuronal differentiation of embryonic

NSPCs, while LA has no such effect [106]. DHA and ARA may be synthesized in these experiments because embryonic NSPCs express enzymes that are necessary for the synthesis of DHA and ARA from ALA and LA [105]. It is possible that these enzymes regulate the metabolism of *n*-3 and *n*-6 PUFAs in the developing brain to regulate proliferation and differentiation of embryonic NSPCs.

n-3 and *n*-6 PUFAs actually affect NSPCs *in vivo*. We have previously shown that, by feeding DHA-rich diet to mother rats, there were no detectable effects on the proliferation of postnatal NSPCs in the SGZ of their offspring. However, it is reported that DHA is actually incorporated into the brain of offspring via the mother's breast milk [90], and another group has found that oral administration of DHA promoted adult neurogenesis in the hippocampus of rats fed with a fish oil-deficient diet over three generations [101]. It is also reported that by feeding *n*-3 PUFAs-rich diet to aged rat, immature neurons in the dentate gyrus was increased [107]. This is because age-related decrease of phospholipids [108] may partially be compensated by feeding *n*-3 PUFAs-rich diet. It is also known that feeding an *n*-3 PUFAs-deficient diet to pregnant rats causes inhibition or delay of neurogenesis in the embryonic brains of pups [109]. Regarding the effects of ARA, we have previously shown that supplementation of ARA to rat pups through mother's breast milk by feeding ARA-rich diet to mother rats promotes the proliferation of postnatal NSPCs in the SGZ [90]. These data suggest that DHA is necessary but not sufficient for regulating NSPCs in physiological condition but that ARA is sufficient to affect NSPCs even in the physiological condition.

3.3. Metabolites of n-6 PUFAs. Like *n*-6 PUFAs, their metabolites also influence NSPCs. *n*-6 PUFAs are metabolized into various substances [105, 110], including prostaglandins (PGs). PGs are strong lipid mediators and are known to have various functions in the regulation of NSPCs. E-type prostaglandin 2 (PGE_2) is synthesized from ARA by cyclooxygenases (COXs) and microsomal PGE synthase-1 and functions by binding to PGE_2 receptors, EP1 to EP4. EP3 is expressed in adult NSPCs [111, 112], and an EP3 agonist promotes the proliferation of adult NSPCs in the SGZ [113]. D-type prostaglandin 2 (PGD_2) is also synthesized from ARA by COXs and two types of PGD synthase and is nonenzymatically metabolized into 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), which also promoted the proliferation of cultured embryonic NSPCs and postnatal NSPCs in the hippocampus [114]. The fact that PGD_2 is the most abundant PG in the brain [115] suggests the importance of 15d-PGJ_2 function in NSPCs. Thus, mediators derived from ARA have significant roles in the regulation of NSPCs.

3.4. Fatty Acid Binding Proteins. Fatty acids taken from diets are delivered to various organs, but fatty acids need to be bound to proteins within the aqueous cytoplasm and blood plasma. This is because the solubility of fatty acids in aqueous solution is extremely low. Albumin can facilitate PUFA transport in blood plasma [116], and fatty acid binding proteins (Fabps) are intracellular carriers that accommodate PUFAs [117]. Among Fabps, Fabp3 (H-Fabp), Fabp5 (E-Fabp, K-Fabp, or S-Fabp) and Fabp7 (BLBP or B-Fabp) are the members expressed in the brain. Fabp3 is not expressed in the embryonic brain but appears in the adult brain [118]. Fabp5 is expressed in NSPCs in the embryonic brain and in the SGZ of the dentate gyrus in the hippocampus as well as in neurons in the cerebral cortex and in astrocytes [119–121]. Fabp7 is also expressed in NSPCs located in the VZ of the embryonic brain and in the SGZ of the dentate gyrus in the hippocampus and in astrocytes [121–124]. Among these Fabps, Fabp3 and Fabp5 bind to ARA [125, 126], while Fabp5 and more preferentially Fabp7 bind to DHA [127–129]. Due to these multiple Fabps, neural cells have access to various types of PUFAs at an adequate level.

The function of Fabps in the NSPCs has been studied by analyzing genetically altered mice. Fabp3 is not expressed in the embryonic brain and in the adult NSPCs [118], suggesting no function in NSPCs. Fabp5 and Fabp7 are strongly expressed in the embryonic brain, but no detectable abnormalities are reported in the gross anatomy of the brain of *Fabp5* and *Fabp7* KO mice [120, 130]. However, we have previously reported that the proliferation of postnatal NSPCs in the SGZ was decreased in both *Fabp5* and *Fabp7* KO mice [121, 124]. More severely reduced proliferation of NSPCs in the SGZ is observed in *Fabp5/7* double KO mice [121]. In addition, acute knockdown of Fabp7 promotes precocious neuronal differentiation, suggesting that Fabp7 is necessary for the maintenance of NSPCs [131]. These data show that Fabps, the intracellular carriers of PUFAs, have important roles in NSPCs.

3.5. Cholesterol. Cholesterol is one of the well-studied steroids in nutritional research. Cholesterol is an essential structural component of the cell membrane and forms lipid rafts interacting with various proteins to generate specific cholesterol-based membrane microdomains. These domains are important in membrane traffic and signal transduction [132]. Cholesterol also serves as a precursor for the steroid hormones and bile acids and is also a component of lipoproteins, that is, carriers for various lipids. About 25% of unesterified cholesterol is concentrated in the CNS [133]; the brain and spinal cord are the organs that contain the most abundant cholesterol among all organs, suggesting their importance in neural functions.

Roles of cholesterol in the regulation of NSPCs are poorly understood despite its significant functions on synaptogenesis [134]. The amount of cholesterol dramatically increases during cortical development [135], and an apical plasma membrane protein, prominin-1, of embryonic NSPCs in the VZ directly interacts with membrane cholesterol [136], suggesting that cholesterol has important roles in neural development. Conditional ablation of cholesterol biosynthesis in embryonic NSPCs leads to angiogenesis by increased vascular endothelial growth factor (VEGF) expression in embryonic NSPCs [137]. This may be a mechanism to compensate for the ablation of endogenous cholesterol, suggesting that cholesterol has essential roles in NSPCs. Regarding the roles of exogenous cholesterol, it is reported that the proliferation

of adult NSPCs in the SGZ is decreased followed by feeding a high-cholesterol diet without increasing calorie intake [138]. These data suggest that appropriate biosynthesis/intake of cholesterol is necessary for the integrity of NSPCs.

3.6. Fat-Soluble Vitamins. Fat-soluble vitamins including vitamin A and E are important nutrients and also regulate the conditions of NSPCs. Vitamin A is well known to be necessary for visual functions and regulation of some genes including αB-crystallin and fibroblast growth factor 8 [139]. Regarding their effects on NSPCs, it is reported that the injection of an excess dose of retinoic acid (RA), an active form of vitamin A, to mice significantly reduced the proliferation of adult NSPCs in the SGZ and SVZ, suppressed adult hippocampal neurogenesis, and disrupted the ability to perform a spatial radial maze task [140]. Depletion of RA in adult mice, on the other hand, leads to significantly decreased neuronal differentiation and reduced neuronal survival within the granular cell layer of the dentate gyrus [141]. RA can restore adult hippocampal neurogenesis in retinoid-deficient rats [142]. These data suggest that a suitable dose of RA is essential for NSPCs.

Vitamin E is a group of compounds with well-known antioxidant functions. Supplementation of α-tocopherol, the most important compound of vitamin E, inhibits the proliferation of adult NSPCs in SGZ, conversely promoting neurogenesis and enhancing the neuronal survival in the dentate gyrus [143]. On the contrary, vitamin E deficiency in rats causes increased proliferation of adult NSPCs in SGZ and reduced neuronal survival [144]. These data clearly show that vitamin E promotes neuronal differentiation of NSPCs and survival of neurons.

3.7. Confounding Factors in Nutritional Research. Not only nutritional contents but also calorie intake, meal frequency, and meal hardness do affect proliferation and differentiation of NSPCs. Restriction of calorie intake increases the numbers of newly generated cells in the dentate gyrus of the hippocampus as a result of increased cell survival [145]. Extending the time between meals without reducing calorie intake also increases adult hippocampal neurogenesis [146]. In addition, cell proliferation in SGZ is decreased followed by feeding powder diet compared to by feeding solid diet [147]. These parameters can be confounding factors that affect basal characters of NSPCs. There are more possible confounding factors that may affect NSPCs, including taste and smell of food, because these factors play important roles in regulating food intake. Researchers on nutritional studies should keep in mind these potential secondary effects.

4. Conclusions

Embryonic NSPCs are essential for neural development and adult NSPCs are important for various neural functions, including cognition and mood. It is now becoming clearer that lipid nutrition has a significant impact on neural development and brain functions. Modulating proliferation and differentiation of NSPCs by diet could be an easily controllable intervention that may prevent neurodevelopmental disorders, cognitive decline during aging, and various kinds of psychiatric disorders. Indeed, *n*-3 PUFAs have ameliorative/preventive effects on patients with schizophrenia [148–151], mood disorders [152–154], and posttraumatic stress disorder [155–157]. A recent report has shown that ARA may potentially have a therapeutic effect on autistic patients [158]. Although effects of lipid nutrition are well focused, mechanisms by which lipid nutrition modulates NSPCs are poorly understood. Fatty acids serve as ligands for several G-protein-coupled receptors. It is recently reported that one of such receptors, that is, GPR40, is necessary for DHA-inducing neuronal differentiation of embryonic NSPCs [103]. GPR40-dependent phospholipase activation may thus be a possible signaling pathway of DHA. Further studies are necessary for comprehensive understanding of the effects of lipid nutrition.

Conflict of Interests

The authors have no potential conflict of interests.

Acknowledgments

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Review Article

Are Mesenchymal Cells Indeed Pluripotent Stem Cells or Just Stromal Cells? OCT-4 and VSELs Biology Has Led to Better Understanding

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Stem cells have excited researchers because of their potential to regenerate. However, which stem cells will be the best candidate for regenerative medicine remains an enigma. Compared to pluripotent stem cells with associated risks of immune rejection and teratoma formation, adult stem cells especially the mesenchymal stem cells (MSCs) are hyped to be a suitable alternate since they also exhibit pluripotent properties. This review shows that there is a subpopulation of pluripotent very small embryonic-like stem cells (VSELs) among MSCs culture. The two populations differ from each other in expression pattern of OCT-4. VSELs exhibit nuclear OCT-4A, whereas the MSCs have cytoplasmic OCT-4B, similar to our earlier findings in testis and ovary. Pluripotent VSELs with nuclear OCT-4A exist in various adult body organs, and the immediate progenitors express cytoplasmic OCT-4B which is eventually lost as the cell differentiates further. To conclude it is essential to discriminate between nuclear and cytoplasmic OCT-4 expression and also to acknowledge the presence of VSELs.

1. Introduction

Stem cells represent a novel cell type in the body which has the potential to regenerate any worn out tissue and maintain tissue homeostasis. Stem cells can be multiplied in large numbers *in vitro* and may serve to replace the damaged cells for regeneration rather than the existing means of managing diseases by treating the damaged cells with drugs. Stem cells are broadly classified based on their source into embryonic (hESCs) and adult (ASCs) stem cells. Embryonic stem cells are pluripotent in nature and can be differentiated into 200 odd cell types in the body belonging to the three germ layers, namely, ectoderm, endoderm, and mesoderm. On the other hand adult stem cells are isolated from adult body tissues and are multi- to unipotent in nature. Since the initial isolation of hES cell lines [1], there has been a divide amongst the embryonic and adult stem cell biologists. It has been the endeavor of the adult stem cell biologists to demonstrate that ASCs are equally good compared to hES cells, and thus hES cell research is not required (because of associated ethics since spare human embryos are used and manipulated). In January 2013, hES cell biologists were greatly relieved, when US Supreme Court refused to hear a case that could have prohibited government funding for hES cells [2]. Various approaches have been used to demonstrate that ASCs can replace hES cells. In particular with the ability to reprogram adult somatic cells to pluripotent state by iPS technology, the lobby against hES cells has become still more strong. Another issue that has been highlighted is that mesenchymal stem cells are pluripotent and besides the differentiation into mesoderm can also transdifferentiate into ectoderm and endoderm [3] and is the focus of this special issue.

Mesenchymal stem cells (MSCs) are spindle-shaped-plastic-adherent cells that can be isolated from the fetus, extra embryonic tissues; and adult organs including bone marrow and several other body tissues. MSCs were first described by Friedenstein and group [4] as hematopoietic supportive mesenchymal stromal cells of bone marrow. Owen and Friedenstein [5] proposed that these cells may be termed mesenchymal stem cells as they had the ability to differentiate into lineages of mesenchymal tissues including bone, cartilage, tendon, ligament, marrow stroma, adipocytes, dermis,

muscle, and connective tissue. However, whether they are a true, stem cell still remains controversial. The names mesenchymal stem or stromal cells are interchangeably used in the literature. The International Society for Cellular Therapy (ISCT) has recommended that these spindle-shaped, plastic-adherent cells be termed, mesenchymal stromal cells [6]. It has been proposed that a yet unidentified stem cell may exist amongst the MSCs, but MSCs themselves must be termed mesenchymal stromal cells [7]. The recent literature suggests that MSCs are a crucial component of the niche for the HSCs in the bone marrow [8, 9].

MSCs undergo lineage-specific differentiation into mesoderm, but the ability to transdifferentiate into other lineages remains controversial. Various groups have published that MSCs can transdifferentiate into ectodermal and mesodermal lineages including hair [10], pancreatic islets [11, 12], hepatocytes [13], and neurons [14, 15]. Greco et al. [16] have further shown that a similar regulatory mechanism for OCT-4 exists among ES cells and MSCs. However, this remains highly controversial especially because the functional properties of MSCs transdifferentiated into ectoderm and endoderm are not as expected. Similarly Osonoi et al. [17] reported that human dermal fibroblasts are able to differentiate directly to all 3 germ layer derivatives that is, neurons (ectodermal), skeletal myocytes (mesodermal), and insulin-producing cells (endodermal). They exhibit nestin, desmin, and insulin when exposed to specific cocktail of growth factors. Thus it is felt that achieving transdifferentiation on the basis of immunolocalization or presence of transcripts may not suffice. Rather, evidence needs to be generated regarding the functional maturation—which has not yet been achieved.

There are two main facets of stem cells biology that have indeed baffled researchers and have led to this confusion about the functional attributes of MSCs. These include (i) OCT-4 biology and (ii) presence of a subpopulation of pluripotent very small ES-like stem cells (VSELs) amongst MSCs.

2. Oct-4 Biology and Pluripotency

Oct-4 is the most crucial POU domain transcription factor responsible for maintaining the self-renewal and pluripotent properties of stem cells including inner cell mass, embryonic stem cells, embryonic germ cells, and embryonic carcinoma cells. Oct-4, Nanog, Sox2, and FoxD3 together form an interconnected autoregulatory network to maintain ES cells pluripotency and self-renewal [18]. Oct-4-deficient mice do not develop beyond blastocyst stage due to lack of pluripotent inner cell mass cells [19]. Oct-4 is downregulated with loss of pluripotency, and knockdown of Oct-4 in ES cells results in differentiation [20, 21]. It has two major isoforms Oct-4A and Oct-4B of which only Oct-4A is responsible for the pluripotent state, whereas no biological function has been associated with Oct-4B isoform [22]. Atlasi et al. [23] reported another Oct-4 spliced variant which is primarily expressed in the pluripotent stem cells and is downregulated following differentiation; however, its function is still not clear [24]. It becomes crucial to discriminate between the various isoforms while concluding pluripotent state of a cell [23, 25]. But stem cell biologists have overlooked this aspect during their studies, have reported Oct-4 in several nonpluripotent cell types, and have resulted in a great deal of confusion [24, 26]. Similarly, there was a lot of excitement recently when various groups reported derivation of pluripotent ES-like cultures from adult testicular biopsies in mice [27–30] as well as in men [31–33] by spontaneous reprogramming of adult spermatogonial stem cells without any genetic modification. However, Warthemann et al. [34] have shown that false-positive antibody signals for OCT-4A in testis-derived cells may have led to erroneous data and misinterpretations.

Oct-4 has been reported in several somatic cell types, placenta, amniotic and cords-derived cells, and also in primary tumor tissues (refer to Supplemental Table 1 in [35]). Zangrossi et al. [36] demonstrated the presence of Oct-4 in peripheral blood and thus challenged whether OCT-4 should really be a marker for pluripotency. Greco et al. [16] showed that OCT-4 functions through similar pathway in human MSCs and ES cells. However, all these reports studied Oct-4 and failed to discriminate between the alternatively spliced Oct-4 transcripts.

In an attempt to clarify the confusion between ASCs and ESCs with respect to Oct-4 expression, Lengner et al. [35] deleted Oct-4 in several tissues with rapid turnover including intestine, bone marrow, hair follicle, liver, or CNS but found no effect on tissue maintenance or injury-induced regeneration. Thus they concluded that Oct-4 expressing cells are not required for maintaining homeostasis in adult body organs. They further discussed that somatic OCT-4 expression could be due to nonspecific staining since the amount of mRNA was very low in somatic cells compared to the ES cells and invariably amplified after 30–40 cycles of PCR amplification.

However, their concluding statement is rather intriguing. They do not deny presence of Oct-4 in adult body tissues, but the levels are very low compared to the ES cells. This is very true for the pluripotent very small ES-like stem cells (VSELS) in adult body tissues.

3. Pluripotent Stem Cells in Adult Body Tissues

Very small embryonic-like stem cells (VSELs) represent a very promising group of stem cells which have the potential to bring together embryonic and adult stem cell biologists. These are pluripotent stem cells in adult body tissues. They exhibit pluripotent characteristics including nuclear Oct-4 albeit at very low level compared to hES cells. However, they can be isolated from autologous source and do not form teratoma in mice (thus all the three major issues associated with hES cells including using spare human embryos to derive hES cell lines, immune rejection, and risk of teratoma formation are taken care of). They are easily mobilized in response to any injury, maintain life-long homeostasis [37, 38], and are also considered as embryonic remnants responsible for various cancers in the body [39], as proposed 150 years ago

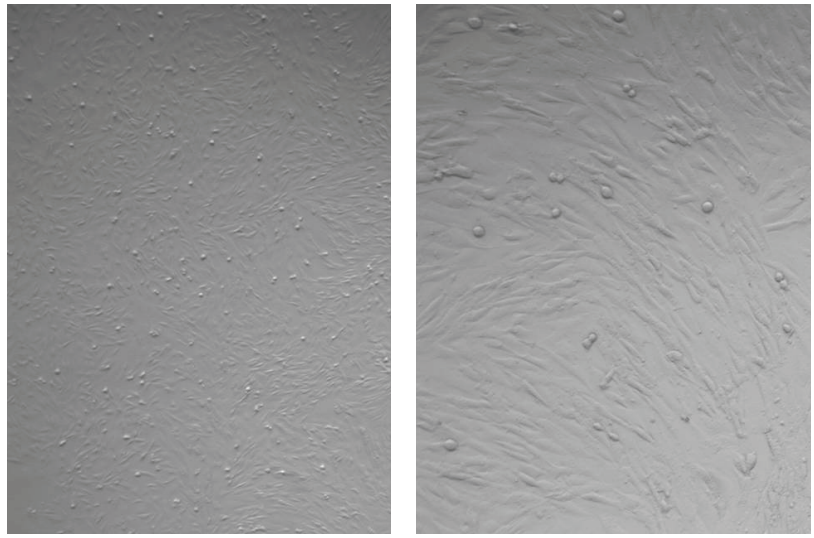

Figure 1: Bovine bone marrow culture to propagate mesenchymal cells. Note that the culture comprises a subpopulation of spherical cells along with the MSCs. These small round cells are possibly the VSELs.

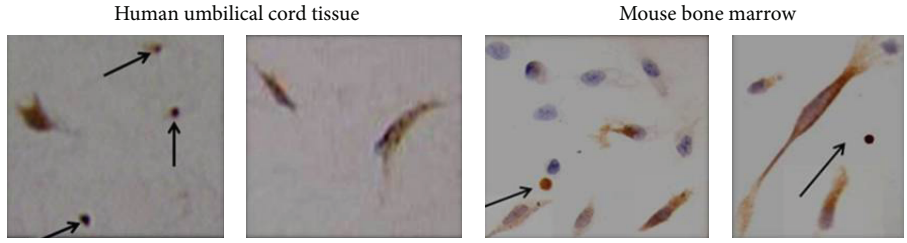

Figure 2: Immunolocalization of OCT-4 in MSCs and VSELs in human umbilical cord tissue sections and mouse bone marrow smears. Note that the round spherical VSELs have nuclear OCT-4, whereas the MSCs have cytoplasmic OCT-4.

by Rudolf Virchow and Julius Conheim. Pioneering work done by Professor Ratajczak and his group have shown that pluripotent, VSELs exist in various adult body tissues [40] and are possibly the primordial germ cells or their precursors which rather than migrating only to the gonadal ridges during early embryonic development migrate to various body organs and persist throughout life.

The confusion in the literature about presence of Oct-4 in adult body tissues is actually because of VSELs. VSELs with nuclear OCT-4 exist in various tissues and give rise to the tissue-specific progenitors which further differentiate into tissue-specific cell types. As the VSELs start differentiating, OCT-4 is observed in the cytoplasm and as the cells differentiate further, it is eventually lost. Our work on mammalian gonads has shown that indeed VSELs with nuclear OCT-4 and their immediate progenitors spermatogonial stem cells (SSCs) in testis [40] and ovarian germ stem cells (OGSCs) in the ovary have cytoplasmic OCT-4 [41]. We used a polyclonal antibody against OCT-4 which detects expression for both the isoforms (i.e. nuclear and cytoplasmic) and has shown that VSELs have nuclear Oct-4, and once differentiation is initiated in the progenitors, OCT-4 is cytoplasmic. Q-PCR analysis clearly shows the abundance of Oct-4B over Oct-4A. In order to show presence of pluripotent VSELs in the adult mammalian gonads, we have always shown the presence of Oct-4A rather than Oct-4. We also reported the presence of VSELs in the discarded pellet of RBCs during volume reduction step while processing cord blood and bone marrow [42] and also in MSCs culture (Figure 1).

Umbilical cord tissue, especially Wharton's jelly and bone marrow, is considered as a rich source of MSCs. Immunohistochemical studies of Wharton's jelly clearly show the presence of a subpopulation of VSELs amongst the MSCs (Figure 2), [42]. Similarly, early passages of MSCs from mouse bone marrow show the presence of VSELs as a distinct subpopulation (personal observations). Interestingly OCT-4 showed nuclear expression in Wharton's jelly VSELs and was cytoplasmic in the MSCs. Similarly, Taichman et al. [43] demonstrated that VSELs could be on top of hierarchy for mesenchymal stem cells (MSCs) in mice. We made a case for VSELs present in the mammalian testis [44] that may actually give rise to the ES-like colonies during testicular

tissue cultures [27–33]. Observations made by Lengner et al. [35] are indeed true because Oct-4 is expressed at very low levels in the VSELs (detected only after >35 cycles during RT-PCR) compared to ES cells (detected after 20–25 cycles during RT-PCR), and the immediate progenitors that is, the adult stem cells that exist in various adult tissues, express cytoplasmic Oct-4 which is eventually lost as cells become more committed. Berg and Goodell [45] coauthored a preview on the Lengner study and correctly summarized in the first sentence that "absence of evidence is not evidence of absence" or stated another way "one cannot prove a negative." They also hinted to the existence of a stem cell population that was not tested in the studies reported and now we understand that it was possibly the VSELs.

Nayernia et al. [46] first reported that BM stem cells/MSCs can transdifferentiate into male germ cells both *in vitro* and *in vivo*. They transplanted BM cells into busulphan treated mice and observed colonization and proliferation but no differentiation beyond premeiotic spermatocytes stage. After this several groups have reported restoration of testicular function by transplanting MSCs. Lue et al. [47] transplanted GFP-tagged BM cells into the testicular interstitium and tubules of wild type mice and reported that the transplanted cells differentiate into Leydig cells, Sertoli cells, and also into germ cells. Similarly, Aziz et al. [48] also reported that bone marrow-derived MSCs when transplanted into the rete testis of busulphan-treated azoospermic rats transdifferentiate into spermatids and spermatocytes. Sabbaghi et al. [49] studied the ability of BM derived MSCs in revival of sperm in rat model for testicular torsion. They have reported that transplantation of MSCs via rete testis can revive spermatogenesis. Cakici et al. [50] also recently reported that fertility is restored in azoospermic rats by injecting adipose-derived MSCs. But this whole body of the literature is confusing because these studies fail to acknowledge the presence of VSELs in mammalian testis which are indeed resistant to busulphan treatment. VSELs are also resistant to damage induced by radiation because of their quiescent nature [51]. VSELs persist in busulphan treated testis and possibly differentiate into germ cells/sperm in the presence of growth factors/cytokines secreted by the transplanted MSCs [52].

To conclude, we propose that MSCs indeed arise from VSELs [53] in agreement with earlier reports by Taichman et al. [43] and are multipotent implying that they can give rise to various mesodermal cell types. Their pluripotent properties implying transdifferentiation are questionable and whatever minimal transdifferentiation that is reported may actually be due to the existing subpopulation of VSELs. The very presence of MSCs in so many diverse body tissues forces us to think that they actually represent a highly specialized ground substance or the microenvironment (source of growth factors and cytokines) for the VSELs and their progenitors to maintain life-long tissue homeostasis and are capable of immune modulation. The growth factors and cytokines secreted by the MSCs keep the VSELs under quiescent state and maintain normal proliferation and differentiation. But with increased age, MSCs function is compromised resulting in uncontrolled proliferation of stem cells at any level resulting in increased incidence of cancers. If VSELs function is disrupted the tumors are more embryonic in nature and more lethal. Nature of the tumors will vary if more committed progenitors function gets disrupted due to the altered secretome of the niche providing cells. Thus the interaction of MSCs with VSELs and the tissue-committed stem cells "progenitors" and age related changes in the MSCs secretome warrants further investigations.

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Research Article

Aldehyded Dextran and ε-Poly(L-lysine) Hydrogel as Nonviral Gene Carrier

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Background. The expression term of the gene transfected in cells needs to belong enough inorder to make a gene therapy clinically effective. The controlled release of the transfected gene can be utilized. The new biodegradable hydrogel material created by 20 w/w% aldehyded dextran and 10 w/w% ε-poly(L-lysine) (ald-dex/PLL) was developed. We examined whether it could be as a nonviral carrier of the gene transfer. *Methods*. A plasmid (Lac-Z) was mixed with ald-dex/PLL. An *in vitro* study was performed to assess the expression of Lac-Z with X-gal stain after gene transfer into the cultured 293 cells and bone marrow cells. As a control group, PLL was used as a cationic polymer. *Results*. We confirmed that the transfection efficiency of the ald-dex/PLL had a higher transfection efficiency than PLL in 293 cells (plasmid of 2 μg: ald-dex/PLL 1.1%, PLL 0.23%, plasmid of 16 μg: ald-dex/PLL 1.23%, PLL 0.48%). In bone marrow cells, we confirmed the expression of Lac-Z by changing the quantity of aldehyded dextran. In the groups using ald-dextran of the quantity of 1/4 and 1/12 of PLL, their transfection efficiency was 0.43% and 0.41%, respectively. *Conclusions*. This study suggested a potential of using ald-dex/PLL as a non-carrier for gene transfer.

1. Introduction

Recently, many studies about gene therapy have been published. One of the achievements in gene therapy is safe and effective expression of the gene in the body. The naked DNA is the safe but rapidly degraded by nucleases, and it shows a poor cellular uptake. Success of gene therapy largely relies on gene delivery vectors [1, 2]. Although viral vectors have good transfer efficiency, the consequent immunogenic side effect is not negligible. On the other hand, advantages of safe nonviral carriers include an ability to introduce DNA into nondividing cells, avoidability of integration into the chromosome, lack of infective risks, and an expense potentially lower cost than viral vectors. However, nonviral carriers display poorer transfer efficiency than viral vectors [3]. Therefore, nonviral gene carriers such as cationic polymer [4], cationic lipid [5], and polysaccharide [6] have been developed to improve this weak point [1].

A cationic polymer and lipid form complexes with DNA by electrostatic interactions between positively charged amine of the polycations and negatively charged phosphate groups of the DNA. This can condense DNA into a relatively small size via ionic interactions, which is important for gene transfer because a small size is favorable for cellular uptake [4, 7]. In addition, the interaction between the complexes and negatively charged cell membranes can enhance DNA uptake by the cells [3]. These help to increase the transfection efficiency.

Recently, a wide range of materials has been investigated as a delivery vector of plasmid DNA. In the cationic polymer, poly(L-lysine) (PLL) is a well-known nonviral gene carrier that is biodegradable and nonantigenic. Nevertheless, it is

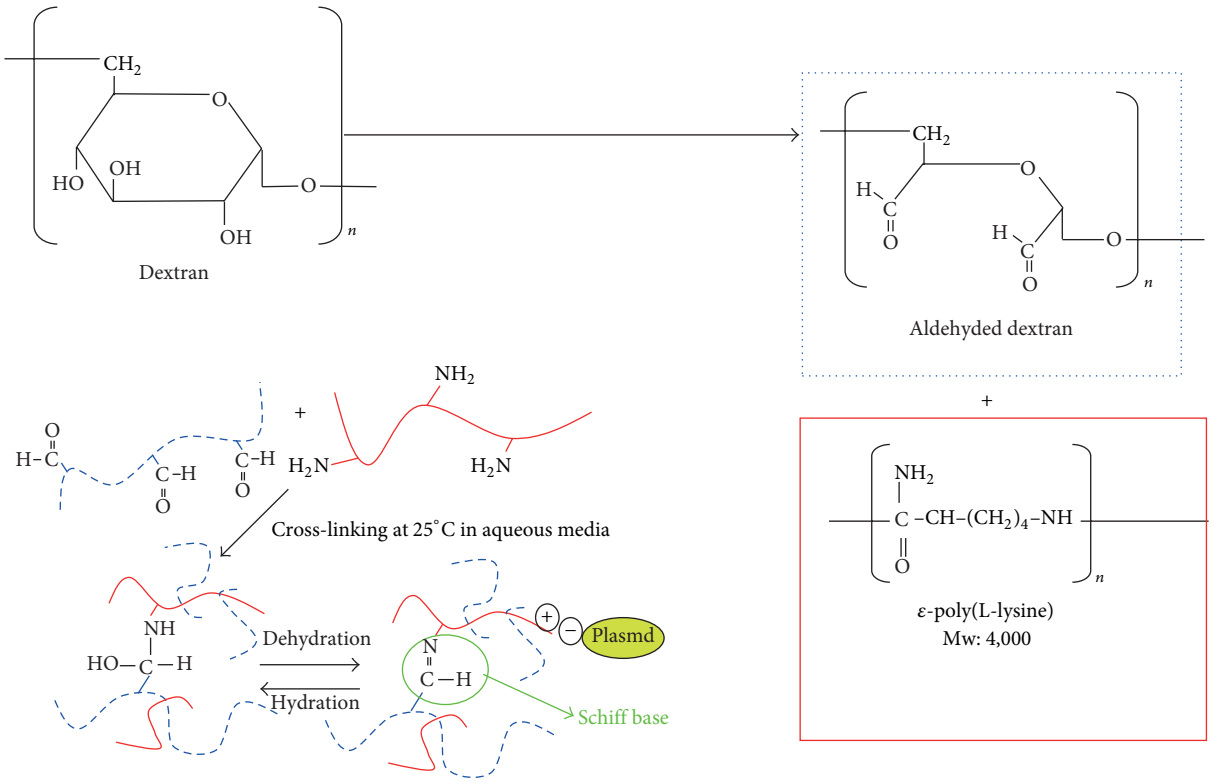

Figure 1: The chemical structure and cross-linking of the new biodegradable hydrogel composed of aldehyded dextran/PLL and plasmid DNA. After the mixture of the solution, gel formation proceeds on the basis of Schiff base formation. Plasmid DNA interacts with PLL by electrostatic interactions between positively charged amine of the PLL and negatively charged phosphate groups of the plasmid DNA.

toxic and tends to bind nonspecifically to the surface of all mammalian cells [8]. Therefore, it is relevant to modify PLL in order to be a safe and effective nonviral gene carrier [9]. Furthermore, the expression term of the gene transfected in a cell needs to be long enough in order to make a gene therapy clinically effective. The technique of controlled release of the transfected gene is utilized to lengthen the expression term by controlling the expression level and the term of gene. Until now, various controlled release techniques of gene have been reported [10–13]. Recently, a novel self-biodegradable bioadhesive hydrogel material has been developed. It was created by mixing aldehyded dextran and ε-PLL (ald-dex/PLL). A gel is formed after the mixture, which can control the degradation speed by changing the concentrations of acetic anhydride in PLL [14]. These characteristics are useful for controlled release of gene, since PLL can combine with DNA.

Of further note, mesenchymal stem cells (MSCs) have a high potential of proliferation and differentiation. The growth factors stimulating and inducing MSCs differentiation are indispensable for MSCs to differentiate into the desired cells [15]. Establishment of a gene transfer technique for MSCs enhances gene therapy and tissue engineering using MSCs [16]. In this study, we used the ald-dex/PLL to transfer the gene into MSCs and examined whether it can be used as a nonviral gene carrier of the gene transfer *in vitro*.

2. Materials and Methods

As a nonviral carrier of gene transfer, 10 w/w% PLL (Mw = 4,000) which contained 2 w/w% acetic anhydride and 20 w/w% ald-dex (Mw = 75 KDa) which was prepared by introducing the aldehyde group into dextran with an oxidizer were used.

A plasmid DNA named pCAGGS-lacZ which causes the cytoplasmic expression of *E. coli* β-galactosidase was used. The plasmid vectors were grown in *Escherichia coli* DH5 α and prepared with a Qiagen Plasmid Giga Kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's instructions. To verify the identity and purity of the plasmid vectors, agarose gel electrophoresis was performed after restriction endonuclease digestion. The plasmid DNA concentration was determined using a UV/visible spectrophotometer (DU-530, Beckman, Fullerton, CA, USA).

To assess the effect of transfection on mammalian cells, 293 cells obtained from the RIKEN Cell Bank (Tsukuba, Japan) and normal rat bone marrow cells obtained from the TAKARA BIO Japan were used. The cells were maintained in DMEM containing 5% fetal bovine serum (FBS) and penicillin as well as streptomycin (PS). Cell cultures were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Upon confluence, 1.4×10^5 cells per well were reseeded into 12 well plates and incubated for 24 hours.

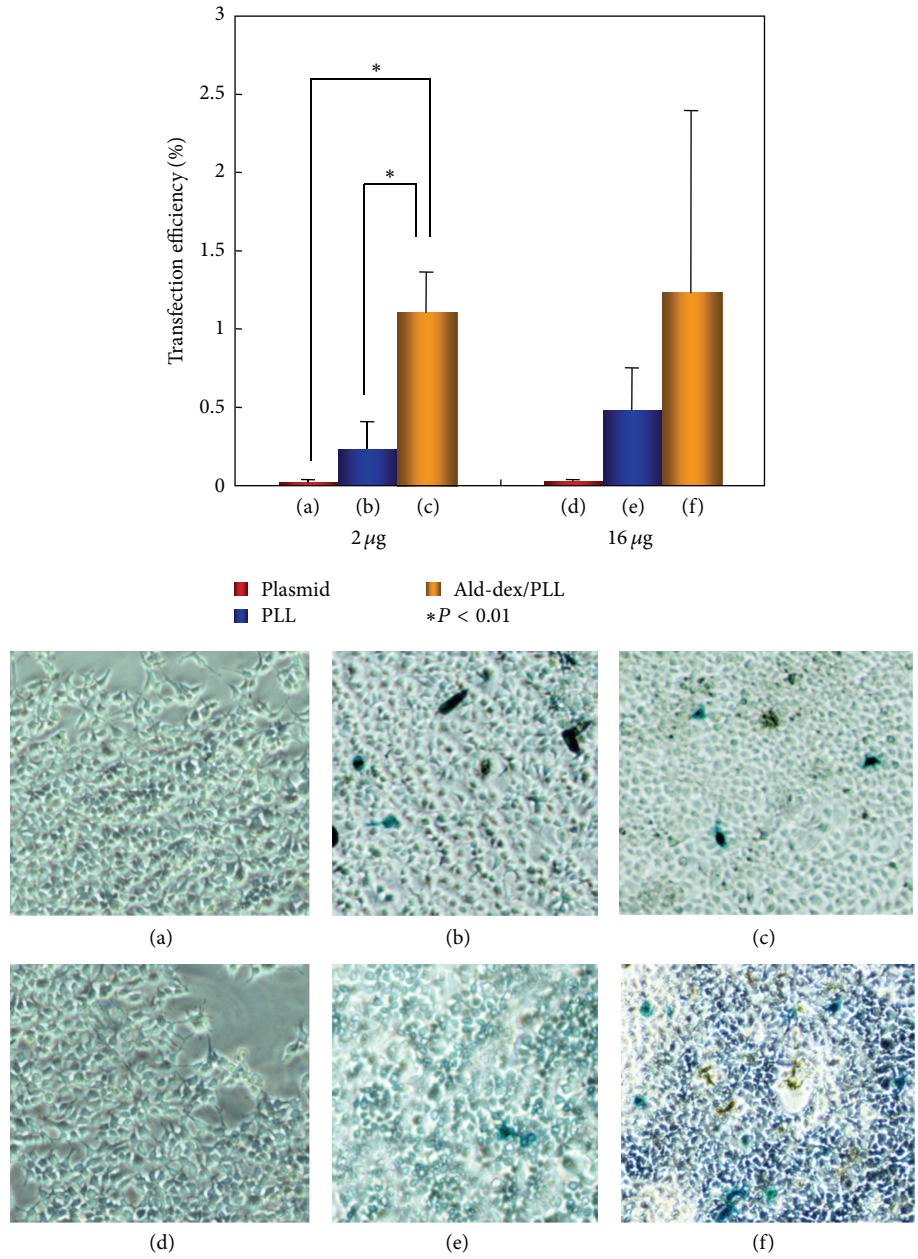

FIGURE 2: Gene transfection into 293 cells. (a), (b), and (c): results of gene transfection in plasmid of 2 μg. (d), (e), and (f): results of gene transfection in plasmid of 16 μg. (a) and (d) were carried out by only plasmid, and (b) and (e) were carried out by using PLL as a gene carrier, for the purpose of controls. (c) and (f) were carried out by using ald-dex/PLL as a gene carrier.

The cases included 293 cells using the same amount of ald-dex and PLL mixed with plasmid DNA in DMEM, whilst the controls used only PLL with plasmid DNA in DMEM. The concentrations of plasmid DNA were 2 μg and 16 μg, respectively. Both were incubated at 25°C overnight. In the cultures of bone marrow cells, ald-dex of the amount of 1/1, 1/4, and 1/12 of PLL and PLL were mixed with plasmid DNA (concentration: 2 μg) in DMEM and incubated at 25°C overnight.

After the cells were about 80% confluent, the medium was changed to DMEM supplemented with PS without FBS. The solution of the complexes was applied and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2 for 24 hours. In the cultures of 293 cells, only plasmids were applied as the controls. After 24 hours' transfection, the medium was changed to a new medium containing FBS. After another 24 hours, X-gal stain was performed to examine the transfection efficiency. The cells were fixed for 5 minutes in phosphate-buffered saline (PBS) containing 2% formaldehyde and 0.2% glutaraldehyde at the room temperature. They were subsequently washed with PBS and stained for 2 hours at 37°C in a 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)

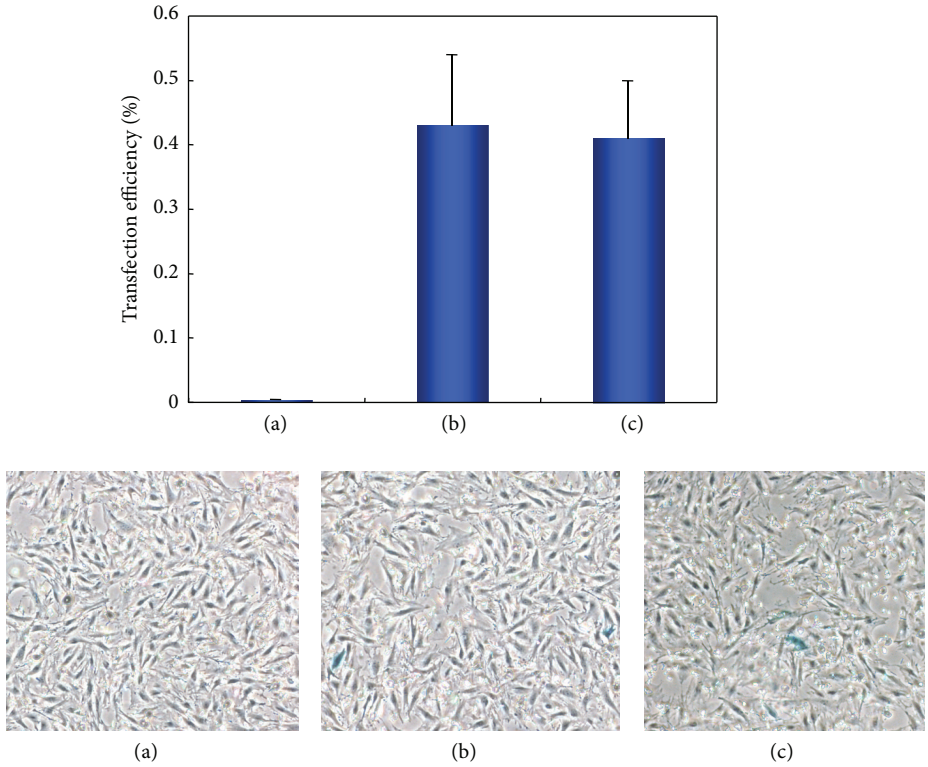

Figure 3: Gene transfection into MSCs. (a) Transfection with ald-dex (1/1 of PLL) and PLL. (b) Transfection with ald-dex (1/4 of PLL) and PLL. (c) Transfection with ald-dex (1/12 of PLL) and PLL.

staining solution containing 1 mg/mL X-gal, 2 mM MgCl2, 5 mM, K3Fe (CN)6, and 5 mM K4Fe (CN)6·3H2O in PBS (pH 7.4). The experiments were repeated three times except the experiments using only PLL, which were repeated four times.

3. Results and Discussion

Ald-dex/PLL hydrogel was formed by Schiff base formation between oxidized and aldehyded dextran and PLL [14]. The chemical structure and cross-linking are displayed in Figure 1. PLL has a sufficient number of primary amines with positive charges to interact with negatively charged phosphate groups of DNA. PLL/DNA complexes have a higher tendency to form precipitates [1, 17]. Previous studies reported that PLL has modified hydrophilic dextran with a reductive amination reaction between amino groups of PLL and reductive ends of dextran in order to increase the solubility of PLL/DNA complexes in aqueous media. The dextran chains do not disturb the electrostatic interaction between PLL and DNA [17]. The schema of ald-dex/PLL and plasmid DNA complex is shown in Figure 1. The complexes interact with negatively charged cell membranes and are taken into cells via endocytosis [1].

In the 293 cells, on the second day after lacZ gene transfer, we found that X-gal positive cells were present in all receiving transfections expect only plasmid transfection (Figure 2). In the groups with 2 μg plasmids, transfection efficiency of PLL and ald-dex/PLL reached to 0.24 ± 0.17% and 1.10 ± 0.25%, respectively ($P < 0.05$ versus PLL). In the groups with 16 μg plasmids, transfection efficiency of PLL and ald-dex/PLL reached to 0.48 ± 0.27% and 1.23 ± 1.16%, separately ($P > 0.05$). These demonstrated that ald-dex/PLL hydrogel could be used as a gene carrier. A higher transfection efficiency could be achieved by using ald-dex/PLL compared with using only PLL.

Subsequently, we performed the gene transfer to MSCs existing in bone marrow cells. In the bone marrow cells, X-gal positive cells were not seen in the group using the same amount of ald-dex and PLL, whilst the specific cells were present in other groups (Figure 3). Transfection efficiency of the groups using ald-dex of 1/4 and 1/12 of PLL reached 0.43 ± 0.11% and 0.41 ± 0.09, respectively ($P > 0.05$). This study demonstrated the feasibility of transferring a gene into MSCs by decreasing the amount of ald-dex in ald-dex/PLL. An earlier study has suggested that the degree of grafting and length of graft chains had an influence on physicochemical properties of DNA in a complex. PLL-graft-dextran copolymers having a lower degree of grafting or shorter dextran chains could interact firmly with the DNAs and condense DNA [17]. This agreed with our findings. Furthermore, it has been reported that the PLL graft copolymers showing cell specific polysaccharide chains could perform cell specific delivery and express a foreign gene *in vivo* [18]. This study found different results between 293 cells and MSCs, since the uptake of complexes into cell is related to the cell specificity.

The novel self-biodegradable bioadhesive hydrogel, ald-dex/PLL, which could be degraded by hydrolysis in the body was developed. The favorable characteristics of ald-dex/PLL

included a high bonding strength, a high flexibility, and a low cytotoxicity. Moreover, the onset time of hydrogel formation can be controlled by changing the amount of aldehyde introduced into dextran. The *in vitro* degradation speed of hydrogel can also be controlled by changing acetic anhydride concentration in the PLL solution [14].

A purpose of regeneration therapy is to induce the repair of defective tissues based on the natural healing potential in patients. It is desirable to use growth factors and/or genes to enhance cell proliferation and differentiation. The release technique is indispensable to make this possible. The success of controlled release of growth factors and genes relies on the incorporation of the growth factors and/or genes with appropriate carriers. Upon appropriate incorporation with carriers, the growth factors and/or genes are protected against proteolysis in the body, and consequently the active time can be lengthened [19].

This study has confirmed the feasibility of gene delivery using a novel self-biodegradable hydrogel. The ald-dex/PLL hydrogel showed a possibility to be used as a nonviral carrier for controlled release. Clinically, ald-dex/PLL combining plasmid DNA could be applied to the injured tissue to enhance tissue healing and regeneration. Thus, ald-dex/PLL combining BMP-2 encoding plasmids can be applied to a defect to enhance bone regeneration in case of fractures of a bone. Moreover, ald-dex/PLL hydrogel has been used as a surgical sealant [20]. If ald-dex/PLL combining with growth factor or genes is used as a surgical sealant, curing wounds with the new technique will become effective and feasible.

4. Conclusion

Our results suggest the potential of gene therapy using ald-dex/PLL hydrogel as a nonviral carrier. Furthermore, self-biodegradability and control of degradation speed in the ald-dex/PLL hydrogel indicated future applications in release technology.

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Research Article

The Cannabinoid Receptor Type 1 Is Essential for Mesenchymal Stem Cell Survival and Differentiation: Implications for Bone Health

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Significant loss of bone due to trauma, underlying metabolic disease, or lack of repair due to old age surpasses the body's endogenous bone repair mechanisms. Mesenchymal stem cells (MSCs) are adult stem cells which may represent an ideal cell type for use in cell-based tissue engineered bone regeneration strategies. The body's endocannabinoid system has been identified as a central regulator of bone metabolism. The aim of the study was to elucidate the role of the cannabinoid receptor type 1 in the differentiation and survival of MSCs. We show that the cannabinoid receptor type 1 has a prosurvival function during acute cell stress. Additionally, we show that the phytocannabinoid, Δ^9-Tetrahydrocannabinol, has a negative impact on MSC survival and osteogenesis. Overall, these results show the potential for the modulation of the cannabinoid system in cell-based tissue engineered bone regeneration strategies whilst highlighting cannabis use as a potential cause for concern in the management of orthopaedic patients.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent adult stem cells present in the bone marrow which can differentiate along several lineages, for example, bone, cartilage, and tendon [1]. Musculoskeletal repair relies on a series of orchestrated events that direct the differentiation of MSCs to its progeny, for example, osteoblasts, chondrocytes, and tenocytes. MSCs represent an ideal cell population for use in tissue engineering and regenerative medicine due to their ease of isolation, multipotency, lack of immunogenicity, and immunosuppressive effects [2]. Tissue engineering aims to learn how to induce, modulate and control the differentiation process of MSCs in order to provide therapeutics for musculoskeletal diseases [3]. We have recently shown that the osteogenic and chondrogenic differentiation process may be controlled by specific growth factors [4], hypoxia [5], and biophysical stimulation [6].

The endocannabinoid system is comprised of two G protein-coupled receptors, CB_1 and CB_2, the endogenous ligands anandamide and 2-arachidonoylglycerol, and their degradative enzymes fatty acid amide hydrolase and monoacylglycerol lipase, respectively. In addition, exogenous cannabinoids such as the bioactive lipids isolated from the *Cannabis sativa* plant and synthetic cannabinoids are currently used therapeutically for a number of diseases such as multiple sclerosis [7]. However, phytocannabinoids have a dual toxicity profile with the psychoactive component of cannabis, Δ^9-Tetrahydrocannabinol (Δ^9-THC), inducing cell death in a number of cell types [8–11]. Δ^9-THC is a partial agonist of the CB_1 and CB_2 receptors but displays higher efficacy at CB_1 over CB_2 where it has reported antagonist activity [12].

The endocannabinoid system is an important regulator of bone mass maintenance. In 2005, Idris et al. reported that CB_1 receptor inactivation resulted in increased bone mass and protected against ovariectomy-induced bone loss, an *in vivo* model of osteoporosis [13]. Further investigation of the skeletal phenotype of CB_1 knock-out mice has demonstrated that animals display increased bone mass at 3 months of age,

due to reduced osteoclast activity, but develop age-related osteoporosis by 12 months, due to enhanced adipocyte differentiation [14]. CB_2 receptor agonists increase bone mass by enhancing osteoblast numbers and activity, inhibiting the proliferation of osteoclasts and stimulating fibroblastic colony formation by bone marrow cells [15, 16]. Furthermore, CB_2 regulates bone loss during periods of increased bone turnover also involving the regulation of osteoclast function [17].

The aim of the present study was to elucidate the role of the cannabinoid system in the survival and differentiation of culture-expanded MSCs in the presence of known osteogenic factors: dexamethasone, β-glycerophosphate, and ascorbic acid. The results demonstrate that the CB_1 receptor is upregulated during osteogenic differentiation of MSCs and is essential for the survival of differentiated MSCs. We also show that the psychoactive phytocannabinoid, Δ^9-Tetrahydrocannabinol, has a negative impact on MSC survival and osteogenesis.

2. Materials and Methods

2.1. Culture of Mesenchymal Stem Cells. Three-month-old Wistar rats (250–300 g) were obtained from the Bioresources Unit, University of Dublin, Trinity College. Animals were sacrificed by CO_2 asphyxiation and cervical dislocation in accordance with European guidelines (86/609/EEC). The femur and tibia were dissected free and placed in sterile prewarmed supplemented Dulbecco's modified Eagle's medium (s-DMEM; Sigma-Aldrich, UK). Supplements were 10% foetal bovine serum; 100 U/mL penicillin/streptomycin; 2 mM GlutaMAX; 1 mM L-glutamine; and 1% nonessential amino acids (Invitrogen, Scotland). The femur and tibia were cut at both epiphyses, and bone marrow was flushed into a 50 mL tube using 5 mL s-DMEM and a 25-gauge needle. The suspension was centrifuged (650 $\times$g) for 5 minutes at 20°C, resuspended in 10 mL of s-DMEM, and passed sequentially through 16-, 18-, and 20-gauge needles. The suspension was passed through a 40 μm nylon mesh into a sterile Petri dish and incubated in a humidified atmosphere (95% air and 5% CO_2) at 37°C for 30 min. The supernatant was removed and split between two T75 flasks. Culture media was replaced following 24 hours to remove nonadherent cells. Cells were passaged upon reaching 80–90% confluency to a maximum of 4 passages. The medium was replaced every 3 to 4 days. To induce osteogenesis, cells were treated with osteogenic factors (OF): 100 nM dexamethasone, 10 mM β-glycerophosphate and 50 μM ascorbic acid for the indicated time period (2–5 weeks). These cells are referred to as differentiated cells, whilst cells maintained in regular culture medium are referred to as undifferentiated cells. Additionally, the differentiation capacity of MSCs was investigated and verified using previously described methods for the induction and detection of osteogenesis, chondrogenesis [4], and adipogenesis [18] in bone marrow derived MSCs (see Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2013/796715).

2.2. Drug Treatments. MSCs were incubated with drugs or vehicle for the time indicated in each experiment. The CB_1 receptor antagonist/inverse agonist SR141716 was a kind gift form Dr. David Finn at The National University of Ireland, Galway (original source: The National Institute of Mental Health's Chemical Synthesis and Drug Supply Program). SR141716 was stored as a 10 mM stock solution in DMSO at −20°C and diluted to a final concentration of 1 μM in culture media. Δ^9-THC was obtained from Sigma-Aldrich Company Ltd. and held under license granted by the Irish Department of Health and Children. Δ^9-THC was stored as a 80 mM stock solution in ethanol at −20°C and diluted to a final concentration of 1 μM in culture media.

2.3. RNA Isolation. Total RNA was isolated from MSCs using a NucleoSpin total RNA isolation kit (Macherey-Nagel Inc., Germany) following the manufacturer's instructions. This protocol included a DNase step in order to remove any genomic DNA contamination. Total RNA concentrations were determined by spectrophotometry (NanoDrop Technologies, USA) and stored at −80°C until required for cDNA synthesis.

2.4. cDNA Synthesis. Total RNA concentrations were adjusted to a standard concentration prior to cDNA synthesis. cDNA was generated from 0.5–1 μg total RNA using High Capacity cDNA Archive kit (Applied Biosystems, Germany) following the manufacturer's instructions. The resultant cDNA was stored at −20°C until required for real time PCR.

2.5. Real-Time PCR. Real-time PCR was performed using Taqman Gene Expression Assays (Applied Biosystems, Germany) on an ABI Prism 7300 instrument (Applied Biosystems, Germany). The assay IDs for the genes examined were as follows: CB_1 receptor (Rn00562880_m1), CB_2 receptor (Rn01637601_m1), osteocalcin (Rn00566386_g1), and β-actin (4352340E). Gene expression was calculated relative to the endogenous control (β-actin) and to the control samples to give a relative quantification (RQ) value.

2.6. Cell Viability Assay. Cell viability was determined by quantifying the enzymatic conversion of cell permeable calcein AM (Invitrogen, Scotland) to a fluorescent product by active intracellular esterases. Briefly, MSCs were grown on sterile 96 well plates (6×10^3 cells per well) and treated as indicated in each experiment. Calcein AM solution (2 μM in PBS) was applied to each well and incubated in a humidified atmosphere (95% air and 5% CO_2) at 37°C for 1 hour. Following incubation calcein fluorescence at 530 nm was determined using a microplate reader heated to 37°C (Synergy HT, BioTek Instruments, USA).

2.7. Immunofluorescent Staining for Active Caspase-3 and Apoptotic Nuclei Determination. Following drug treatment, MSCs were fixed in 100% methanol for 5 minutes at −20°C, permeabilised with 0.2% Triton-X100 for 10 minutes, and washed in 3 changes of PBS at room temperature (RT). MSCs were blocked with 30% goat serum overnight at 4°C (Vector Laboratories, USA). Caspase-3 was labelled with a rabbit antiactive caspase-3 (1 : 1000 in 30% blocking buffer;

Promega, England) for 1 hour at RT. Labelled protein was detected with goat anti-rabbit secondary antibody conjugated to biotin (1 : 1500 in 30% blocking buffer; Vector Laboratories, USA) for 1 hour at RT. MSCs were then incubated with avidin-conjugated FITC (1 : 500; Sigma-Aldrich, England) for 1 hour at RT. Nuclei were stained with Hoechst 33258 (1 : 500; Invitrogen, Scotland) for 15 minutes at RT. Coverslips were mounted with mounting medium (Vector Laboratories, USA). Incorporated fluorophores were examined with a confocal microscope (Carl Zeiss, Germany) using appropriate excitation wavelengths and filter sets. The number of abnormal apoptotic nuclei was determined (by a blinded counter) from 10 random fields of view for each treatment group with the n number indicated in each experiment.

2.8. Extracellular Matrix Mineralization Quantification. The specific marker of mineralized bone, hydroxyapatite, was quantified using a commercially available assay kit (Lonza, Switzerland) following the manufacturer's instructions. Briefly, MSCs were grown on sterile 96 well plates (13×10^3 cells per well) and treated as indicated in each experiment. Following treatment, MSCs were washed in PBS (×2) and then fixed in 100% ethanol for 20 minutes at RT. MSCs were incubated with fluorescent staining reagent specific for hydroxyapatite for 30 minutes at RT. MSCs were washed in diluted wash buffer (×3), and fluorescence was read at 518 nm using a spectrophotometer (Labsystems, Finland). In some experiments, MSCs were grown on glass coverslips and stained with the fluorescent staining reagent specific for hydroxyapatite. Nuclei were stained with Hoechst 33258. Labelled hydroxyapatite and nuclei were visualized with a confocal microscope (Carl Zeiss, Germany) using appropriate excitation wavelengths and filter sets.

2.9. Statistical Analysis. Data are reported as the mean ± SEM of the number of experiments indicated in each case. ANOVA followed by a Student Newman-Keuls *post hoc* test was used to determine the statistical significance between groups. For comparisons between relevant treatments, an unpaired Student's t-test was performed.

3. Results

3.1. Increased CB_1 Receptor Expression Is Responsible for MSC Survival during Osteogenesis. As MSCs underwent osteogenic differentiation, a significant increase in CB_1 receptor mRNA expression was observed after 2 weeks of differentiation (6.15 ± 1.28; RQ value, mean ± SEM) compared to undifferentiated MSCs (0.36 ± 0.17; RQ value, mean ± SEM; $P = 0.002$, Student's unpaired t-test, $n = 5$; Figure 1(a)). No change in CB_2 receptor mRNA expression was observed between undifferentiated and differentiated MSCs (supplemental Figure 2).

Since an induction of CB_1 receptor mRNA was evident in MSCs undergoing osteogenic differentiation, we sought to identify whether the induction of the CB_1 receptor was pertinent in the control of any aspect of MSC function and focused our attention on cell survival. Undifferentiated and differentiated MSCs were deprived of serum in the presence or absence of the CB_1 receptor antagonist/inverse agonist, SR141716 (SR1; 1 μM), and cell viability was measured by monitoring the metabolism of calcein AM. In undifferentiated MSCs, fluorescent intensity at 530 nm, a marker of cellular metabolism and viability, was 5.62 ± 0.56 (×10^4 RFU at 530 nm, mean ± SEM), and this was significantly reduced to 1.6 ± 0.69 following serum withdrawal for 24 hours ($P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 5$; Figure 1(b)). In contrast, when differentiated MSCs were exposed to serum withdrawal fluorescence was unaffected, indicating that the differentiated MSCs were able to withstand serum withdrawal. However, in the presence of SR141716 (SR1; 1 μM; 24 hours) the differentiated MSCs were unable to survive following serum withdrawal indicating that the increased levels of CB_1 receptor present in differentiated MSCs are essential for survival. Treatment of differentiated MSCs with SR1 alone had no effect on MSC cell viability indicating that SR1 treatment was not toxic.

In addition, we monitored cell death by assessing the percentage of apoptotic nuclei and the expression of the active form of the proapoptotic protein, caspase-3 (Figures 1(c) and 1(d)). In undifferentiated MSCs, serum withdrawal significantly increased the percentage of apoptotic nuclei from 14 ± 2% to 47 ± 3% (mean ± SEM; $P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 4$; Figure 1(c)) and also increased the expression of active caspase-3 (Figure 1(d)(ii)). However, in differentiated MSCs serum withdrawal evoked significantly less apoptosis (14 ± 1% apoptotic nuclei, mean ± SEM; $P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 4$; Figure 1(c)). In the presence of SR141716 the apoptotic effect of serum withdrawal was restored (43 ± 3% apoptotic nuclei) in the differentiated MSCs. These results provide evidence that the CB_1 receptor in differentiated MSCs is essential for survival following an insult such as serum withdrawal.

3.2. Δ^9-THC Negatively Impacts on MSC Viability and Osteogenic Potential. Given that we have shown an essential role for the CB_1 receptor in the survival of MSCs during stressful stimulus (serum withdrawal) we therefore sought to elucidate if exogenous cannabinoids could interfere with MSC viability and differentiation capacity. Hence, we monitored the effect of exogenous phytocannabinoid Δ^9-THC on the viability and osteogenic capacity of MSCs.

The effect of the Δ^9-THC on the viability of MSCs was determined by assessing the ability of undifferentiated and differentiated MSCs treated with Δ^9-THC to metabolise calcein AM. Treatment with Δ^9-THC (1 μM, 2 weeks) significantly reduced undifferentiated MSC metabolic activity from 3.68 ± 0.83 (×10^4 RFU at 530 nm, mean ± SEM) to 0.88 ± 0.15 ($P < 0.05$, 1-way ANOVA and Newman-Keuls, $n = 5$; Figure 2(a)). In differentiated MSCs treatment with Δ^9-THC (1 μM, 2 weeks) induced a significant decrease in MSC metabolic activity ($P < 0.05$, 1-way ANOVA and Newman-Keuls, $n = 5$; Figure 2(a)). Additionally, treatment of undifferentiated and differentiated MSCs with Δ^9-THC (1 μM, 2 weeks) evoked a significant increase in the % of apoptotic nuclei ($P < 0.001$, 1-way ANOVA and

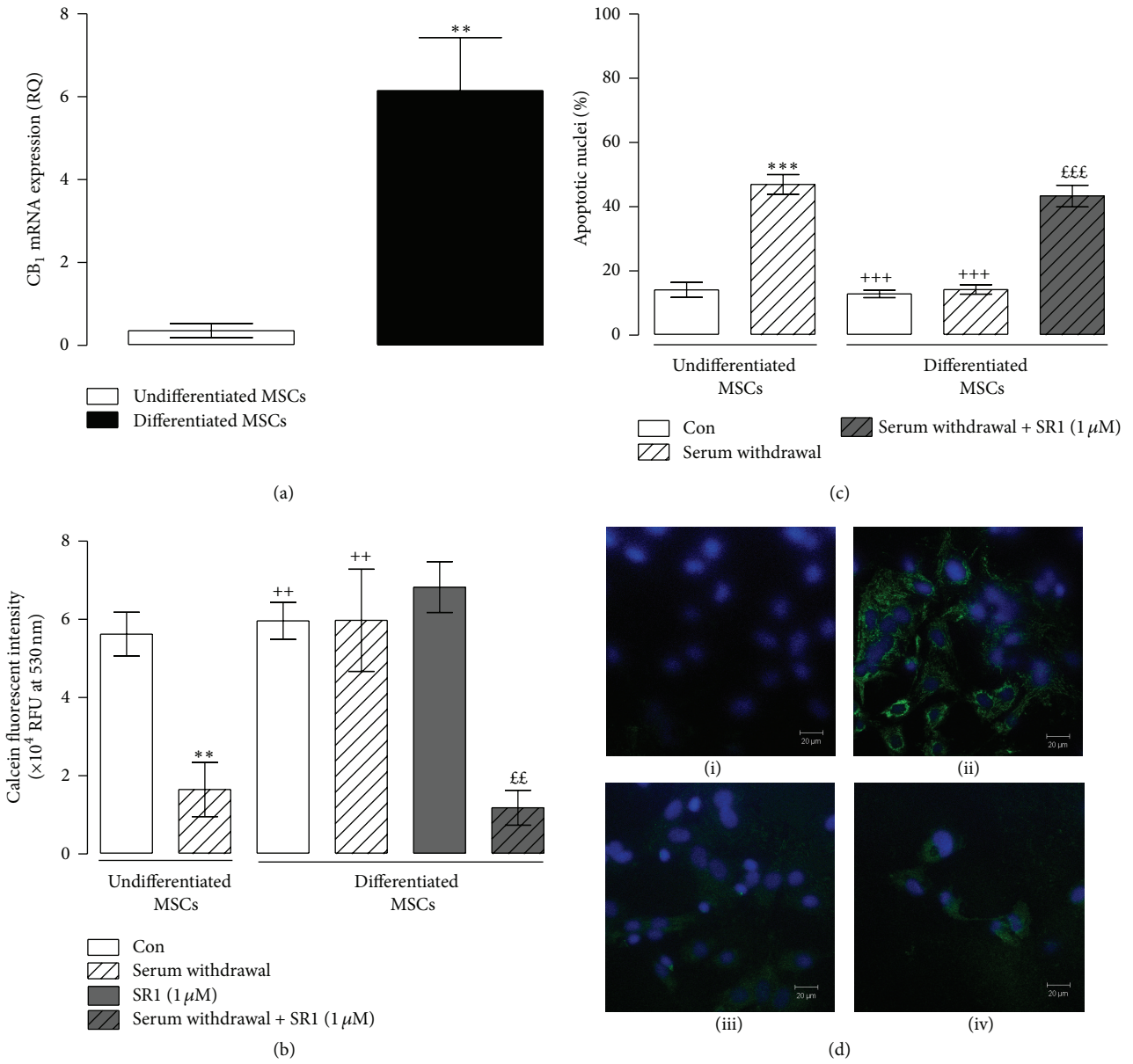

Figure 1: The CB_1 receptor is increased during early osteogenesis and is essential for the survival of differentiated MSCs. (a) Differentiated MSCs displayed a significant increase in CB_1 receptor mRNA expression after 2 weeks of differentiation compared to undifferentiated MSCs ($^{**}P = 0.002$, Student's unpaired t-test, $n = 5$). (b) Serum withdrawal significantly reduced the metabolic function of undifferentiated MSCs (Con; $^{**}P < 0.01$, 1-way ANOVA and Newman-Keuls, $n = 5$). In differentiated MSCs, serum withdrawal had no effect on metabolic function, and serum deprived differentiated MSCs displayed significantly greater metabolic function compared to serum deprived undifferentiated MSCs ($^{++}P < 0.01$, 1-way ANOVA and Newman-Keuls, $n = 5$). Treatment of differentiated MSCs with SR141716 (SR1, 1 μM; 24 hours) blocked the ability of differentiated MSCs to survive serum withdrawal ($^{\pounds\pounds}P < 0.01$, 1-way ANOVA and Newman-Keuls, $n = 5$). (c) Serum withdrawal induced a significant increase in the numbers of undifferentiated MSCs displaying apoptotic nuclei (Con; $^{***}P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 4$) compared to undifferentiated MSCs maintained with serum. Differentiated MSCs survived serum withdrawal compared to serum deprived undifferentiated MSCs ($^{+++}P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 4$). Treatment of differentiated MSCs with SR1 blocked the ability of differentiated MSCs to survive serum withdrawal compared to serum deprived differentiated MSCs ($^{\pounds\pounds\pounds}P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 4$). (d) Representative images of caspase-3 activity in undifferentiated MSCs exposed to control (i) and (ii) serum withdrawal conditions and caspase-3 activity in differentiated MSCs exposed to control (iii) and serum withdrawal in the presence of SR1 (iv).

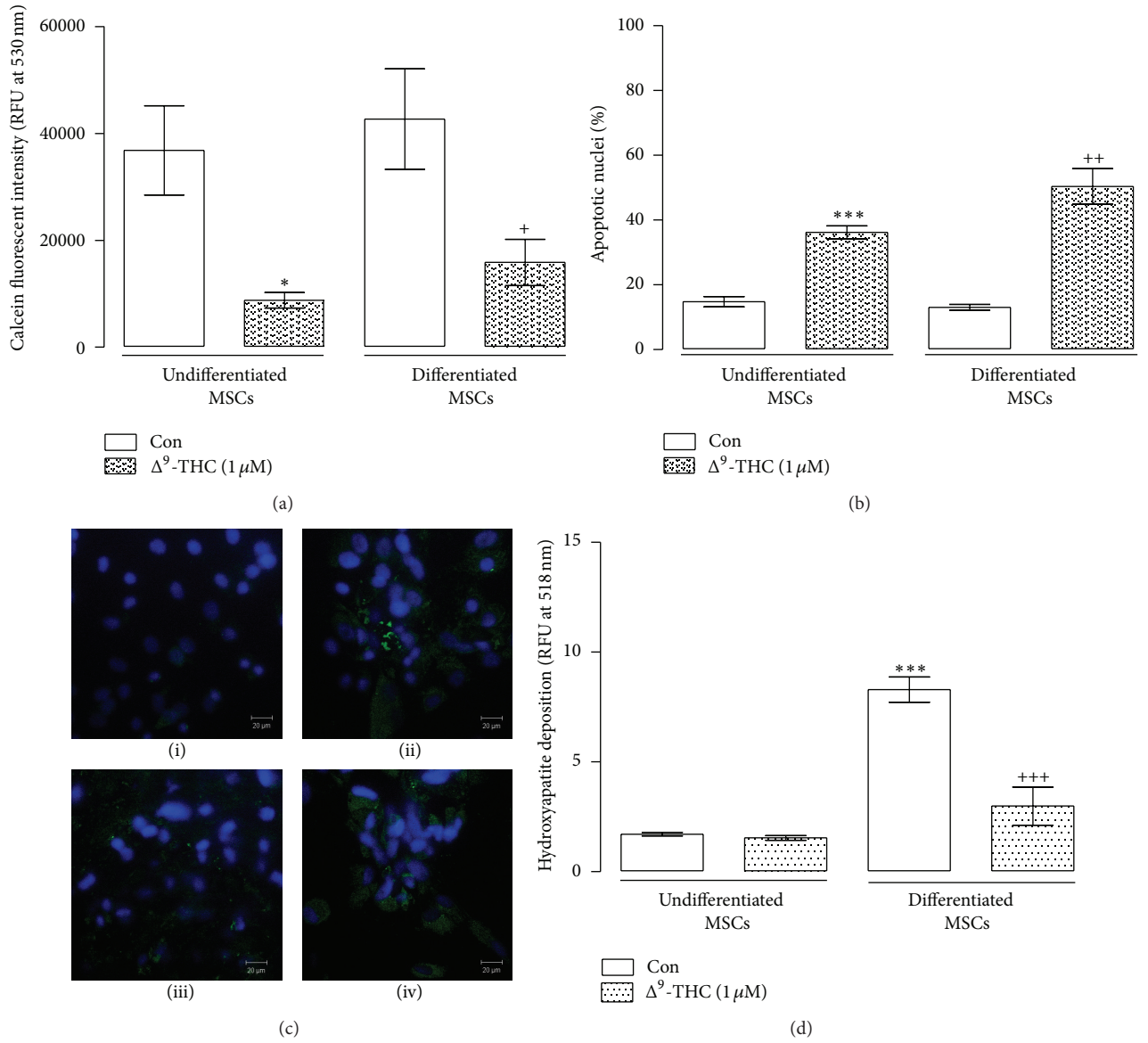

FIGURE 2: Δ^9-THC negatively affects MSC viability and inhibits MSC osteogenesis. (a) Treatment of undifferentiated MSCs with Δ^9-THC (1 μM) significantly reduced viability compared to control undifferentiated MSCs (Con; $^{*}P < 0.05$, 1-way ANOVA and Newman-Keuls, $n = 5$). Also, differentiation of MSCs in the presence of Δ^9-THC significantly decreased viability compared to control differentiated MSCs (Con; $^{+}P < 0.05$, 1-way ANOVA and Newman-Keuls, $n = 5$). (b) Treatment of undifferentiated MSCs with Δ^9-THC induced a significant increase in the percentage of apoptotic nuclei compared to control MSCs (Con; $^{***}P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 6$). Also, differentiation of MSCs in the presence of Δ^9-THC significantly increased the percentage of apoptotic nuclei compared to control differentiated MSCs ($^{++}P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 6$). (c) Representative images of cells stained for active caspase-3 in control undifferentiated MSCs (i), undifferentiated MSCs treated with Δ^9-THC (ii), control differentiated MSCs (iii), and differentiated MSCs in the presence of Δ^9-THC (iv). (d) Differentiation of MSCs in the presence of Δ^9-THC (1 μM) significantly decreased hydroxyapatite deposits compared to control differentiated MSCs ($^{+++}P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 6$).

Newman-Keuls, $n = 6$; Figure 2(b)) and caspase-3 activity (Figure 2(c)).

The effect of the Δ^9-THC on the differentiation of MSCs was determined by monitoring hydroxyapatite deposits in undifferentiated and differentiated MSCs. Deposits of hydroxyapatite were significantly increased from 1.71 ± 0.07 (RFU at 518 nm, mean ± SEM) to 8.30 ± 0.57 in MSCs differentiated with OF ($P < 0.001$, 1-way ANOVA and Newman-Keuls, $n = 6$; Figure 2(d)). However, MSCs differentiated with OF in the presence of Δ^9-THC had reduced osteogenic potential (2.30 ± 0.87, RFU at 518 nm, mean ± SEM; $P < 0.001$ 1-way ANOVA and Newman-Keuls, $n = 6$; Figure 2(d)). These results indicate that the phytocannabinoid Δ^9-THC has a negative effect on osteogenesis by decreasing the survival of both undifferentiated and differentiated MSCs.

4. Discussion

The aim of this study was to examine the role of the CB_1 receptor during the osteogenic differentiation of MSCs harvested from adult Wistar rats. The results demonstrate that the CB_1 receptor is increased during MSC osteogenic differentiation and is essential for the survival of differentiated MSCs during the acute insult of serum withdrawal. We also show that the exogenous phytocannabinoid, Δ^9-THC, reduced MSC survival and differentiation potential of MSCs.

Substantial loss of bone due to trauma, tumour ressection, metabolic bone disease or lack of bone repair due to ageing may require intervention to restore a positive balance to bone metabolism [19]. MSCs represent an ideal adult stem cell for the use in bone repair since strategies for bone regeneration (osteogenesis, osteoinduction, osteoconduction, and osteopromotion) all fundamentally rely on MSCs [20]. We have observed that MSCs produce osteocalcin and extracellular hydroxyapatite deposits (supplemental Figures 1 and 3) confirming the potential of isolated MSCs to become bone forming cells suitable for use in bone tissue engineering strategies in accordance with previously established criteria [21, 22]. The CB_1 and CB_2 receptors are G-protein coupled receptors which are currently being assessed, along with the putative cannabinoid receptor GPR55, as potential modulators of bone mass [23, 24]. It has been previously established that MSCs express CB_1 receptors [12, 14, 15], however, we are the first to show a functional increase of the CB_1 receptor during osteogenesis. We did not observe any increase in CB_2 receptor expression (supplemental Figure 2); however, this may be due to the time point analysed (2 weeks) as expression of the CB_2 receptor has previously been found to be expressed after 3 weeks of osteogenic differentiation in murine bone marrow-derived primary stromal cells [15]. We have also shown that the CB_1 receptor has a functional role in the survival of differentiated MSCs exposed to an acute insult (serum withdrawal), which is an *in vitro* model of the environment surrounding bone fractures or orthopaedic implants. Our results indicate that the CB_1 receptor is required for MSC survival during the early stages of MSC osteogenesis. Successful fracture repair and bone healing around orthopaedic implants rely on favourable biological and mechanical environments in addition to the recruitment and differentiation of MSCs. However, in certain circumstances the local environment can be actively inhospitable to infiltrating MSCs resulting in the failure of bone healing [20, 25]. The CB_1 receptor has been demonstrated to be cytoprotective in many cell types [26, 27]. In our study we show that differentiated MSCs have increased CB_1 receptor and display the ability to survive an acute insult (serum withdrawal) compared to undifferentiated MSCs. Interestingly, Cudaback and coworkers [28] have demonstrated that increased cannabinoid receptor expression changes the coupling of these receptors to specific kinase pathways and the efficacy by which cannabinoid receptor ligands induce the activation of these pathways. Furthermore, they showed that increased CB_1 receptor expression enhanced the efficacy of cannabinoids to regulate the prosurvival AKT pathway whilst low levels of CB_1 receptor expression lead only to the activation of ERK [28]. Furthermore, we have previously shown that activation of the cannabinoid system enhances the survival, migration, and chondrogenic differentiation of MSCs, which are the three key points that determine the success of cell-based tissue-engineered repair strategies [29]. Interestingly, Idris et al. [13] suggest that normal bone formation in CB_1 receptor knock-out mice can be maintained by alternative signalling pathways; however, with increasing age these compensatory mechanisms fail leading to decreased bone formation. Furthermore, the physiological upregulation of the CB_1 receptor with age has been proposed to protect against the development of osteoporosis [13]. Results from our experiments using SR141716 show that the CB_1 receptor is necessary for MSC survival following an acute insult, yet long term (3–5 weeks) CB_1 receptor antagonism results in increased osteogenesis (supplemental Figure 3) indicating a temporal effect of the CB_1 receptor on MSC function. This novel temporal response may reflect a dual role for the CB_1 receptor in MSC physiology: firstly being essential for survival during stress which is of relevance to the inhospitable environment present around areas of bone healing and secondly acting as a brake on osteogenesis, reflective of endocannabinoids having an inhibitory role during osteogenesis. The osteogenic effect of long-term CB_1 receptor antagonism that we observed may be due to enhanced signalling through the CB_2 receptor, since CB_2 receptor signalling leads to expansion of the preosteoblastic pool and increased numbers of osteoblastic colony formation [14, 15]. Furthermore, CB_2 receptor activation attenuates bone loss in an animal model of bone cancer metastases using sarcoma cells [30]. Further studies utilizing CB_1 and CB_2 knock-out animals will be necessary to dissect out the exact role of both receptors and to corroborate our findings. Alternatively SR141716 may be signalling through other receptors such as PPAR-γ [31].

Our results also demonstrate that Δ^9-THC prevents osteogenesis and induces cell death in both undifferentiated and differentiated MSCs. These findings may provide a molecular explanation for the results of Nogueira-Filho and coworkers [32] who showed reduced cancellous bone healing around titanium implants, due to a reduction in bone filling in rats subjected to cannabis smoke inhalation. In contrast, the nonpsychoactive component of cannabis, cannabidiol has been shown to reduce bone resorption during experimental periodontitis in rats due to the reduction in proinflammatory mediators [33]. It has been reported that Δ^9-THC is a mitochondrial inhibitor [34], an effect that may inhibit the survival of MSCs and osteoblasts since mitochondrial function determines the viability and osteogenic potency of these cells [35]. These reports further emphasise the relevance of our observations that Δ^9-THC exposure increases numbers of apoptotic nuclei and induced the expression of active caspase-3 (a proapoptotic downstream signalling protease involved in the mitochondrial intrinsic pathway of apoptosis) in undifferentiated and differentiated MSCs. Thus, Δ^9-THC exposure may lead to a decreased ability of MSCs to differentiate into their mature bone forming progeny due to a lack of cell viability at early stages of osteogenesis which could inturn impact upon the osteogenic potential of MSCs

(supplemental Figure 4). Furthermore we conclude that this effect is specific to a long-term treatment with Δ^9-THC as we have previously published observations showing no deleterious effects following an acute 24-hour Δ^9-THC (1 μM) treatment [29]. This indicates that a long-term exposure to Δ^9-THC may have a negative effect on bone health possibly due to exogenous agonist-induced blockade of CB_1 receptor activation by endocannabinoids. However, further studies need to be carried out to confirm this. These results have important clinical implications for bone repair in cannabis users or self-medicating orthopaedic patients since it has already been clearly established that tobacco and alcohol consumption negatively impacts on bone health [36].

In summary, we have obtained additional insights into the role of the cannabinoid system in the regulation of bone maintenance by investigating the cannabinoid system during MSC osteogenic differentiation. Herein we show that the CB_1 receptor is induced during osteogenic differentiation and that it has a functional role in MSC survival during acute stress. These results are relevant to the successful culturing of osteogenic progenitor cells used in cell-based tissue engineered bone replacement therapies as a cannabinoid based approach may overcome the challenges associated with cell senescence and donor site morbidity present in current tissue engineered applications. Indeed, the concept of priming cells with specific growth factors or receptor specific ligands has been shown to control the differentiation potential and immunomodulatory profile of MSCs [37, 38]. In view of this, our results demonstrate the potential application of cannabinoids to prime MSCs in order to influence their *in vitro* and *in vivo* physiological functions representing an intriguing avenue for further research. We also provide evidence that the phytocannabinoid Δ^9-THC has a negative impact on MSC osteogenesis and survival. This may be a relevant factor which should be considered as a potential source of risk in the rate of clinical success of any bone replacement strategies.

Abbreviations

MSCs: Mesenchymal stem cells
OF: Osteogenic factors
CB: Cannabinoid
SW: Serum withdrawal.

Conflict of Interests

The authors of the paper do not have a direct financial relation with any commercial bodies mentioned in the paper.

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Research Article

Detection of the Hematopoietic Stem and Progenitor Cell Marker CD133 during Angiogenesis in Three-Dimensional Collagen Gel Culture

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We detected the hematopoietic stem and progenitor cell marker CD133 using immunogold labeling during angiogenesis in a three-dimensional collagen gel culture. CD133-positive cells were present in capillary tubes newly formed from aortic explants in vitro. The CD133-positive cell population had the capacity to form capillary tubes. Lovastatin strongly inhibited cell migration from aortic explants and caused the degradation of the capillary tubes. The present study provides insight into the function of CD133 during angiogenesis as well as an explanation for the antiangiogenic effect of statins.

1. Introduction

CD133 was first isolated and cloned in 1997. CD133 expression was originally observed in hematopoietic stem and progenitor cells using a monoclonal antibody called AC133 [1] and neuroepithelial cells using a monoclonal antibody called prominin [2]. Gehling et al. [3] reported that the CD133-positive cell population consists of progenitor and stem cells that not only have hematopoietic potential but also have the capacity to differentiate into endothelial cells. Invernici et al. [4] reported that human fetal aorta contains vascular progenitor cells capable of inducing vasculogenesis and angiogenesis. Barcelos et al. [5] reported that human CD133 progenitor cells promote the healing of diabetic ischemic ulcers by paracrine stimulation of angiogenesis and activation of Wnt signaling.

To grow, solid tumors require a blood supply. They recruit new blood vessels mainly by inducing the sprouting of endothelial cells from external vessels. Recent research in tumor biology shows that, in addition to recruiting vessels from outside the tumor, brain tumors produce endothelial cells for vessel formation within the tumor [6]. Wang et al. [7] reported that a glioblastoma cell population (CD144 and CD133 double positive) differentiated into endothelial cells and formed intracellular vacuolar structures in collagen gel. However, the biological function of CD133 in angiogenesis remains largely unknown.

For in vitro studies of angiogenesis, several culture techniques using matrix structures have been developed, including fibrin and collagen gels [8], Matrigel, collagen, fibrin, and plasma clots [9]. Collagen gel culture has been used widely and effectively for analyzing the biological process of angiogenesis [10–13]. Using a three-dimensional (3D) collagen gel culture, we have conducted electron microscopic studies [14, 15] and immunohistochemical studies of fibroblast growth factor- (FGF-) 2 and FGF-9 [16]. Additionally, we have used 3D collagen gel cultures to test angiogenic and anti-angiogenic agents (TNF-α and thalidomide) [17, 18] to study vascular injury after laser microdissection [19] and for profiling of DNA microarray gene expression during angiogenesis [20]. The 3D collagen gel culture system provides a simple and rapid method to analyze angiogenesis.

In the present study, we detected CD133 by immunogold labeling during angiogenesis in the 3D collagen gel culture system. Here, we show that CD133-positive cell population has a capacity to form capillary tubes.

2. Materials and Methods

2.1. Animals. ICR mice (male, 1 month old, $n = 5$; CLEA Japan, Inc.) and Wistar rats (male, 2 months old, $n = 3$; CLEA Japan, Inc.) were used for the experiment. The mice and rats were maintained according to the guidelines on the care and use of laboratory animals established by Saitama Medical University. These experiments were approved by the Animal Research Committee of Saitama Medical University.

2.2. Collagen Gel Culture of Mouse and Rat Aortae. The collagen culture technique used in the present study was modified from our previous technique [14, 15]. Thoracic aortae were obtained from mice and rats. Under a stereoscopic microscope, fibroadipose tissue and blood were removed from the aortae. The thoracic aortae were then serially cross-sectioned into ~2 mm rings. Four pieces were placed at the bottom of each tissue culture dish (35 mm; $n = 25$), overlaid with an even layer of reconstituted collagen solution (0.3% Cellmatrix type IA, Nitta Gelatin, Tokyo, Japan), and allowed to gel at 37°C for approximately 10 min. After the gels formed, they were overlaid with Ham's F-12 medium (Invitrogen Corp., Carlsbad, CA, USA), containing 20% fetal bovine serum (FBS), 1% nonessential amino acids, 100 units/mL penicillin, and 100 mg/mL streptomycin (Invitrogen Corp., Carlsbad, CA, USA), and cultured for 14 days in an incubator (95% air/5% CO_2). The medium was replaced three times a week starting from day 3. Capillary tube formation was observed using a phase contrast microscope during the culture period. These experiments were performed three times.

2.3. Phase-Contrast Microscopy and Time-Lapse Imaging. Standard phase-contrast images were collected by using a phase-contrast inverted microscope (Nikon TE2000, Japan) and a CCD camera (ORCA-ER, Hamamatsu Photonics, Japan). For time-lapse experiments, the aortic rings were cultured as described above. Cells that grew out from the aortic rings were visualized using a phase-contrast inverted microscope equipped with a stage that was preheated to 37°C. The cells were maintained under 5% CO_2 in a culture chamber during image acquisition, and images were recorded at 5 min intervals using an Aquacosmos imaging system (Hamamatsu Photonics, Japan).

2.4. Transmission Electron Microscopy. The cultured aortic rings were fixed in 0.1 M phosphate buffer (pH 7.2) containing 2.5% glutaraldehyde for 1 hour and then fixed in 0.1 M phosphate buffer (pH 7.2) containing 1% OsO_4 for 1 hour. The rings were dehydrated in graded ethanol, embedded in epoxy resin, cut into ultrathin sections, and stained with uranyl acetate and lead citrate. The stained ultrathin sections were observed under a transmission electron microscope (JEM-1010, Tokyo, Japan).

2.5. Rhodamine-Phalloidin and Lectin Histochemistry. After fixation in 4% paraformaldehyde/PBS, the cultured aortic rings were stained with rhodamine-phalloidin (Invitrogen Corp., Carlsbad, CA, USA) to determine the presence of F-actin, and FITC-conjugated endothelial-cell-specific tomato lectin (*Lycopersicon esculentum*, EY Labo, CA, USA), which selectively binds to fucose residues that are present on the endothelial cell surface, was used to label endothelial cells [21].

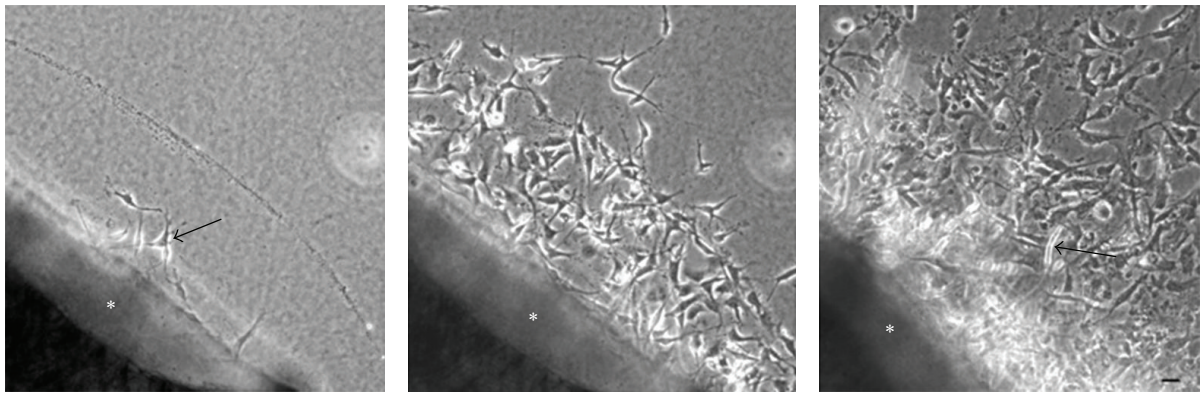
2.6. Immunohistochemical Detection of CD133. The cultured aortic rings were fixed in 4% paraformaldehyde/PBS. For the detection of CD133 on the cell surface, the rings were incubated overnight with CD133 antibody (rabbit polyclonal, Abcam, Tokyo, Japan) after treatment with 1% skim milk/PBS for 30 min, and then they were incubated with Alexa Fluor 488- and Nanogold (1.4 nm)-conjugated goat anti-rabbit IgG (Nanoprobes, Inc., Yaphank, NY) for 1 hour. The Nanogold signal was enhanced using GoldEnhance EM (Nanoprobes) at room temperature for 3–5 min for electron microscopy and 20–25 min for light microscopy.

2.7. Effect of Lovastatin (Mevinolin) on Angiogenesis. Before and after tube formation, the effect of lovastatin (mevinolin from *Aspergillus* sp.) was tested. Lovastatin (mevinolin, M2147) supplied by Sigma has an empirical formula of $C_{24}H_{36}O_5$ and is 2-methyl-1,2,3,7,8,8a-hexahydro-3,7-dimethyl-8-[2-(tetrahydro-4-hydroxy-6-oxo-2H-pyran-2-yl)-ethyl]-1-naphthalenyl ester butanoic acid. It is a white crystalline powder that is insoluble in water. A stock solution was prepared by dissolving it in 100% ethanol at a concentration of 12.3 mM [22]. Two different sets of experiments were designed as follows.

Before Tube Formation. Rat aortic rings were cultured in 35 mm dishes as described above. After 24 hours, aortic rings were cultured with 12.3 mM lovastatin or without lovastatin. Cultures were maintained at 37°C under 5% CO_2 in a humidified incubator.

After Tube Formation. Rat aortic rings were cultured in 35 mm dishes as described above. After 10 days, lovastatin was added to the culture medium. Cultures were maintained at 37°C under 5% CO_2 in a humidified incubator.

2.8. Endothelial Cell Scraping. In a separate set of experiments, the trimmed thoracic aorta was cultured as follows. To visualise the intimal surface directly, the thoracic aorta was everted with a procedure that sequestered the adventitial cells and possible remnant microvessels of periaortic soft tissue inside the aortic tube [23]. The endothelial cells were scraped from the everted aorta with a sterile cotton swab. The everted aorta with/without endothelium was cut into small pieces and cultured in the same manner as previously described, and followed by 10% formalin fixation and Giemsa staining.



(a) (b) (c)

Figure 1: Phase-contrast microscopy. (a) After 2 days of cultivation, phase-contrast microscopy revealed fibroblastic cells (arrow) outgrown from a mouse aortic explant (∗) into a three-dimensional collagen gel. (b) After 5 days of cultivation, phase-contrast microscopy showed numerous fibroblastic cells outgrown from an aortic explant (∗). (c) After 7 days of cultivation, phase-contrast microscopy showed a tubular structure protruding (arrow) from an aortic explant (∗) into a three-dimensional collagen gel. (a), (b), and (c): Scale bar = 20 μm.

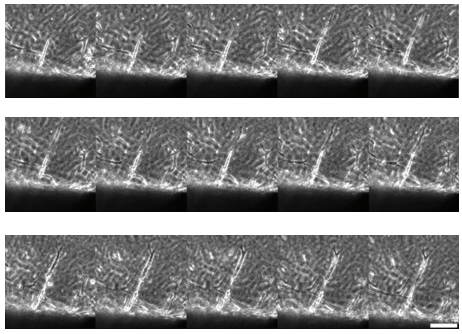
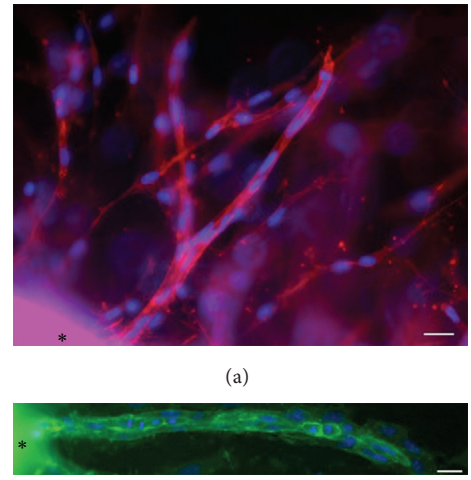


Figure 2: Time-lapse imaging of additional sprouts emerging and extending in a sequential manner from the leading edges of a newly formed capillary tube from the mouse aortic explant. Selected sequence from a time-lapse movie focusing on a single sprout. Note the protrusion of lamellipodia and continued migration of the leading cell. Scale bar = 50 μm.



(a)

(b)

Figure 3: Rhodamine-phalloidin and lectin histochemistry. (a) After 11 days of cultivation, fluorescence microscopy showed capillary tubes that are stained with rhodamine-phalloidin for F-actin (purple-red) and 4′,6-diamidino-2-phenylindole (DAPI) nucleic acid stain (blue). The asterisk (∗) shows the mouse aortic explant. Scale bar = 20 μm. (b) After 11 days of cultivation, the capillary tubes are strongly positive for FITC-conjugated endothelial-cell-specific tomato lectin staining (yellowish-green) and DAPI nucleic acid stain (blue). The asterisk (∗) shows the aortic explant. Scale bar = 20 μm.

3. Results

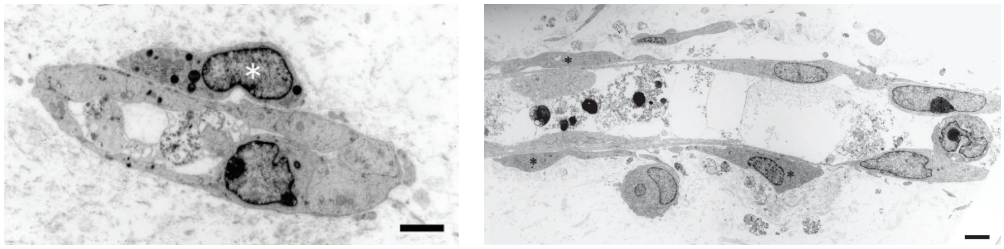
3.1. Capillary Tube Formation

3.1.1. Phase-Contrast Microscopy. Microscope examination after as little as 2 days of culture revealed the presence of migrating cells proximal to the aortic ring in the collagen gel. These cells were spindle-shaped and their longitudinal axes were radially orientated toward the stump of the aortic ring (Figures 1(a) and 1(b)). After a 7-day culture period, capillary sprouts were recognizable (Figure 1(c)), although lumen formation was not observed in these early capillary structures.

3.2. Time-Lapse Imaging of Capillary Tube Formation. Time-lapse imaging was used to visualize the dynamic process of capillary tube formation from the aortic ring. Additional sprouts emerged and extended in a sequential manner from the leading edges of newly formed capillary tubes (Figure 2).

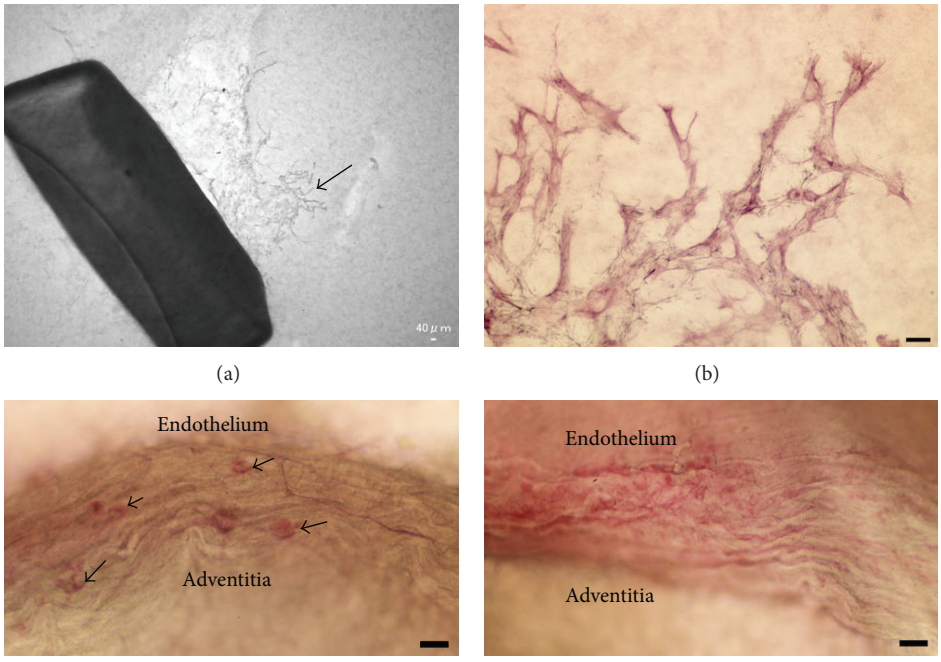
3.3. Rhodamine-Phalloidin and Lectin Histochemistry. After 10 to 14 days in culture, elongated capillary tubes with branches were observed. Capillary tubes in the collagen gels were observed by rhodamine-phalloidin staining (Figure 3(a)). Capillary tubes that formed in the collagen gels were also strongly positive for tomato lectin (Figure 3(b)).

3.4. Transmission Electron Microscopy. As demonstrated by cross-sectioning, endothelial cells of the capillary tubes formed tight contacts with each other, and pericyte-like cells



(a) (b)

Figure 4: Transmission electron microscopy. (a) Electron microphotograph of a newly formed capillary tube from the mouse aortic explant in the collagen gel. A cross-section shows a capillary tube with a lumen that contains cell debris. Pericyte-like cells (∗) surround the tube. The edges of the cells are in contact with each other. Scale bar = 2 μm. (b) Electron microphotograph of a longitudinal section of a capillary tube. Pericyte-like cells (∗) surround the tube. Scale bar = 2 μm.



(a) (b)

(c) (d)

Figure 5: Immunohistochemistry of CD133. (a) In the early stages of culture (4-day culture), CD133-positive cells (arrow) were detected among cells migrating from the rat aortic explant. (b) Enlarged image of migrating CD133-positive cells. Scale bar = 20 μm. (c) CD133 expression (arrows) was found in the smooth muscle layer and near the adventitia. Scale bar = 20 μm. (d) CD133 expression was also found in the endothelium and the smooth muscle layer. Scale bar = 20 μm.

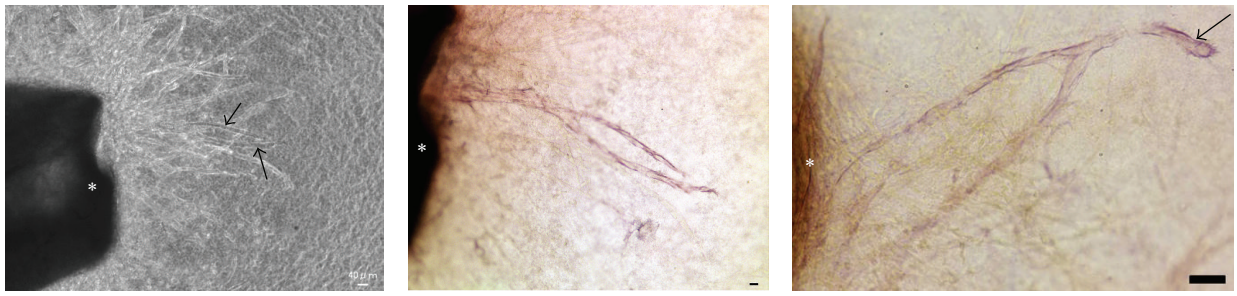
were present on the outside of the endothelial cells (Figure 4(a)). The endothelial cells did not show any pores or gaps. Typical gap junctions and tight junctions were not observed. Cell organelles were present in large numbers, particularly in the thicker endothelial cells. Longitudinal sectioning revealed that endothelial cells and pericyte-like cells made close contact (Figure 4(b)).

3.5. Immunohistochemical Detection of CD133. In the early stages of culture, CD133-positive cells were detected among cells migrating from the aortic ring (Figures 5(a) and 5(b)). CD133 expression was also found within the aortic wall (Figures 5(c) and 5(d)). In the later stages of culture, capillary tubes formed. CD133-positive cells were present in a tube-like pattern (Figures 6(a) and 6(b)), CD133 expression was found in both the tip and stalk regions. The leading edge of the capillary tube was strongly positive for CD133 (Figure 6(c)). Electron microscopic observation revealed CD133 expression in cells located on the bottom of the collagen gels (Figure 7).

3.6. Effect of Lovastatin (Mevinolin) on Angiogenesis

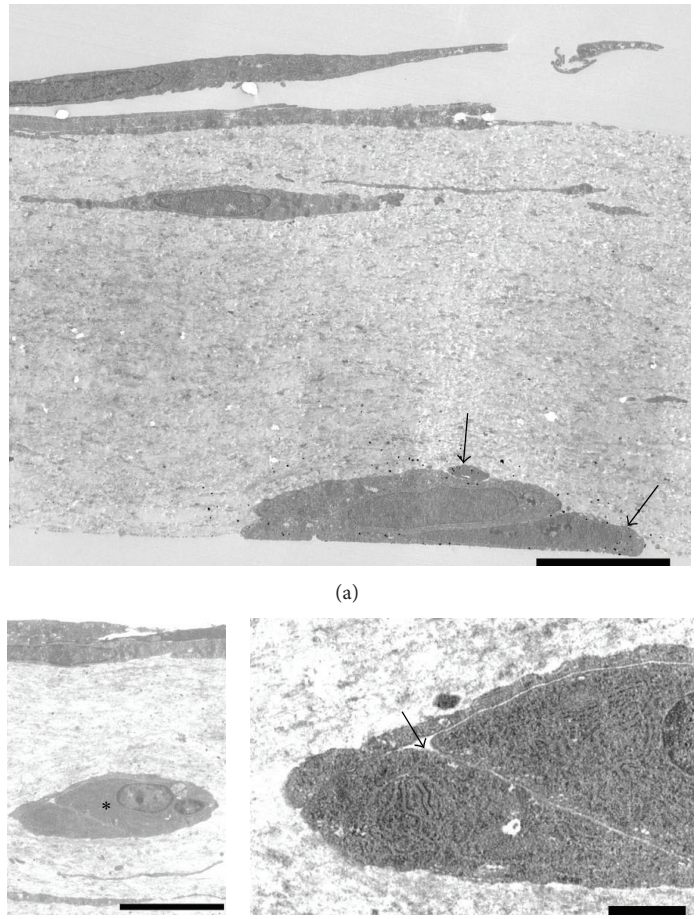
Before Tube Formation. When aortic rings were cultured with lovastatin, cell migration was strongly inhibited relative to the control (Figures 8(a) and 8(b)).

After Tube Formation. Lovastatin treatment induced the degradation of newly formed capillary tubes (Figures 9(a), 9(b), and 9(c)). Cell-cell adhesion was diminished, and the morphology of many CD133-positive cells changed to an oval



(a) (b) (c)

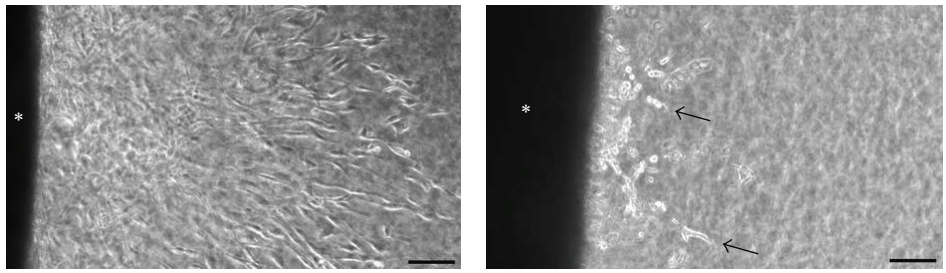
Figure 6: CD133 immunoreactivity in capillary tubes. (a) In the later stages of culture (9-day culture), capillary tubes formed. Phase-contrast microscopy showed that many tubes were formed from the rat aortic explant. Arrows indicate CD133-positive tubes among CD133-negative tubes. Rat aortic explant (∗). (b) CD133-positive cells were present in a tubular pattern. Photograph showing CD133-positive tubes indicated by arrows in (a). Rat aortic explant (∗). Scale bar = 20 μm. (c) CD133 expression was clearly found in the tip region (arrow) of the tube rather than the stalk region near the rat aortic explant (∗). Scale bar = 20 μm.



(a)

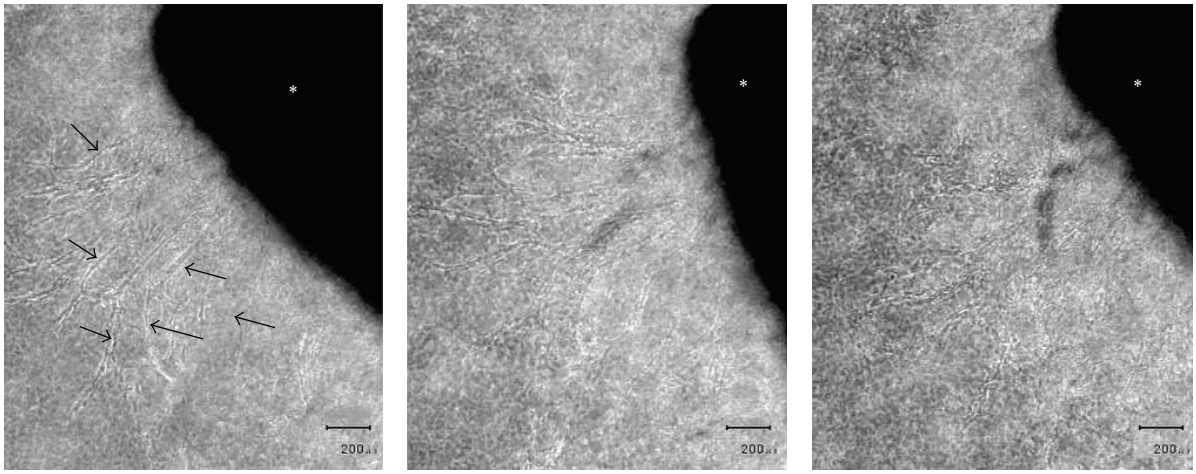
(b) (c)

Figure 7: Immunoelectron microscopy of CD133. (a) At the bottom side of the collagen gels, CD133-positive cells made contact with each other (arrows). Cell organelles were sparse in the cells. Scale bar = 5 μm. (b) CD133-negative cells made contact with each other in the middle layer of the collagen gels (∗). Scale bar = 5 μm. (c) Enlargement of (b) (∗). These cells formed intercellular vacuolar structures (arrow). Cell organelles, such as the rough endoplasmic reticulum, were rich in these cells. Scale bar = 1 μm.



(a) (b)

Figure 8: Effect of lovastatin (mevinolin) before tube formation. (a)-(b) When rat aortic rings were cultured with lovastatin, cell migration (arrows) was strongly inhibited relative to the control. (a) = control, (b) = lovastatin, aortic explant (∗). Scale bar = 100 μm.



(a) (b) (c)

Figure 9: Effect of lovastatin (mevinolin) after tube formation. (a)–(c) After tube formation, lovastatin treatment induced the degradation of newly formed capillary tubes (arrows). (a) Before Lovastatin treatment; (b) after 24 hours; (c) after 48 hours; rat aortic explant (∗). Scale bar = 200 μm.

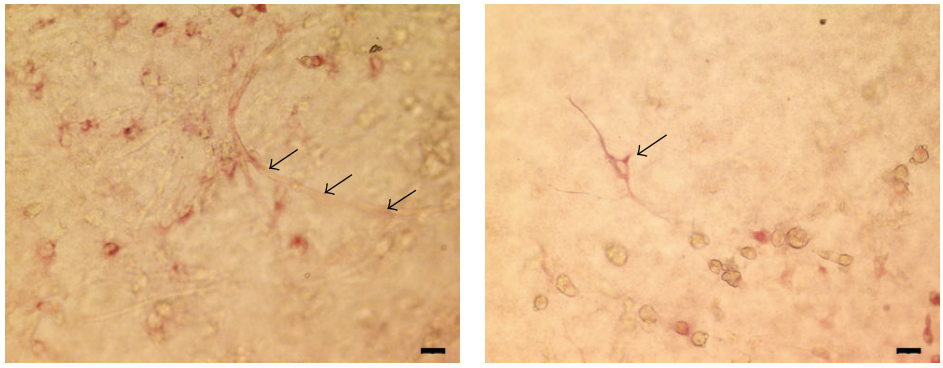
shape (Figure 10(a)), although some polygonal cells with cell processes maintained their morphology (Figure 10(b)).

3.7. Effect of Endothelial Cell Scraping. For the everted aorta with intact epithelium, spindle-shaped cells migrated into the collagen gels, and capillary tube formation occurred in a similar manner (Figure 11(a)). After endothelial cell scraping, spindle-shaped cells migrated into the collagen gels from the everted aorta, even without the presence of epithelial cells. However, capillary tube formation did not occur (Figure 11(b)).

4. Discussion

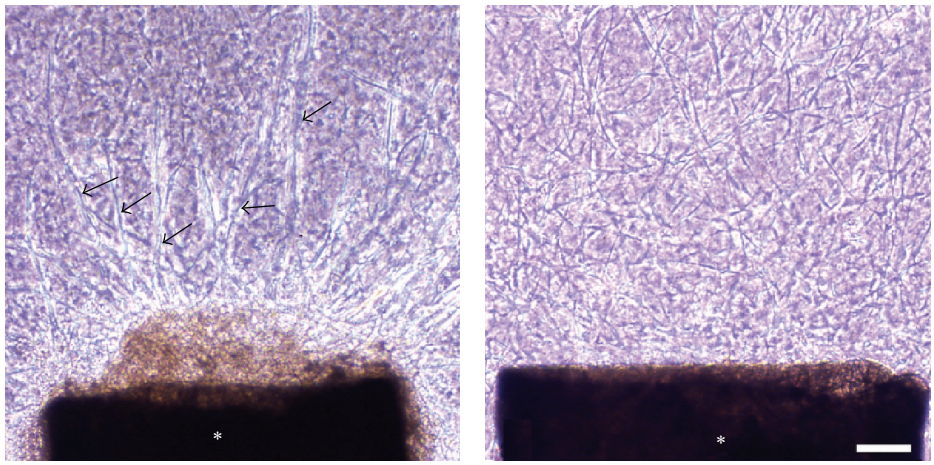
Recently, LS-7 (amino acid sequence: LQNAPRS), which is a specific binding peptide that targets mouse CD133, was screened and identified for the first time using phage-displayed peptide library technology [24]. However, the biological function of CD133 remains unclear. CD133 expression is not restricted to the neuroepithelial and hematopoietic stem and progenitor cells in which it was originally observed; it also extends to several epithelial and nonepithelial cell types. CD133 is also widely used as a marker for cancer stem cells (CSCs) in many different types of solid tumors including colon [25, 26], brain [27, 28], skin [29], pancreatic [30], liver [31–33], and prostate [34] tumors. Wang et al. [7] and Ricci-Vitiani et al. [35] presented evidence that tumor-derived endothelial cells arise from tumor stem-like cells. Wang et al. [7] found that a glioblastoma cell population that could differentiate into endothelial cells and form intracellular vacuolar structures in collagen gels was enriched in cells expressing CD133. Although the possibility of endothelial differentiation of tumor cells has been suggested in lymphoma, myeloma, chronic myeloid leukemia, breast cancer, and neuroblastoma [36–40], the angiogenic activity of CSCs has not been investigated in other types of tumors. Because glioblastoma is one of the most vascular-rich tumors, further investigation is needed to evaluate the differentiation of CSCs into endothelial cells.

In the present study, we demonstrated that CD133-positive cells were present in the newly formed capillary tubes. Wang et al. [7] suggested that the differentiation of CSCs into endothelial cells may be mediated by signaling pathways



(a) (b)

Figure 10: (a) Cell-cell adhesion was diminished, and many CD133-positive cells adopted a round morphology. Arrows indicated the degradation of capillary tubes. Scale bar = 20 μm. (b) Some polygonal cells with cell processes maintained their morphology (arrow). Scale bar = 20 μm.



(a) (b)

Figure 11: (a) A control culture from the rat aorta explant. Many capillary tubes (arrows) are present. (b) A deendothelialized rat aortic explant that has spindle-shaped cells migrating into collagen gels. However, capillary tube formation did not occur. Rat aortic explant (∗), Giemsa stain. Scale bar = 200 μm.

involving two proteins: vascular endothelial growth factor (VEGF) and notch. Wang et al. [7] proposed that notch regulates the initial differentiation of cancer stem cells to endothelial progenitor cells, whereas VEGF selectively affects the differentiation of endothelial progenitors to tumor-derived endothelial cells. We showed that the leading edge of the capillary tube was strongly positive for CD133, which suggests that CD133-positive cells are involved in the elongation and/or branching of capillary tubes. Mature endothelial cells do not express CD133. CD133 is expressed by endothelial precursors and rapidly lost upon differentiation into mature endothelial cells [41]. It is therefore likely that the newly formed capillary tubes that consist of CD133-positive cells are immature. Soda et al. [42] reported that hypoxia-inducible factor-1 (HIF-1) is an important enhancer of EC differentiation of tumor cells and that the formation of tumor-derived endothelial cells is independent of VEGF. Hypoxic conditions may have thus resulted in the formation of CD133-positive capillary tubes at the bottom of the collagen gels.

The present study showed that CD133-positive cells were also present within aortic explants. Zengin et al. [43] reported the existence of endothelial precursor and stem cells in a distinct zone of the vascular wall that are capable to differentiate into mature endothelial cells, hematopoietic cells, and local immune cells such as macrophages. This zone has been identified to be loculated between the smooth muscle and the adventitial layer of the adult human vascular wall. Progenitor cells isolated from the adventitia of both murine and human blood vessels have the potential to form endothelial cells, mural cells, osteogenic cells, and adipocytes. These progenitors appear to cluster at or near the border zone between the outer media and inner adventitia [44]. After scraping the epithelium, spindle-shaped cells migrated into the collagen gels from both ends of the everted aorta. However, capillary tube formation did not occur. In this aortic culture, it is likely that the primary source of newly formed capillary tubes is the intimal endothelial cells. Nicosia [12] also noted that rat carotid artery explants failed to generate an angiogenic

response when completely deendothelialized with a balloon catheter, whereas control arteries with an intact intimal endothelium produced microvessels from their resected ends. At the present time, it is unclear whether the primary source of newly formed capillary tubes is derived from the distinct zone between the smooth muscle and the adventitial layer or from the intimal endothelial cell layer. Further studies are needed to clarify these issues.

The present study also showed that lovastatin strongly inhibited cell migration from the aortic explant. The mechanism of inhibition of cell migration is considered as follows. Lovastatin is a potent inhibitor of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase. The inhibition of HMG-CoA reductase, which is involved in lipid metabolism, causes the lipids necessary for the normal membrane functioning to become defective, and further impairment is seen in their adhesive properties. The adhesion of lipids is mediated through integrins, which are necessary for cell motility and migration through the extracellular matrix in the process of invasion [22]. Clinical data indicate that statin-treated patients have diminished intraplaque angiogenesis [45], which suggests that statins have angiostatic effects in vivo. It is also intriguing that statins have been reported to reduce the growth and spread of many cancers [46, 47], which may be related to inhibition of angiogenesis [48]. The present study also showed that lovastatin cased the abrogation of cell-cell adhesion and degradation of capillary tubes. Khaidakov et al. [48] suggested that statins through VE-cadherin stimulation modulate cell-cell adhesion and diminish the ability of cells to proliferate and migrate. Recently, Koyama-Nasu et al. [49] reported that CD133 interacts with plakoglobin (also known as c-catenin), a desmosomal linker protein. They further demonstrate that knockdown of CD133 by RNA interference (RNAi) results in the downregulation of desmoglein-2, a desmosomal cadherin, and abrogates cell-cell adhesion and tumorigenicity of clear cell carcinoma of the ovary stem cells. In addition, we reported that the cholesterol chelating agent, methyl-β-cyclodextrin, diminished cell adhesion by decreasing desmosomes and intercellular digitations. A decrease in the cholesterol level may perturb CD133 membrane localization [50]. Modulation of cell-cell adhesion may help to explain the degradation of the capillary tubes. The present study thus provides insight into the function of CD133 during angiogenesis and provides an explanation for the anti-angiogenic effect of statins.

5. Conclusions

The CD133-positive cell population has the capacity to form capillary tubes. The present study provides a useful method for determining the function of CD133 during angiogenesis.

Conflict of Interests

The authors report no conflict of interests concerning the materials or methods used in this study or the findings specified in this paper.

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Review Article

Intrinsic Ability of Adult Stem Cell in Skeletal Muscle: An Effective and Replenishable Resource to the Establishment of Pluripotent Stem Cells

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Adult stem cells play an essential role in mammalian organ maintenance and repair throughout adulthood since they ensure that organs retain their ability to regenerate. The choice of cell fate by adult stem cells for cellular proliferation, self-renewal, and differentiation into multiple lineages is critically important for the homeostasis and biological function of individual organs. Responses of stem cells to stress, injury, or environmental change are precisely regulated by intercellular and intracellular signaling networks, and these molecular events cooperatively define the ability of stem cell throughout life. Skeletal muscle tissue represents an abundant, accessible, and replenishable source of adult stem cells. Skeletal muscle contains myogenic satellite cells and muscle-derived stem cells that retain multipotent differentiation abilities. These stem cell populations have the capacity for long-term proliferation and high self-renewal. The molecular mechanisms associated with deficits in skeletal muscle and stem cell function have been extensively studied. Muscle-derived stem cells are an obvious, readily available cell resource that offers promise for cell-based therapy and various applications in the field of tissue engineering. This review describes the strategies commonly used to identify and functionally characterize adult stem cells, focusing especially on satellite cells, and discusses their potential applications.

1. Introduction

Stem cells are primordial cells common to all multicellular organisms and retain two distinctive properties: (1) the ability to self-renew through mitotic cell division and thus remain in an undifferentiated state and (2) the ability to differentiate into specific cell types [1, 2]. When a stem cell divides, each new cell has the potential either to remain a stem cell or become another type of cell with a more specialized function, such as a muscle cell, a blood cell, or a brain neuronal cell. Recent studies in the field of therapeutics suggest that stem cells will become a major focus in organ transplantation and replacement of lost tissue [3]. Stem cells can be categorized as totipotent, pluripotent, and multipotent, depending upon their differentiation potential [4, 5]. Totipotent stem cells arise through the fusion of an egg with a sperm and differentiate into embryonic and extraembryonic cell types. Pluripotent cells are the descendants of totipotent cells and can give rise to most of the tissues necessary for embryonic development.

Embryonic stem (ES) cells are pluripotent, meaning that they can differentiate into all lineages of the primary three germ layers [6]: ectoderm, endoderm, and mesoderm, which are distinguished by their pluripotency and capability for indefinite self-renewal. Pluripotent stem cells originate as an inner cell mass within a blastocyst. The blastocyst contains three distinct areas: the trophoblast, which is the surrounding outer layer that later becomes the placenta, the blastocoel, which is a fluid-filled cavity within the blastocyst, and the inner cell mass, which becomes the embryo proper. ES cells

can be created from cells taken from the inner cell mass. Because these cells represent such an early stage of development, they have the ability to become cells of any tissue type (except for the whole embryo itself), making them pluripotent. ES cells generate more than 220 cell types in the adult body, while adult stem cells are multipotent and can only produce a limited number of cell types [7].

Induced pluripotent stem (iPS) cells are generated by reprogramming a differentiated somatic cell into a pluripotent ES cell using defined factors (Oct4/c-Myc/Klf4/Sox2) [8]. iPS cells appear to be an ideal substitute for ES cells, and many efforts have been made to improve methods of iPS cell generation and for understanding the reprogramming mechanism as well as the nature of iPS cells. The most important contribution of iPS cells to medicine may be the possibility of establishing personalized iPS cells for clinical applications without the need to harvest allogeneic human ES cells from embryos or deal with nuclear transfer [9]. The generation of patient-specific iPS cells for studies of genetic background and disease mechanisms is also useful approach for the screening of new drugs. Such customized iPS cells generated from patients can also be studied *in vitro* or *in vivo* as models for the pathogenesis of specific diseases [10]. One issue that hinders the clinical use of human ES cells is the lack of identical genetics between donor cells and recipients. This issue can be resolved using iPS cell. However, iPS cells generated from patients harboring genetic disorders cannot be applied for cell therapy, as iPS cell technology reprograms epigenetic, but not genetic, information in somatic nuclei. Several technologies have been developed for genome editing using disease-specific iPS cell lines [11, 12], and further elucidation of safety concerns and the mechanisms behind the differences in genetic background is required.

2. Adult Stem Cells

Pluripotency distinguishes ES cells from adult stem cells, which retain multipotency. Adult stem cells are undifferentiated cells contained throughout the body and divide to replenish dying cells and regenerate damaged tissue [13, 14]. They are also known as somatic stem cells. Adult stem cells have a close relationship with the surrounding tissue and the environment. Their niche is a specialized cellular microenvironment that provides them with the support needed for self-renewal [15, 16]. To ensure this, stem cells undergo two types of cell division. Symmetric division gives rise to two identical daughter cells both endured with stem cell properties. Asymmetric division produces only one stem cell and a progenitor cell with limited self-renewal potential [17, 18]. Progenitors can undergo several rounds of cell division before terminally differentiating into a mature cell.

Bone marrow is the major source of adult stem cells. Hematopoietic stem cells can give rise to all blood cell types including both the myeloid (monocytes and macrophages, neutrophils, basophils, eosinophils, erythrocytes, megakaryocytes/platelets, and dendritic cells) and lymphoid (T cells, B cells, NK cells, and some dendritic cells). Bone marrow stromal stem cells are progenitors of skeletal tissue components such as bone, cartilage, hematopoiesis-supporting stroma, and adipocytes. Mesenchymal stem cells are multipotent stem cells that can differentiate into a variety of cell types *in vitro* or *in vivo*. Adult mesenchymal stem cells have been used in preclinical models for tissue engineering of bone, cartilage, muscle, marrow stroma, tendon, fat, and other connective tissues [19]. Although therapeutic applications are potentially acceptable, a rigorous understanding of mesenchymal stem cells requires a better definition of what such stem cells are, since currently there are no specific markers that can reliably discriminate between mesenchymal stem cells and others (e.g., fibroblasts).

Muscle-derived stem cells have been shown to differentiate into myogenic, osteogenic, chondrogenic, adipogenic, and hematopoietic cells, similarly to mesenchymal stem cells [20]. In contrast, satellite cells that are committed myogenic stem cells are more restricted to skeletal muscle lineage [21]. Although satellite cells are considered to be the major stem cell source in skeletal muscle, many studies suggest that non-satellite cells exhibit myogenic capacities [22–28]. Mesenchymal stem cells are able to display a skeletal muscle phenotype under appropriate conditions [29]; however, details of the mechanism of transdifferentiation remain elusive.

3. Muscle-Specific Adult Stem Cells—"Satellite Cell"—and the Development

Muscle tissue represents an abundant, accessible, and replenishable source of adult stem cells. Skeletal muscle accounts for a large proportion of total body weight, being over 30% for women and around 38% for men [30]. Myofibers are the basic cellular unit of skeletal muscle, and the syncytial myofiber is formed in the embryo through fusion of many myoblasts. Skeletal muscles are formed from the paraxial mesoderm, surrounding the neural tube, which separates into blocks known as somites. Dorsally, somites differentiate into epithelial dermomyotome, which then develops into myotome (the source of limb muscle), into dermatome (a specific region for nerve reception supplied by sensory neurons), and ventrally into mesenchymal sclerotome. The nuclei of myofibers originate from the myotomal somitic or lateral plate somatic mesoderm depending on the anatomical tissue location [31].

Cells within the dermomyotome exhibit specific expression patterns of Pax3 and Pax7. In the mouse embryo, Pax3(+) or Pax7(+) muscle progenitor cells in the dermomyotome enter the myotome in the central compartment of the somite from embryonic day 10.5 (E10.5). Initially, Pax3 and Pax7 are expressed ubiquitously throughout the dermomyotome. During embryonic development, Pax3(+)/Pax7(+) positive cells continually generate fetal myoblasts, which can be identified by their expression of Myf5 (the earliest marker of the myogenic lineage). Although the expression of Pax3 gradually decreases in the dorsomedial area and becomes predominant in the lateral dermomyotome, the expression of Pax7 becomes concentrated in the dorsomedial region of the dermomyotome [32]. Pax7(+) cells become lineage specific to muscle after E12.5 and occupy the sublaminal spaces of myofibers

around E16.5, and expression of Pax7 is retained by the population of satellite cells (quiescent muscle progenitors; see below). Clonal analyses of satellite cells have suggested that satellite cells are heterogeneous with regard to their self-renewal abilities and the extent of the progeny they generate [33]. Pax7 is considered to be both a specific marker and essential for specification of the adult satellite cell pool [34].

Satellite cells are muscle-specific stem cells identified by their direct attachment to the muscle fibers under the basal lamia (Figure 1) [35]. In adult muscle, satellite cells are in a quiescent state under normal conditions and represent 2.5–6% of all the nuclei of a muscle fiber. However, when activated by muscle injury, they can proliferate, undergo self-renewal, differentiate, and then generate a large number of new fibers within a few days [36]. The basal lamina contains several extracellular matrix proteins, such as collagen, laminin, fibronectin, and proteoglycans, and provides structural support by anchoring satellite cells. Skeletal muscle niches control the signaling of extracellular matrix materials, cell adhesion, and the behavior of satellite cells [37–39]. Identification of the molecular signals of stem cell niches is indispensable for understanding the action and function of satellite cells [39].

As an external stimulatory factor, exercise has a positive effect on the regulation of satellite cells. Several studies have indicated that the number of satellite cells increases after long-term or acute exercise training [40, 41]. This accretion of satellite cells in skeletal muscle is also evident in humans [42]. As a long-term effect of exercise, the trapezius muscle of trained power lifters contains 70% more satellite cells than that of control subjects [43]. The increased number of satellite cells after exercise gradually decreases during detraining, indicating that continuous exercise is required to maintain a rich satellite cell pool in skeletal muscle. Kurosaka et al. reported that the satellite cell pool following endurance training depends on the intensity rather than duration of exercise [44]. In various types of exercise, the effective method to increase and maintain satellite cell pool is investigated [45].

Since skeletal muscle is a flexibly changeable organ that can frequently increase or decrease in strength and mass and regenerate after injury, the stem cells included in the tissue can show dramatic changes in their fate depending on the circumstances of an individual's life. The balance that exists among self-renewal, differentiation, survival, activation, fusion, cell adhesion, and migration supported by these various extracellular signals is crucial for stem cell maintenance and muscle tissue homeostasis.

4. Molecular Mechanisms Regulating Satellite Cells

4.1. Paired Box Transcriptional Factor 7 (Pax7) and the MyoD Family. Satellite cells are a heterogeneous population and demonstrate at least two phases in skeletal muscle turnover: a mitotically quiescent state and an activated proliferative state (Figure 1). Both quiescent and activated satellite cells express a characteristic marker, Pax7 [46]. Quiescent satellite cells express Pax7 alone, whereas activated satellite cells coexpress Pax7, Myf5, and MyoD, which are key transcription factors for myogenic differentiation [46]. Although most Pax7(+)/MyoD(+) activated satellite cells proliferate and differentiate, accompanied by Pax7 downregulation, a small population of Pax7(+)/MyoD(−) satellite cells withdraws from the cell cycle and returns to a quiescent state [47]. Pax7-deficient satellite cells are gradually lost in skeletal muscle due to death or precocious differentiation [34, 48]. In particular, skeletal muscle mass and myofiber diameter are significantly reduced in $Pax7^{-/-}$ mice [49].

Activated satellite cells express Myf5 and MyoD, which are members of the MyoD family of basic helix-loop-helix (bHLH) transcription factors that play essential roles in regulating satellite cell differentiation and skeletal muscle development [36]. Myf5 is a target of the Pax7 transcription factor. Pax7 activates Myf5 expression through recruitment of a histone methyltransferase (HMT) complex, and the activator complex directly methylates histone H3 lysine 4 (H3K4) in the promoter region of Myf5 [50]. Kawabe et al. have reported that Pax7 is a specific substrate of coactivator-associated arginine methyltransferase 1 (Carm1), which is a protein arginine methyltransferase that methylates histone H3, and the methylation of Pax7 by Carm1 leads the recruitment of the HMT complex to the Myf5 locus [51]. Double-positive Pax7(+)/Myf5(+) satellite cells can upregulate MyoD, triggering proliferation [47, 52]. In this context, FoxO3 also contributes to MyoD upregulation by binding to the MyoD promoter region with Pax7 and recruiting RNA polymerase II [53]. The bHLH MyoD transcription factor initiates a differentiation program through association with E proteins (i.e., the E2A gene products, E12, E47, and HEB) by creating a heterodimer for the consensus E-box regulatory sequences on muscle-specific genes (myogenin, which is expressed at a more differentiated stage, Acta1, Lsp1, Mef2c, Tnnc2, Tnni2, Tnnt3, etc.) [54, 55]. For transcriptional activation, MyoD associates with HATs p300 and pCAF, which acetylate histones H3 and H4 [56, 57]. MyoD can also interact with the ATP-dependent chromatin remodeling factor SWI/SNF, leading to activation of the muscle-specific genes [58, 59]. Additionally, Rampalli et al. have demonstrated that MyoD collaborates with Mef2d to trigger the expression of the target myogenin gene [57]. Members of the Mef2 transcriptional regulator family are expressed in most tissues and play a critical role during myogenesis, with Mef2d being the skeletal muscle-specific isoform [60]. Mef2d accelerates the differentiation of skeletal muscle together with MyoD [61]. MyoD acts as master regulator of myogenesis to access and remodel chromatins and to induce the active transcription of muscle-specific genes.

4.2. Notch Signaling Pathway. One candidate for regulating the quiescent state of satellite cells is the Notch signaling pathway, whose activity has been shown to regulate cell fate and proliferation in a variety of tissues [58, 59, 62, 63]. Binding of Notch receptors to their DSL ligands (Delta/jagged, Serrate, or Lag2) releases the Notch intracellular domain (NICD) [64]. NICD is translocated into the nucleus and binds to recombining binding protein-Jκ (RBP-Jκ) [65], which is a key mediator of Notch signaling and acts downstream of Notch receptors [66]. RBP-Jκ inhibits transcription target genes

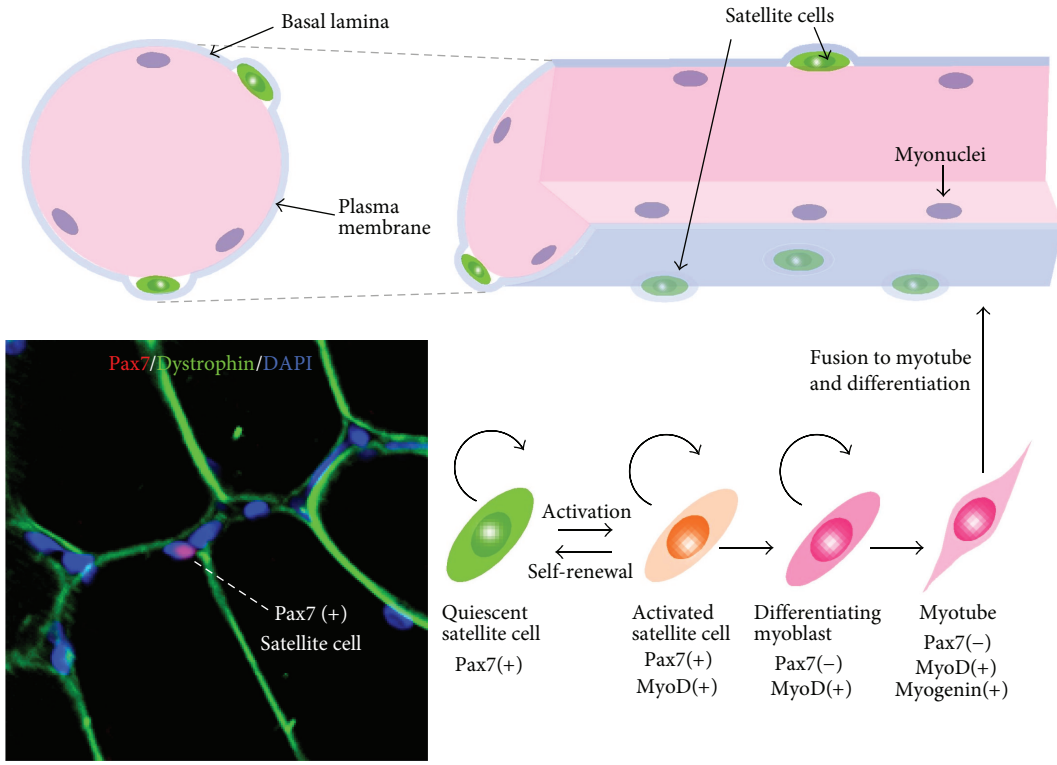

Figure 1: The self-renewal, activation, and differentiation of satellite cells in adult skeletal muscle. Satellite cells reside adjacent to the plasma membrane under the basal lamina on the surface of the myofiber. The nuclei of myofiber (myonuclei) are positioned at the periphery of the cell. Satellite cells are activated upon receiving various external stimuli and differentiate together with the upregulation of MyoD. Quiescent and activated satellite cells express a characteristic marker, Pax7. Immunohistochemical detection of Pax7 (red), dystrophin (green) and DAPI (blue) of adult rat skeletal muscle is shown (left bottom).

by binding transcriptional corepressors in the absence of Notch signaling [67], while binding to NICD and displacing corepressors, leading to transcriptional activation in the presence of Notch [68].

Notch signaling regulates the proliferation and differentiation of activated muscle satellite cells [69]. Notch3 is expressed by quiescent satellite cells [70], and disruption of Notch3 results in loss of regulation of satellite cell proliferation [71]. Deletion of RBP-Jκ in satellite cells specifically leads to their depletion through loss of their ability to regenerate after muscle injury [72, 73]. Whereas RBP-Jκ-deficient satellite cells proliferate before fusion, most of them differentiate without the first division and fuse with adjacent myofibers, resulting in satellite cell depletion in muscles. Hes1, Hey1, and HeyL, which are downstream factors of Notch signaling, are highly expressed in quiescent satellite cells. Members of both the Hes and Hey families of bHLH repressors are induced by Notch. Hes1 or Hey1 inhibits the expression of MyoD via the formation of inactive Hes1/MyoD or Hey1/MyoD heterodimers [74, 75] and blocks the differentiation of satellite cells into myoblasts. Inhibition of myogenesis by Notch is critical for the expansion of the undifferentiated stem cell population, and expression of the target genes for Notch signaling contributes to regulation of the quiescence of satellite cells.

4.3. Wingless-Type MMTV Integration Site Family (Wnt) Signaling Pathway. Wnt is a family of highly conserved secreted signaling molecules that play an essential role in the development and function of a variety of tissues. Wnt proteins typically bind to Frizzled receptors (Fzd) located in the plasma membrane [76]. The binding of Wnt and receptors activates β-catenin/TCF/LEF transcriptional complexes. β-Catenin, which is subunit of the cadherin protein complex and acts as an intracellular signal transducer, associates with its own degradation complex, resulting in its ubiquitin-dependent degradation [77]. However, when Wnt binds to Fzd receptors, β-catenin can translocate into the nucleus and bind members of the TCF and LEF family of transcription factors resulting in activation of target gene transcription [78]. This pathway through β-catenin is referred to as canonical Wnt signaling. In contrast, there are noncanonical Wnt signaling pathways that transmit signals through Rac/Rho activation, leading to cytoskeletal remodeling and induction of Jun target genes [79] and the PKC-calcium-dependent pathway [80].

It has been clarified that Wnt signaling is an important factor in the regulation of myogenesis, because of its influence on expression of the MyoD family. Wnt1 induces the expression of Myf5, whereas Wnt7a or Wnt6 preferentially activates MyoD in explant cultures of mouse paraxial mesoderm [81].

Wnt/β-catenin signaling has been shown to initiate the differentiation of satellite cells by replacing Notch signaling (Figure 2) [82]. Recently, Han et al. reported that R-spondin, which is an activator of the canonical Wnt signaling pathway leading to activation of β-catenin-dependent gene transcription, positively regulates myogenic differentiation [83]. Furthermore, Wnt7a regulates the self-renewal of satellite cells via noncanonical Wnt signaling [84]. These findings suggest that both the Wnt canonical and noncanonical signaling pathways play various roles in embryonic and postnatal skeletal muscle development including the cell fate choice of satellite cells.

4.4. Other Factors. Sox8 is a member of the Sox proteins, which play fundamental roles in developmental and differentiation processes in a variety of tissues [85], and Sox8-deficient mice show a reduction of overall body weight in postnatal life [86]. Schmidt et al. have demonstrated that Sox8 is confined to satellite cells and is downregulated during differentiation in parallel with downregulation of Sox9. Overexpression of Sox8 or Sox9 inhibits myotube formation and leads to an obvious reduction in the expressions of MyoD and myogenin [87], suggesting that Sox8 is a negative regulator of skeletal muscle differentiation and, like Pax7 or Notch/Delta, ensures the maintenance of a proper pool of satellite cells.

It has been shown that hypoxia influences the function of satellite cells. Gustafsson et al. have demonstrated that hypoxia maintains myoblasts in an undifferentiated state by activating the Notch signaling pathway [88]. Liu et al. have also shown that hypoxia promotes Pax7 expression in satellite cells through activation of the Notch signaling pathway [89], suggesting that oxygen levels in satellite cells play a role in maintaining a balance between quiescence and activation.

Nitric oxide (NO) also regulates the state of satellite cells. Wozniak and Anderson have reported that the concentration of NO regulates the balance between quiescence and activation of satellite cells on myofibers. Satellite cells maintain a quiescent state in the presence of a normal concentration of NO, whereas injury activates satellite cells by altering the concentration of NO by stretching fibers or through dysfunction of NO synthase, eventually leading to the release of hepatocyte growth factor/scatter factor (HGF) from the extracellular compartment [90]. HGF acts as an activator of satellite cells and plays an essential role during the early phase of the repair process. NO-dependent satellite cell activation via HGF mediates many aspects of the inflammatory response, and further research is necessary to gain a better understanding of the processes of muscle healing and regeneration.

5. Effects of Aging and Myogenic Disorders on Satellite Cells

5.1. Satellite Cells and Aging. Regenerated myofibers contain central nucleus (Figure 3). It is important for satellite cells to maintain their regeneration potential in order to prevent any decrease of skeletal muscle mass during aging. The number of satellite cells declines in aged rodent and human skeletal muscles [91, 92]. The age-related decrease in the satellite cell population is one important cause of the sarcopenia, degenerative loss of muscle mass, strength, and frailty associated with aging. These observations suggest that maintenance of satellite cell number and function is important for allowing the advance of sarcopenia.

Skeletal muscle has vigorous regeneration potential. Once skeletal muscle has been subjected to severe mechanical, chemical, or toxic stimulation, a proportion of myofibers are broken down and the resulting debris is subjected to phagocytosis by leukocytes such as neutrophils and macrophages (Figure 3). Satellite cells migrate to site of injury, where they rapidly proliferate and differentiate. Finally, skeletal muscle regeneration occurs through fusion of myoblasts into myofibers. Deletion of Pax7(+) cells disrupts this regeneration process [48], indicating the indispensable role of the Pax7 transcription factor in satellite cells. It has been reported that inhibition of leukocytes infiltration into injured skeletal muscle induces incomplete muscle regeneration and severe fibrosis [93]. Therefore, the environment surrounding satellite cells and the associated conditions also affect the regeneration process.

5.2. Changes in the Proliferation of Satellite Cells with Aging. It is well known that aging reduces the function of satellite cells, especially their proliferation potential. For instance, Schultz and Lipton have revealed that the number of satellite cells and the proliferation rate of isolated satellite cells decline with advancing age [94]. Carlson and Conboy recently indicated that the percentage of BrdU-positive satellite cells was reduced in aged mice relative to that in young mice under the same culture conditions [95]. In addition, many studies have indicated that aging severely affects the proliferation potential of satellite cells [96–98]. This decrease is associated with the muscle atrophy referred to as sarcopenia. The age-related decline in the proliferative ability of satellite cells impairs the regeneration potential of skeletal muscle.

The activation of satellite cells after muscle injury is controlled by Notch signaling, which is triggered by a rapid increase in the expression of Delta (a Notch ligand). In aged muscle, Delta fails to become upregulated after injury. Young muscles show significant upregulation of Delta upon injury, with Delta being expressed on the surface of satellite cells. Disruption of Notch signaling leads to a decrease in the number of satellite cells in aged skeletal muscle after injury [99]. Using heterochronic parabiosis, Conboy et al. have demonstrated that systemic factors in the blood of young mice can rejuvenate aged satellite cells and rescue the regeneration potential of aged mouse skeletal muscle [100]. Moreover, aged serum has been shown to reduce the percentage of Notch-positive satellite cells isolated from aged mice. BrdU-positive aged satellite cells can be increased by exposure to serum from young mice, and this upregulation is hampered when Notch signaling is inhibited. These results suggest that the dysfunction of Notch signaling induced in satellite cells by aged serum negatively affects their proliferation potential. Liu et al. have reported that satellite cell-specific constitutive Notch activation increases the number

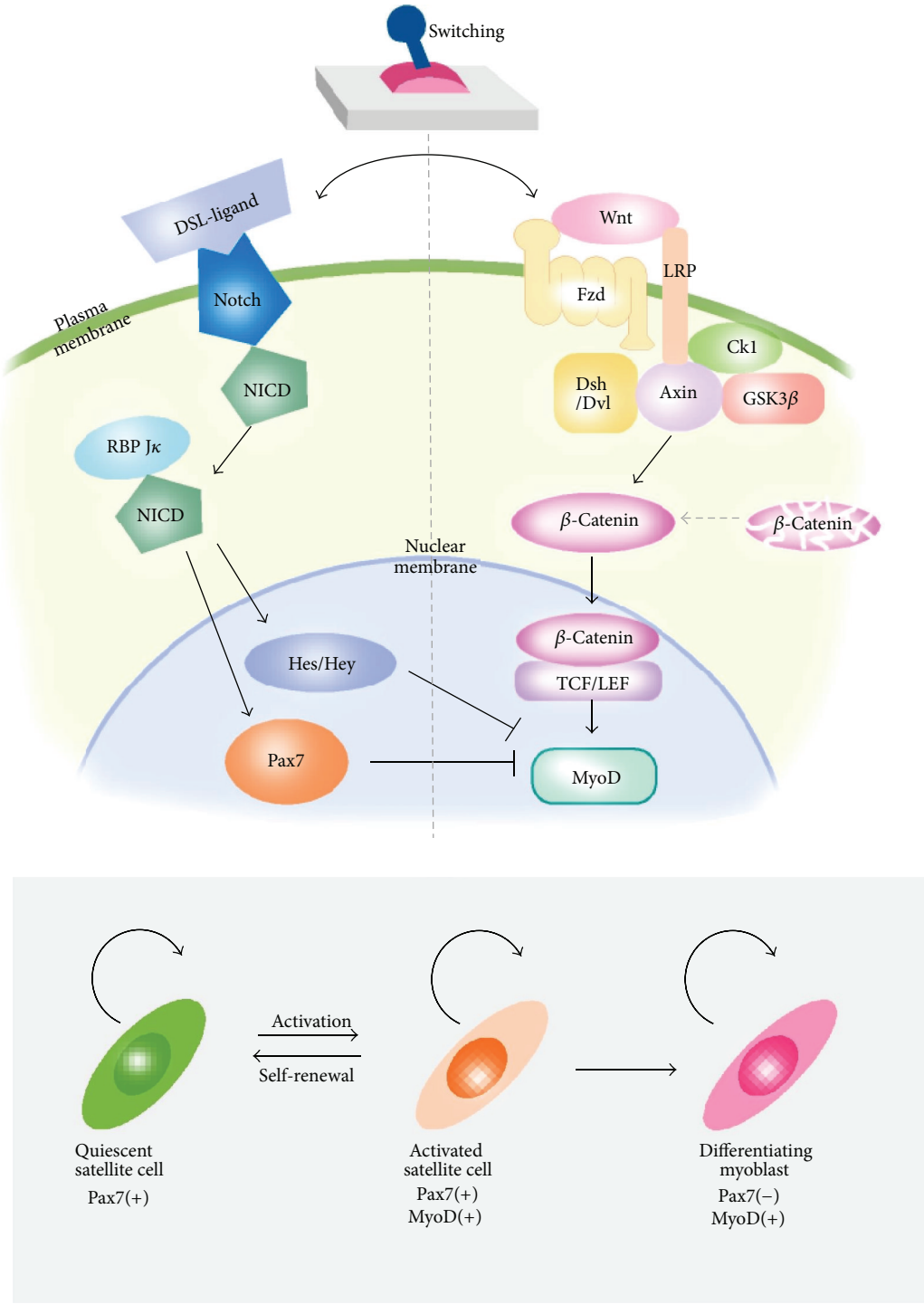

Figure 2: Regulatory switch between quiescent and activated states of satellite cells. Notch and Wnt signaling antagonize each other to define the state of satellite cells. Upon binding of DSL ligands (Delta) to Notch receptor, released Notch intracellular domain (NICD) translocates to the nucleus. After associating with RBP-Jκ, the complex activates the transcription of target genes, such as Hes, Hey, and Pax7 to maintain satellite cells in quiescent state (left). When the "molecular switch" turns from the quiescent state to the activation state by Wnt proteins (right), the Wnt signal transduction activates β-catenin/TCF/LEF transcriptional complexes. The transcription complex triggers the expression of target MyoD gene and positively regulates myogenic differentiation.

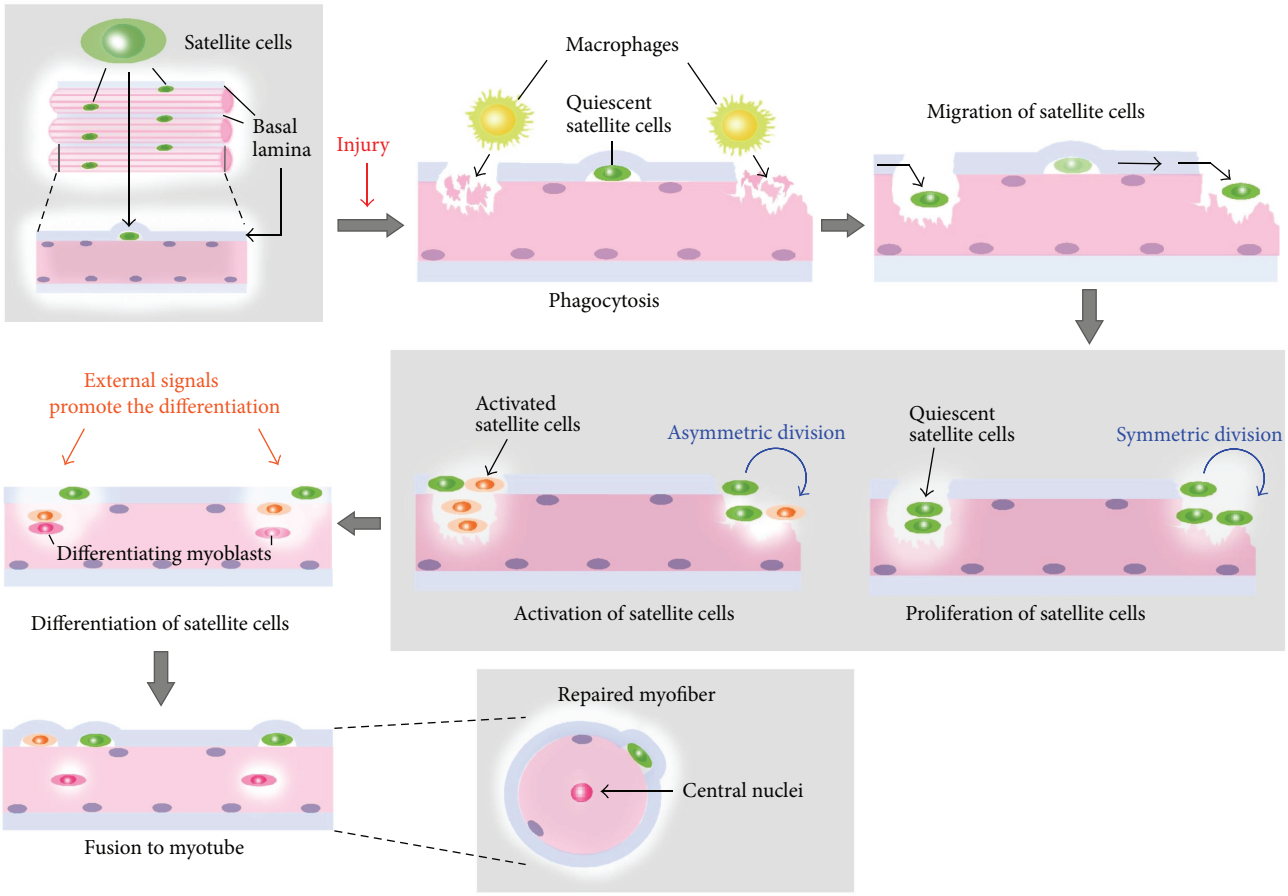

FIGURE 3: Satellite cell-mediated muscle regeneration upon injury. Damaged muscle fiber induces activation of quiescent satellite cells that reside between the plasma membrane and basal lamina. Macrophages digest myofiber debris at the damaged site (phagocytosis). Satellite cells migrate and proliferate through symmetric and asymmetric divisions. During the process, various external signals promote the differentiation of satellite cells and their fusions to myotube.

of satellite cells both *in vivo* and *in vitro* [89]. Additionally, Bjornson et al. have reported that satellite cell-specific Notch inhibition disrupts the regeneration of skeletal muscle and decreases the number of satellite cells after injury *in vivo* [72], suggesting that Notch inhibition reduces the satellite cell pool in skeletal muscle and that this is responsible for disruption of skeletal muscle regeneration.

Although the concentration of Notch is normal in aged muscle and aged satellite cells retain functional Notch receptors, Notch activation is impaired due to a lack of the Notch ligand, Delta [99, 101]. Since Delta is a regulator of satellite cell proliferation through Notch signaling, any alteration of Delta would modify the process of muscle repair. After muscle injury, satellite cells are activated and undergo explosive proliferation at the affected site (Figure 3). During this phase, activated Notch stimulates the proliferation and self-renewal of satellite cells, and these renewed satellite cells are the source of the subsequent explosive proliferation. Therefore, Notch inactivation by aging disrupts muscle regeneration due to a decline of the original satellite cell pool.

During proliferative activation of satellite cells induced by upregulation of Delta/Notch, transforming growth factor (TGF-β) signaling antagonizes this process. Carlson et al. reported that higher levels of TGF-β were expressed in aged than young satellite cell niches [102]. Increased expression of TGF-β during aging led to activation of phosphorylated SMAD (pSmad) and the subsequent signal transduction upregulated cyclin-dependent kinase (CDK) inhibitors, such as p15, p16, p21, and p27, via physical competition between Notch and pSmad3 on the promoters of CDK inhibitors. Increased expression of TGF-β with aging suppresses the proliferation potential of aged satellite cells, and a neutralizing antibody against TGF-β rescues this potential [97]. Both lack of Notch activation and upregulation of TGF-β synergistically inhibit satellite cell proliferation, resulting in deficient muscle repair and a decline of regeneration with aging.

Another aging-related potential factor that acts as an extrinsic stimulus of satellite cells is Wnt. The downstream target of Wnt signaling, axis inhibition protein 2 (Axin2), is expressed at high levels in satellite cells derived from aged muscle, indicating a progressive increase of Wnt signaling during aging [96]. Injection of Wnt3a into young regenerating muscle after injury results in increased deposition of

connective tissue, and exogenous Wnt induction also reduces cellular proliferation in young regenerating muscles that have a similar phenotype to aged muscle. These findings indicate that a low level of Wnt signaling is important for generating adequate levels of myogenic progenitors. Notch promotes proliferation and self-renewal of satellite cells as described above and also prevents their differentiation, that is, maintaining them in an undifferentiated state, by inhibiting Wnt signaling via induction of GSK-3β and degradation of β-catenin [103, 104]. Conversely, GSK-3β retains the ability to phosphorylate Notch-1 and Notch-2 [105, 106], suggesting that precise timing of the transition from Notch to Wnt and reciprocal control of the working stage from satellite cells to skeletal myoblast cells, respectively, is important for stem cell maintenance and the triggering of differentiation (Figure 2). The switch from stem cell proliferation to differentiation without loss of the original satellite cell pool is essential for the effective repair, regeneration, and aging of skeletal muscles.

5.3. Muscle Fibrosis and Myogenic Disorders. After skeletal muscle injury, the repair process is initiated by release of growth factors and cytokines. Macrophages and fibroblasts that increase the production of extracellular matrix components migrate and proliferate. When normal regeneration occurs, these components are gradually degraded. Fibrous scar tissue is generated after muscle injury to fill the surface of the damaged area to facilitate regeneration. Although fibroblasts contribute to the repair response of tissues to injury by secreting extracellular matrix proteins such as collagen, fibrinogen, and fibronectin, continuous fibrosis is a pathological process in a variety of vital organs. Once fibrous scar tissue is overproduced in skeletal muscle, the muscle function becomes weaker. Pathophysiologic fibrosis due to accumulated extracellular matrix impairs muscle strength and can cause fibrotic diseases such as chronic myopathy and muscular dystrophy. In these conditions, fibrosis inhibits the diffusion of nutrients to myofibers [107]. Myostatin, also known as GDF-8, a member of the TGF-β family, is expressed in skeletal muscle and acts as an inhibitor of muscle growth by prohibiting the proliferation and differentiation of satellite cells, and thus deletion of myostatin causes muscle hypertrophy and hyperplasia [108]. Zhao et al. have reported that myostatin stimulates muscle fibroblast proliferation and expression of extracellular matrix proteins [109]. Persistent exposure to the inflammatory response increases the level of TGF–β1 [110], and this inhibits the activation of satellite cells and impairs myogenic differentiation [111].

Alexakis et al. showed that undifferentiated satellite cells express type I collagen, suggesting that satellite cells have the potential to adopt a fibroblastic phenotype [112]. Brack et al. reported that overexpression of Wnt3a resulted in abnormal extracellular matrix deposition and that aged mouse serum increased the population of nonmyogenic cells and fibronectin expression in satellite cells [113]. Since the aging-related fibrosis-converting phenomenon was diminished by induction of an inhibitor of Wnt3a, the Wnt signaling is also an important factor for induction of muscle fibrosis. On the other hand, quiescent satellite stem cells have shown to express the Wnt-receptor Fzd7, and Wnt7a significantly promotes the symmetric expansion of satellite stem cells (a 2-fold increase in the number of Pax7(+) satellite cells) via the planar cell polarity (PCP) pathway of noncanonical Wnt signaling [114]. Ectopic Wnt7a enhances muscle regeneration, suggesting an effect differing from that of Wnt3a, which causes fibrosis. These results suggest that there is cross-talk between Wnt7a and PCP and that Wnt3a/β-catenin signaling defines the fate of satellite stem cells, self-renewal, myogenesis, regeneration, and fibrosis during aging.

Macrophages, which are a vital component of the immune system, also play a role in muscle repair as they clear myofiber debris in regenerating and dystrophic muscle. Depletion or impairment of macrophages causes fibrogenesis in dystrophic muscle [93, 115]. Segawa et al. showed that reduction of macrophage infiltration using clodronate liposomes increased muscle fibrosis after injury, indicating that aging-induced dysfunction of immunity leads to fibrosis. Macrophages release the profibrotic molecules TGF-β, which activates fibroblasts to generate extracellular matrix. Excessive and persistent deposition of fibrinogen in the extracellular matrix inhibits the repair of myofibers, and fibrinogen accumulation is well correlated with advancing age [116]. Such deposition accelerates muscle inflammation and fibrosis. Further studies to reveal the mechanisms and molecules regulating inflammation and the promotion of fibrosis might provide an effective strategy for repair and healing of muscle injuries.

6. Transdifferentiation of Muscle-Derived Stem Cells

Satellite cells are considered to have specific unipotential for the myogenic lineage. While Pax7(+) satellite cells are committed stem cells for myogenic cells, stem-cell-like populations within skeletal muscle can differentiate through multiple pathways to form a variety of cell types and tissues (Figure 4). It is important to distinguish between defined satellite cells and muscle-derived stem cells (including side populations other than Pax7(+) cells). Muscle-derived stem cells that reside potentially as one of the origin of satellite cells [21] retain a high degree of flexibility and an intrinsic ability to exhibit multiple lineages.

6.1. Smooth Muscle. Unlike skeletal muscle, smooth muscle is an involuntary muscle found in the walls of blood vessels, the gastrointestinal tract, the bladder, or the uterus. Its structure differs from that of skeletal muscle in that it lacks visible cross-striations. Hwang et al. have reported that skeletal muscle-derived stem cells are able to differentiate into the smooth muscle lineage *in vitro* in response to vascular endothelial growth factor (VEGF) when cocultured with a feeder layer of smooth muscle cells. Two days of coculture of skeletal muscle-derived stem cells on a layer of smooth muscle cells converted them to alpha-smooth-muscle-actin- (αSMA-) positive cells [117]. Nolazco et al. have demonstrated that skeletal muscle-derived stem cells can transdifferentiate to the smooth muscle lineage when implanted into the rat

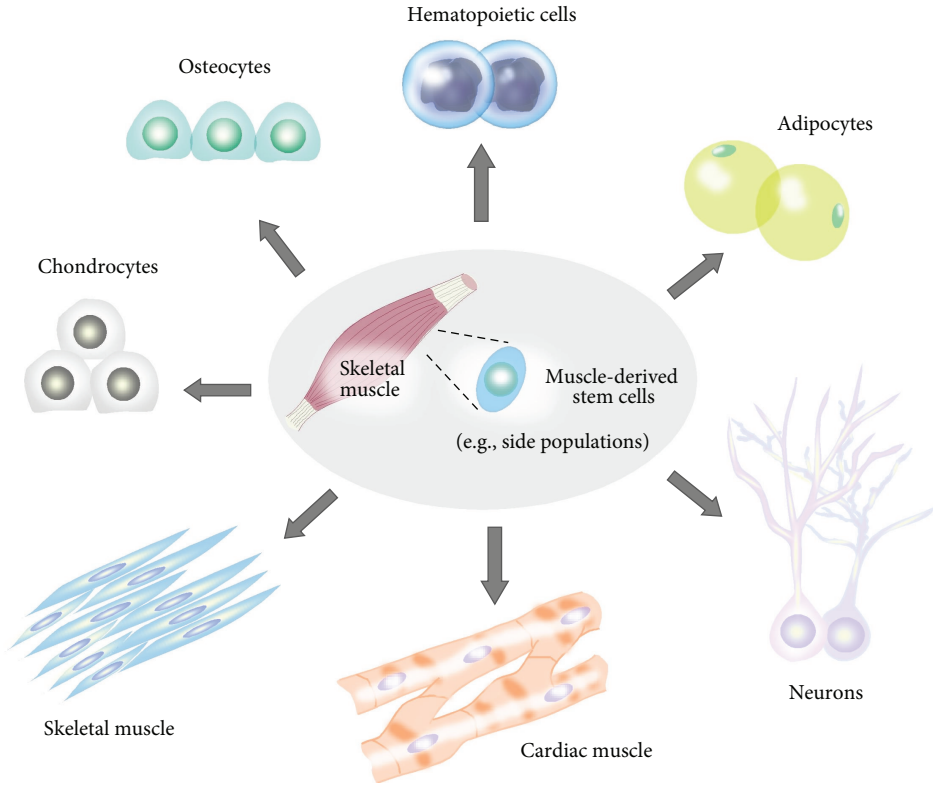

FIGURE 4: *Trans*-differentiation of muscle-derived stem cells. Possible *trans*-differentiation pathways of muscle-derived stem cells. Satellite cells retain a high intrinsic ability for *trans*-differentiation into multiple lineages (cardiac muscle, skeletal muscle, hematopoietic cells, adipocytes, neurons, chondrocytes, and osteocytes).

corpora cavernosa and can correct aging-related erectile dysfunction, showing expression of αSMA, calponin 1, and smoothelin [117, 118]. Injection of skeletal muscle-derived cells into the urinary tract resulted in the formation of new myofibers [119]. Ho et al. have reported that skeletal muscle-derived stem cells grown on small-intestinal submucosa generated smooth-muscle cells expressing αSMA, calponin, and smoothelin [120]. These studies suggest that autologous transplantation of skeletal muscle-derived stem cells within a smooth muscle environment (niche) may be an effective approach for the treatment of smooth muscle injury.

6.2. Cardiac Muscle. Cardiac muscle is one of three major types of muscles (skeletal, cardiac, and smooth muscles) and is an involuntary type of striated muscle. The cardiomyocytes that compose cardiac muscle have a large number of mitochondria, myoglobin (an oxygen-storing pigment) for providing nutrients and oxygen, and show anaerobic metabolism. In contrast to skeletal muscle cells, cardiomyocytes are ischemia intolerant. Since the heart lacks functional repair mechanisms, a number of studies have investigated muscle-derived stem cells (skeletal muscle) for cardiac repair [121, 122].

Taylor et al. reported that autologous implantation of skeletal myoblasts in cryoinjured rabbit hearts improved systolic and diastolic function [121]. Skeletal myoblasts express N-cadherin and connexin43, which are major components of the gap junction, but these are downregulated as the cells terminally differentiate. Cardiomyocytes are electromechanically coupled by cell-cell junctions, the intercalated disks, including gap junctions for electrical communication between the cells [123]. The β-adrenergic agonist isoproterenol increases the synchronized beating rate between muscle-derived stem cell (skeletal muscle) grafts and cardiomyocytes, and heptanal (a gap junction blocker) inhibits contraction and restricts individual cardiomyocytes to their intrinsic pacemaker frequency [124]. Tamaki et al. have reported that freshly isolated CD34(+)/CD45(−) cells derived from skeletal muscle were converted to cardiomyocytes in a myocardial infarction model (left ventricle, LV) and that transplantation resulted in significant functional recovery (LV function: percentage of fractional shortening, regional wall motion score, ejection fraction, etc.). In a phase I clinical study, Herreros et al. have examined autologous skeletal myoblast transplantation in 12 patients with old myocardial infarction (MI) undergoing coronary artery bypass surgery. Okada et al. have also reported that transplantation of human muscle-derived stem cells (skeletal muscle biopsy samples from three human subjects) into an acute MI model in NOD/SCID mice significantly improved survival and engraftment, stimulated angiogenesis, and improved LV function. Transplantation of muscle-derived stem cells (skeletal muscle) was more effective than that of committed skeletal myoblasts, suggesting

that high intrinsic and feasible regenerative ability of muscle-derived stem cells is important for MI repair [125]. These results suggest the usefulness of autologous cellular cardiomyoplasty using muscle-derived stem cells from skeletal muscle.

6.3. Osteogenic Lineage. Myoblasts derived from skeletal muscle treated with bone morphogenetic proteins (BMPs) or adipogenic-inducing agents differentiate into osteocytes or adipocytes [126, 127]. Katagiri et al. demonstrated that BMP-2 and TGF–β1 inhibited myotube formation in C2C12 cells (myoblast cell line originating from muscular tissue satellite cells) and downregulated the expression of MyoD and myogenin [128]. C2C12 cells induced by BMP-2 alone differentiated into osteoblast lineage. Chalaux et al. have shown that JunB contributes to the inhibition of myogenesis by BMP-2 and TGF–β1. Namiki et al. further demonstrated that BMP-2-dependent osteoblast differentiation was transduced via BMPR-IA (BMP receptor), and Akiyama et al. also showed that the transduced expression of BMPR-IB exhibited osteoblast-specific phenotypes in C2C12 cells [129, 130]. Lee et al. also reported that muscle-derived stem cells became hypertrophic and expressed the bone-specific marker osteocalcin/BGLAP under *in vitro* culture conditions with BMP-2 [131]. However, when BMP-2 was absent, myogenesis with myotube formation occurred. Kawasaki et al. demonstrated that BMP-2 inhibited myotube formation using primary cells extracted from human muscle tissue (pectoralis major, gluteus maximus, and adductor magnus) [132]. Yamamoto et al. demonstrated that Smad1 and Smad5, which mediate BMP signaling, were involved in the process of myogenic inhibition and the induction step of osteoblast differentiation [133].

Transplantation of muscle-derived stem cells has been attempted for repair of defects in bone. Muscle-derived stem cells transduced with viral vectors encoding BMP induced bone formation and improved bone healing [134, 135]. The transplanted muscle-derived stem cells responded to the secreted BMP-2 in an autocrine manner. Musgrave et al. reported that primary cells derived from human skeletal muscle could be used to produce bone in SCID mice [136]. Injection of the human cell expressing BMP-2 into the hind-limb muscle of the SCID mice caused ectopic osteogenesis within a few weeks. When these cell grafts are employed for orthopedic applications, extracellular matrix scaffolds support the process. Usas et al. demonstrated that delivery of BMP4-secreting muscle-derived stem cells was able to induce osteogenesis in mice if used with collagen gel, fibrin sealant, and gelatin sponge carriers and showed that a gel scaffold was more suitable for bone formation than sponge material [137]. Up to now, orthopedic treatment involving engraftment of allografts supplemented with demineralized bone matrix or vascularized bone grafts has been limited because of the reduced osteogenic capacity of the donor bone-forming cells. Muscle-derived stem-cell-based regenerative approaches for bone formation are potentially attractive as they exploit the intrinsic ability of muscle stem cells as a replenishable cell source for autologous transplantation.

6.4. Adipogenic Lineage. In aged mice, muscle shows a decrease of regenerative and proliferative capacity, with consequent loss of muscle mass, and myoblasts express higher levels of adipocyte lineage genes (adipose-specific FABP, C/EBPα, and PPARγ), although a fully differentiated adipocyte phenotype is not achieved. Muscle-derived stem cells exposed to adipogenic inducers *in vitro* differentiate into adipocytes with a characteristic polygonal morphology and lipid-filled vacuoles in their cytoplasmic fractions. Replacement of muscle tissues by adipose tissues has been demonstrated in mutant mice ($MyoD^{-/-}$: $Myf5^{-/-}$), and adipogenic potential has been examined in not only murine [138] but also human studies [139]. The process of adipogenesis is enhanced by the insulin sensitizer rosiglitazone [140]. Reagents (long-chain fatty acids and/or thiazolidinediones) that activate the peroxisome proliferator-activated-receptor- (PPAR-) γ could cause upregulation of genes involved in fatty acid uptake, storage, and metabolism in skeletal muscle tissues [141]. PPARγ is required for insulin responsiveness of fat cells, and the expression levels of (age-related) adipogenic transcription factors determine the size of fat cells, and their capacities to store lipid and insulin respond to insulin. Elevated PPARγ expression in skeletal muscle increases insulin sensitivity [142], and knockout of PPARγ in skeletal muscle using a Cre-loxP system results in glucose intolerance and insulin resistance [143].

Aguiari et al. have reported that muscle-derived stem cells differentiate into adipogenic lineage upon exposure to high levels of glucoses, which in turn induces reactive oxygen species (ROS) and activation of the downstream effector kinase, PKCβ [144]. ROS are byproducts of normal cellular metabolism that can cause cellular damage through oxidation of lipids, and PKCβ plays a role in signaling that connects ROS with mitochondrial targets. These data suggest that *trans*-differentiation from muscle-derived stem cells to adipose lineage reflects oxidative stress, and thus converted cells are considered to be damaging cell populations in the age-related organ dysfunction induced by ROS. Elevated superoxide accelerates age-associated muscle atrophy through mitochondrial dysfunction, causing irreversible cell injury and death, and these changes to muscle tissue are responsible for the pathogenesis of sarcopenia [145, 146]. Oxygen concentration also modulates the *trans*-differentiation of muscle-derived stem cells to adipogenic lineages [147]. Further identification of the signals, cellular stage and specification, and molecular mechanism that underlie the process of *trans*-differentiation into adipose lineage may provide a deeper understanding of the intrinsic abilities of muscle-derived stem cells in nature and also the pathogenesis of sarcopenia. Muscle-derived stem cells retain the capacity to enter either a static myogenic differentiation pathway or a mesenchymal cell-like widely varied alternative differentiation pathway.

6.5. Neuronal Lineage. The function of skeletal muscle is intimately dependent on the central and peripheral nervous systems. Functional linkage between skeletal muscle and the nervous system at neuromuscular junction is necessary for the normal function of various organs. Although a number

of methods have been explored, muscle-derived stem cells can be induced to adopt neuronal lineages [148–152]. Arsic et al. reported that muscle-derived stem cells (skeletal muscle) began to express N-CAM, β-tubulin III, and GFAP [153]. Alessandri et al. isolated muscle-derived stem cells from human brachioradialis muscle of 12 patients and demonstrated that these cells differentiated into skeletal muscle fibers with a smooth-muscle cell phenotype (expression of smooth-muscle actin) and a neuronal phenotype (expression of β-tubulin III, GFAP, S100) *in vitro* [148]. Vourc'h et al. also showed that a particular population of CD34(+)/CD45(−)/CD90(+) cells isolated from adult skeletal muscle by rapid cell sorting gave rise to a significant number of cells entering the neuronal lineage [154]. Kwon et al. reported that valproic acid (VA), a histone deacetylase inhibitor used in the treatment of epilepsy, bipolar disorder, led to differentiation of muscle-derived stem cells toward neuronal lineages. Recently, Kang et al. demonstrated that fibroblast growth factor (FGF) and ethosuximide, which is used clinically to treat absence seizures in humans, induced neuronal differentiation of muscle-derived stem cells by showing immunohistochemically positive cells for TuJ1, NeuN, and neurofilaments M and H [150]. Further studies to reveal the mechanistic background of the conversion into the neuronal lineage may reveal further potential applications of muscle-derived stem cells, which can easily regenerate and consequently be rapidly expanded *ex vivo*, for repair of neuronal injuries and treatment of neuronal disorders.

6.6. Hematopoietic Lineage. Muscle-derived stem cells also have the ability to differentiate into hematopoietic cell lineages. Bellayr et al. showed that muscle-derived stem cells differentiated into hepatocyte lineage with liver regeneration ability [155]. Stem cell populations within skeletal muscle were capable of not only muscle regeneration but also hematopoietic engraftment (bipotent lineage potential) [22, 156–158]. Farace et al. reported that muscle-derived stem cells showed hematopoietic activity more than 10-fold than that of bone marrow giving rise to myeloid, T, B, and natural killer cells [159]. Regarding the origin of the muscle-derived stem cell showing myogenic and hematopoietic lineages, there are possibilities that hematopoietic stem cells are the plastically circulating stem cells or that primitive pluripotent stem cells in adult tissues [160, 161], for example, very small embryonic-like cells (VSEL), contribute to give rise to multiple lineages [162, 163]. If the yield and sensitivity of blood-forming human muscle-derived stem cells could be improved further, skeletal muscle would become a useful alternative cell source for autologous bone marrow transplantation, especially for patients with aplastic anemia.

6.7. Chondrogenic Lineage. Muscle-derived stem cells have been shown to have the capacity to generate cartilage. Articular cartilage has a limited healing capacity because of its poor vascular supply. Transforming growth factor β (TGF-β), BMPs, insulin-like growth factor 1 (IGF-1), and basic FGF can improve chondrocyte proliferation and extracellular matrix synthesis. Subpopulation of myogenic progenitor cells, accumulating in the callus tissue of bone fractures, has been shown to express the cartilage marker collagen II in a mouse model of fracture healing [164]. L6 myoblasts and C2C12 myoblasts were able to differentiate into chondrocytes when treated with demineralized matrix or BMP-2 [165]. These data suggest that muscle progenitor cells have intrinsic ability to undergo chondrogenic differentiation in specific circumstances and that this process may be important for cartilage regeneration [166].

Transplantation of stem cell populations is an attractive approach for more efficient repair of articular cartilage defects. Adachi et al. have reported that skeletal muscle-derived stem cells promote cartilage repair when used with collagen gel [167]. Huard's group has reported monolayers of BMP-4-expressing muscle-derived stem cells from type II collagen-positive colonies, suggesting that chondrogenesis and TGF-β further promote *trans*-differentiation [168, 169]. The same group has also demonstrated that intracapsular injection of muscle-derived stem cells expressing BMP-4 and soluble Flt-1 is effective for repairing articular cartilage after induction of osteoarthritis and that platelet-rich plasma can promote collagen synthesis, thus increasing the therapeutic potential through inhibition of chondrocyte apoptosis [170]. The combination of IGF-1 and TGF–β1 (IGF-1/TGF–β1), TGF–β2/BMP-7, TGF–β2/BMP-6, TGF–β2/BMP-2, and TGF–β2/IGF-1 enhanced the conversion of muscle-derived stem cells into chondrogenic lineage [171, 172]. Cairns et al. have reported that Nkx3.2 plays a central role in the chondrogenic differentiation during the step at which Sox9 promotes chondrogenesis and inhibits myogenesis [173]. Further clarification of the intracellular molecular mechanisms, effective scaffolds, activating signaling molecules, and growth factors operating during the *trans*-differentiation of muscle derived-stem cells or myoblasts into the chondrogenic lineage may accelerate their utility for tissue engineering aimed at cartilage repair.

6.8. Angiogenic Lineage. Restoration of the vascular network for the exchange of oxygen, carbon dioxide, nutrients, and waste products is essential for muscle regeneration. Therefore, for muscle regeneration, every muscle construct is connected to a vascular system, and angiogenesis is controlled in a spatiotemporally coordinated manner. Angiogenesis is controlled largely by hypoxia-driven transcriptional upregulation and secretion of vascular endothelial growth factor (VEGF), and the expression of VEGF, angiopoietin 1/2, monocyte-chemoattractant-protein- (MCP-) 1, and their receptors (VEGFR, VEGFR2, etc.) strongly increases after injury [174]. Skeletal muscle-derived cells express the vascular endothelial markers VE-cadherin, VEGF-R2 (VEGF receptor), and smooth muscle α-actin [175, 176]. Bryan et al. have reported that VEGF expression is mediated by MyoD, since the VEGF promoter contains three tandem CANNTG consensus MyoD binding sites [177]. Furthermore, VEGF-null ES cells exhibit impaired myogenesis compared with wild-type ES cells, suggesting that VEGF retains an important role in the skeletal lineage differentiation program.

Interestingly, undifferentiated C2C12 myoblast cells express VEGF-R1 and VEGF-R2 at low levels, and the expression levels of the VEGF receptors are upregulated upon differentiation of skeletal muscle [177]. These data suggest that myotube hypertrophy is coupled with both VEGF stimulation and also receptor activation and that the myogenic differentiation program is tightly regulated together with angiogenesis in an autocrine manner. Furthermore, there is a possibility that cancer patients undergoing long-term anti-VEGF therapy may suffer impairment of muscle regeneration as a side effect. Further research might shed further light on the possible clinical application of skeletal muscle-derived stem cells for reconstructive vascularization.

The adult skeletal muscle compartment is a complex organ because of the diversity of its lineages and its intrinsic potential for the *trans*-differentiation of muscle-derived stem cells. Muscle-derived stem cells can also act as mediators by releasing angiotrophic, neurotrophic, chondrogenic, hematopoietic, adipogenic, osteogenic, and other growth factors, thus supporting and further activating endogenous mechanisms for regeneration. Regenerative therapies using muscle-derived stem cells hold promise for the treatment of various diseases/disorders and also for injury repair. On the other hand, recent studies have indicated that somatic cells can be reprogrammed into iPS cells for clinical application. Skeletal muscle-derived cells themselves retain high utility as a cell resource for reconstructive tissue engineering but are also a useful and efficient source for the establishment of iPS cells because of their highly flexible intrinsic abilities and replenishable properties.

7. iPS Cells from Skeletal Muscle Cells

Muscle satellite cells are responsible for the robust regeneration capacity of adult skeletal muscle. Satellite cells derived from skeletal muscle are capable of repopulating the stem cell pool, implying that they retain direct potential for the therapy of degenerative muscle disorders. After exercise or muscle injury, large numbers of muscle fibers are newly generated within a short period. Skeletal muscle cells are attractive as a source of iPS cells since they can be replenished easily. Although the efficiency of the reprogramming of somatic cells, such as fibroblast, into iPS cells is generally quite low, adult neural stem cells or hematopoietic stem cell cells reprogram efficiently [178, 179]. Therefore, muscle-derived stem cells or (myogenic) satellite cells are thought to be feasible cell source for iPS generation.

Polo et al. have reported that freshly isolated skeletal muscle precursors can be reprogramed to iPS cells effectively [180]. Established iPS lines gave rise to differentiated teratomas, and all tested lines supported the development of chimeric animals after blastocyst injection. Tan et al. have also demonstrated that skeletal muscle precursor cells (CD45(−)/Mac1(−)/Sca1(−)/β1-integrin(+)/CXCR4(+) satellite cells) and Sca1(+) mesenchymal progenitors from skeletal muscle can be reprogrammed into iPS cells with greater efficiency than differentiated CXCR4(−) cells using clonal assays and a second-generation inducible reprogramming system [181]. This indicates that pluripotency can be induced more efficiently in stem cells than in their more differentiated progeny. Furthermore, Watanabe et al. have reported that iPS cells can be generated from fully committed myogenic cells (MyoD-positive primary myoblasts) by retroviral transfer of four factors (Oct4/c-Myc/Klf4/Sox2) [182]. The muscle-derived iPS cells exhibited characteristics similar to those of ES cells and formed embryoid bodies and teratomas and contributed to the production of chimeric mice and their offspring, demonstrating their potential to develop into all three germ layers as well as into germ cells.

Importantly, continuous expression of the MyoD gene inhibited the step of reprogramming into iPS cells, since MyoD expression alone can program many nonmuscle cells to undergo differentiation into the myogenic lineage [183]. The efficiency of iPS cell generation was much higher when muscle from $MyoD^{-/-}$ mice was used for iPS production. Since Oct4 prevents the expression of MyoD, ectopic expression of Oct4 in myoblasts immediately shuts down MyoD gene expression. Lang et al. have reported that Oct4 also suppresses the expression of Pax7 by binding to the regulatory region of the Pax7 gene [184]. Moreover, Oct4 is able to bind to the upstream regulatory region of Cdx2 and Cldn4, genes that are specific for trophectoderm, to repress their expression [185]. Oct4 binds at the transcriptionally inactive Myf5 locus in ES cells [186], but recent ChIP-Seq analysis has shown that Oct4 does not bind to MyoD or the Pax7 locus in ES cells [187]. These data suggest that other transcription factor(s) containing a POU domain or cofactor protein(s) of Oct4 may negatively regulate MyoD expression indirectly. A consistent notion is that Oct4 is required for the initial reprogramming step in the induction of pluripotent stem cells from muscle cells. Although early passage iPS cells tend to retain an epigenetic memory of their somatic cell of origin, which reinforces biased commitment potential [188], iPS cells generated from different cell types in skeletal muscle show equal pluripotency. Adult stem cells reprogram more efficiently than terminally differentiated cells. Further studies aimed at identifying the molecular mechanism that prevents reprogramming from muscle tissues would facilitate effective generation of iPS cells for disease modeling, drug discovery, and cell therapy.

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Review Article

Human Cardiospheres as a Source of Multipotent Stem and Progenitor Cells

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Cardiospheres (CSs) are self-assembling multicellular clusters from the cellular outgrowth from cardiac explants cultured in nonadhesive substrates. They contain a core of primitive, proliferating cells, and an outer layer of mesenchymal/stromal cells and differentiating cells that express cardiomyocyte proteins and connexin 43. Because CSs contain both primitive cells and committed progenitors for the three major cell types present in the heart, that is, cardiomyocytes, endothelial cells, and smooth muscle cells, and because they are derived from percutaneous endomyocardial biopsies, they represent an attractive cell source for cardiac regeneration. In preclinical studies, CS-derived cells (CDCs) delivered to infarcted hearts resulted in improved cardiac function. CDCs have been tested safely in an initial phase-1 clinical trial in patients after myocardial infarction. Whether or not CDCs are superior to purified populations, for example, c-kit$^+$ cardiac stem cells, or to gene therapy approaches for cardiac regeneration remains to be evaluated.

1. Introduction

Myocardial infarction (MI) and the subsequent development of congestive heart failure are the leading cause of mortality in industrialized countries. MI causes a sudden and dramatic loss of contractile heart muscle cells, or cardiomyocytes, healing with scarring. The surviving cardiomyocytes undergo hypertrophy and the heart remodels. These adaptive mechanisms are detrimental in the long run, eventually leading to pump failure. Hence, there is a pressing need for reconstituting contractile cardiac tissue after acute MI as well as in chronic heart failure, for example, in dilated cardiomyopathy. In principle, this goal could be achieved by using two general approaches, namely, by exogenous delivery of cardiomyocytes or other cell types with a potential for cardiac differentiation, or by stimulating endogenous cardiomyogenesis through appropriate small molecules or nucleic acids, either individually or in combination.

Early claims of transdifferentiation of murine bone marrow- (BM-) derived hematopoietic stem cells (HSCs) into cardiomyocytes after delivery to infarcted mouse hearts [1] were questioned by subsequent studies [2, 3]. However, these negative results did not prevent clinical studies of cell therapy for ischemic heart disease from being initiated [4–10]. A majority of the clinical trials utilized autologous BM-derived mononuclear cells delivered either into the target coronary artery or directly into the peri-infarct region [5–10]. Additional cell types that have been tested clinically in patients after MI include autologous skeletal myoblasts [11, 12], both autologous and allogeneic BM-derived mesenchymal stem cells (MSCs) [13], purified BM-derived populations such as CD133$^+$ cells [4, 14], autologous BM-derived MSCs pretreated *ex vivo* with molecules that stimulate cardiomyogenic specification [15], autologous adipose tissue-derived cells [16], as well as stem and progenitor cells derived from the heart itself [17, 18]. Almost ten years after the initiation

of randomized, controlled clinical trials of BM cell therapy for cardiac regeneration, it must be recognized that results have been inconsistent, and that the overall improvement of cardiac function in MI patients has been modest [19–21]. The optimal timing of cell transplantation, the delivery technique, and the most effective cell type remain to be defined. It also has been shown that reduced cell functionality in old patients and in those with advanced cardiovascular disease or comorbidities limits the benefits of autologous cells [22]. Hence, an unresolved paradox persists between robust effects of cell therapy in animal models and modest benefits in patients. In principle, cardiac-derived stem and progenitor cell populations may offer major advantages over extracardiac cell sources, as cardiac progenitors might be more prone to differentiate along the cardiomyocytic and vascular lineages and to survive in the myocardial environment [23, 24]. Most recently, two phase-1 clinical trials of autologous cardiac stem cell therapy in patients after acute MI have shown that these approaches are both safe and promising [17, 18].

2. *Ex Vivo* Tissue Explant Cultures, "Spheres," and Stemness

The first report that cardiac progenitor cells can be clonally expanded from murine and human myocardial biopsy specimens and form "spheres" *in vitro* came from Messina et al. [25]. Surgical atrial appendage specimens placed in the primary *ex vivo* tissue culture spontaneously shed a heterogenous cell population, the cellular outgrowth. Remarkably, we have observed that cardiac explants keep shedding cells for more than one year in the *ex vivo* culture [26], providing direct evidence for the existence of cells within the tissue explant that are able to proliferate in long term even in the absence of blood supply. When cultured in cardiosphere- (CS-) forming medium (a base medium supplemented with basic fibroblast growth factor, epidermal growth factor, cardiotrophin-1, thrombin, and B27 as a serum substitute) and the nonadhesive substrate poly-D-lysine, the cellular outgrowth gives rise to CSs (Figure 1(a)). Spheres are self-assembling, multicellular, and floating cell clusters. Sphere-forming cells may lose, in part, anchorage-dependent growth. First described in neural stem cells [27], spheres have been considered—or named, at least—as a characteristic feature of stemness. However, it is now well recognized that sphere formation is not sufficient to establish stemness [28, 29]. In fact, spheres can be either clonal or nonclonal. Decreasing cell density in culture dishes typically increases the proportion of clonal spheres, which result from clonal cell proliferation. By contrast, nonclonal spheres result from both proliferation and cell aggregation.

3. CSs in Rodents

The early cellular outgrowth from murine cardiac explants forms a layer of fibroblast-like cells on which numerous small round "phase-bright" cells appear with a delay of 1 to 2 weeks [26, 30]. The cellular outgrowth from neonatal mouse cardiac explants is heterogeneous and contains both hematopoietic ($CD45^+$) and nonhematopoietic ($CD45^-$) cells. The latter include differentiation lineage-negative (Lin^-), c-kit^+ (CD117) progenitor cells, endothelial cells and endothelial progenitor cells ($CD31^+$ and/or $CD34^+$), as well as mesenchymal/stromal progenitor cells ($CD90^+$ and $CD105^+$) [26, 30–34]. Davis et al. [33] recently proposed that the $CD45^+$ subset within cellular outgrowths may result from a retained hematologic component, which was minimized by perfusing the heart with heparinized saline before placing the tissue explants in the culture dishes. Approximately 10% of cells shed by mouse cardiac explants during the first few days express c-kit, stem cell antigen-1 (Sca-1), the stem and progenitor cell-related antigen CD34, and the endothelial marker CD31. We have shown that CSs are composed of clonally derived cells that consist of proliferating cells primarily in their core, including a subset of c-kit^+ cells, along with an outer sheet of early committed progenitors and differentiating cells that express cardiac, endothelial, and stromal markers. CSs from transgenic mice expressing a nuclear *lac*Z reporter gene driven by the cardiac-specific cTnI promoter exhibited lacZ expression mainly in the outer sheet [35]. By video microscopy, we have demonstrated spontaneous beating of CSs from neonatal, but not adult, mouse cardiac explants in the absence of coculture with mature cardiomyocytes [26]. Recently, Andersen et al. [34] challenged the view that CS-derived cells (CDCs) are a source of stem cells with cardiomyogenic potential. These authors showed that CSs from neonatal mice may contain small myocardial fragments that detached from the tissue explant, especially when this is not removed from the cell culture, as neonatal mouse explants become less cohesive after prolonged periods of time in culture. To address this question, we used Z/EG transgenic mice in which cardiac-specific expression of Cre-recombinase results in the excision of a *lac*Z gene and activation of expression of the second reporter gene (EGFP) in the heart [36]. Following Cre-recombinase gene transfer into the heart before the initiation of the *ex vivo* tissue culture, EGFP expression was observed in Z/EG cardiac explants but not in their cellular outgrowths, indicating that the latter lacked mature cardiomyocytes resulting from small tissue fragments detaching from the explant [26]. When cardiac explants were cultured in complete MesenCult MSC medium, a commercially available medium developed for MSC cultures, as opposed to standard media utilized in the original protocol [25], a relatively homogeneous population of plastic-adherent cells expressing hematopoietic and monocyte/macrophage markers ($CD45^+$ and $CD14^+$) and exhibiting MSC-like differentiation potential was obtained. At high densities, these cells formed CSs that lost adhesion to plastic and detached from culture dishes [26], even when cultured directly on plastic. These observations suggest that different experimental conditions may result in the preferential expansion of different cell populations from a heterogeneous early cellular outgrowth. Recently, Ye et al. [37] addressed the question whether the $CD45^+$ cells are an essential component in CS formation. They harvested CSs from 1-week post-MI mouse hearts or from healthy hearts. $CD45^+$ cells were depleted from populations of CS-forming cells by immunomagnetic beads. The depletion of $CD45^+$ cells from these populations actually increased the

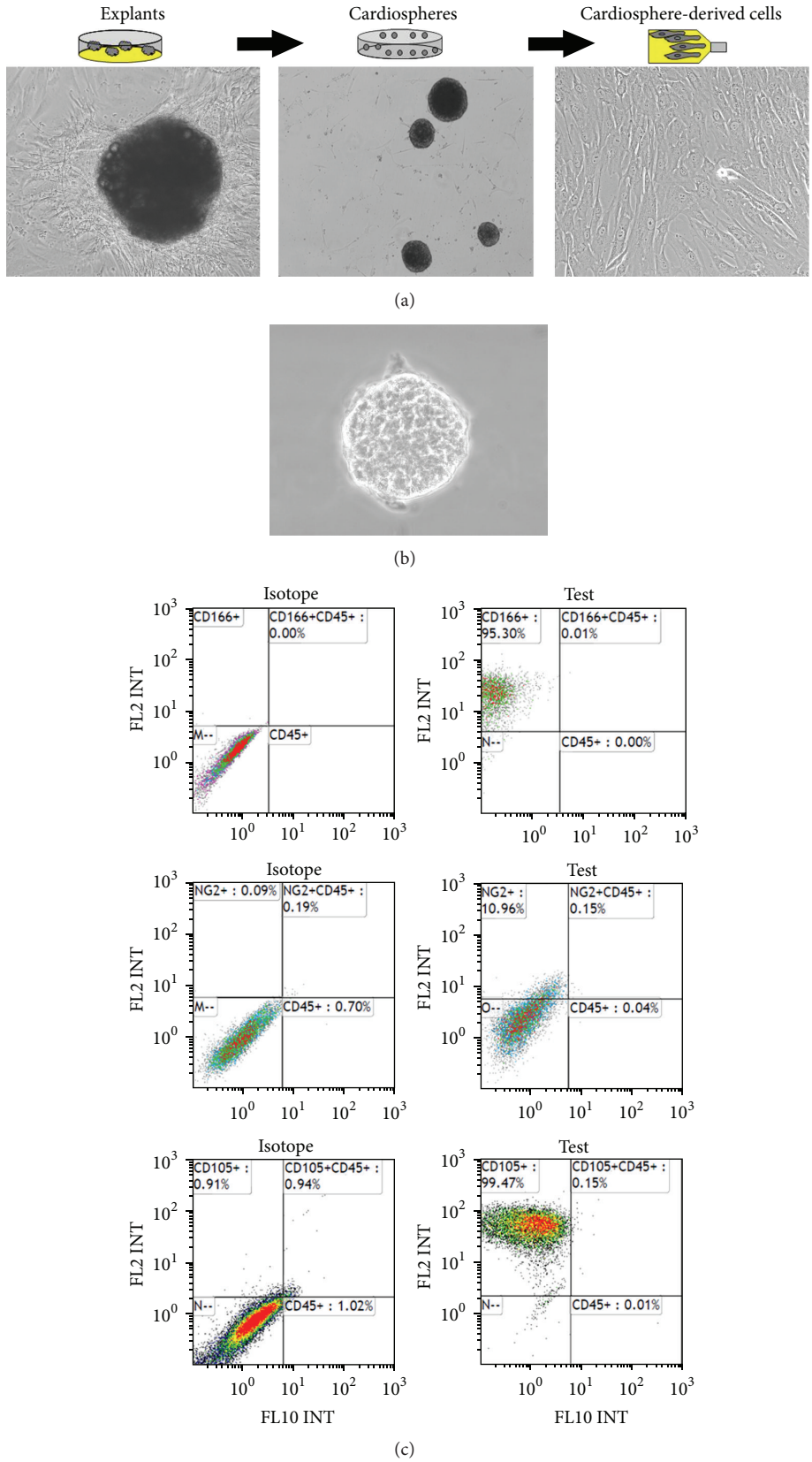

Figure 1: (a) Photomicrographs of a human atrial appendage specimen in the primary culture giving rise to a cellular outgrowth (left panel); CSs (middle panel); CDCs (right panel). (b) High magnification view of a human CS. (c) Flow-cytometric analysis of cell-surface marker expression by CS-forming cells (top to bottom: plots for CD45 versus CD166, CD45 versus NG2, and CD45 versus CD105 expression).

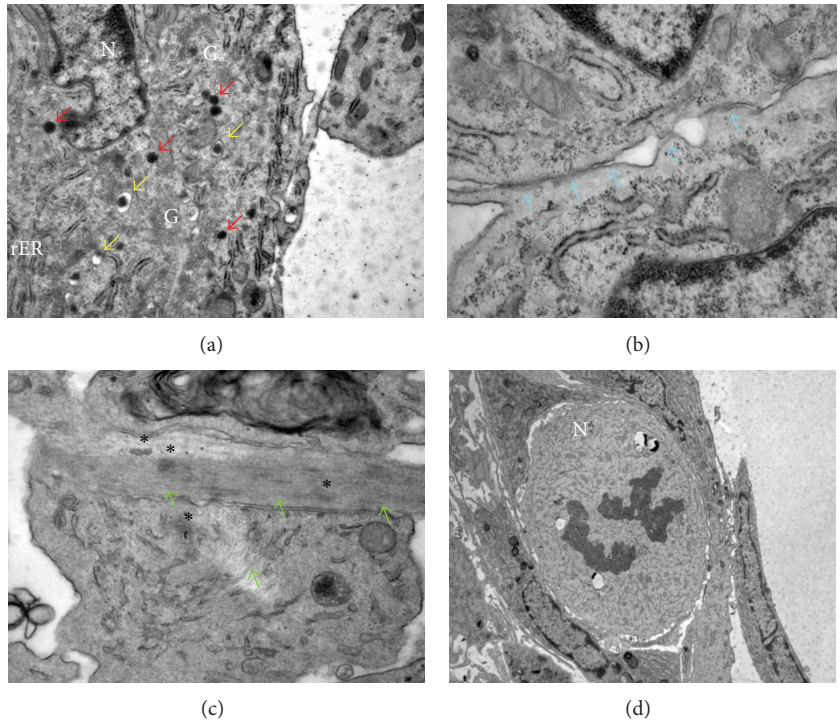

Figure 2: Electron-microscopical analysis of human CS ultrastructure. (a) Secretory granules (red arrows); primary lysosomes (yellow arrows). (b) Intercellular contacts (blue arrows). (c) Intracellular, unorganized thick filaments (green arrows); dense bodies (asterisks). (d) Mitosis (N, nucleus; G, Golgi apparatus; rER, rough endoplasmic reticulum).

formation of CSs compared with nondepleted populations. Purified $CD45^+$ cells from CS-forming cells did not form CSs, indicating that BM-derived $CD45^+$ cells are neither necessary nor sufficient for CS formation.

4. Human CSs Contain Both Primitive Cells and Cells Differentiating into Cardiomyocytes

We have generated human CSs from cells spontaneously shed from cultured surgical atrial appendage specimens from patients undergoing heart surgery for coronary artery disease or heart valve disease. However, CSs can also be obtained from human percutaneous endomyocardial biopsy specimens [38]. CSs placed in a new culture dish disassemble and give rise to a monolayer of CDCs that are clonogenic, can be expanded on fibronectin, and can give rise to a second generation of spheres. CS-forming cells express MSC markers [39] such as CD105 (endoglin, a part of the TGF-β1 receptor complex), CD13 (aminopeptidase N), and CD73 (lymphocyte-vascular adhesion-protein 2), as well as CD166 (activated leukocyte cell adhesion molecule; ALCAM; Figure 1(c)). Subsets of these cells also express NG2 chondroitin sulfate proteoglycan and CD140b (platelet-derived growth factor receptor B), which have been associated with pericytes/perivascular cells and MSCs in many tissues [40]. However, the cellular outgrowth does not express CD45 and CD34.

By electron microscopy, we have provided ultrastructural evidence of the presence of secretory granules, intercellular contacts, mitotic cells, and unorganized thick filaments consistent with cardiac progenitors/precursors within human CSs (Figure 2). In line with previous studies [15, 38], we have shown that human CSs express both early (Nkx2.5 and GATA4) and late (cTnI, α-sarcomeric actinin) cardiac genes (Figure 3). We also have shown that cardiac troponin I and α-sarcomeric actinin in association with sarcomeric structures, as well as connexin 43, are detectable immunocytochemically, most abundantly in the outer layer of CSs. By contrast, cellular outgrowths from cultured cardiac tissue explants, from which CSs are derived, do not express these sarcomeric proteinsg. It has been shown that human cardiac cellular outgrowths cocultured with neonatal rat ventricular myocytes exhibit spontaneous, synchronous beating activity [35]. Moreover, differentiation of human adult CDCs could be stimulated by exposure to extremely low-frequency electromagnetic fields [41]. The CS method has also been used to enrich $c\text{-}kit^+$ [42] and $Sca\text{-}1^+$ cardiac cells [43].

5. Human Cardiospheres Recapitulate Stem Cell Niche Properties *In Vitro*

Anversa et al. [44] first postulated that CSs may recapitulate *ex vivo* several features of cardiac stem cell niches, as described *in vivo*. This notion is supported by data by Li et al. [45]. Expression of connexin 43, a gap junction protein playing a key role for the electric coupling of differentiating cardiac progenitors with the surrounding cells, suggests that the differentiated cells may serve as supporting cells for the more primitive cells. Cells self-assembled into niche-like

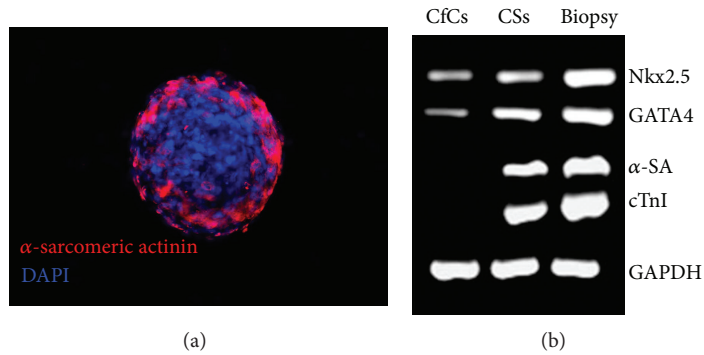

Figure 3: (a) Immunostaining of a human CS showing cells expressing cardiac α-sarcomeric actinin (red) in the outer sheet; nuclear staining with DAPI (blue). (b) PCR expression analysis of early and late cardiac genes by CS-forming cells (CfCs), CSs, and human cardiac biopsy tissue. CfCs express lower levels of early genes (Nkx2.5 and GATA4 transcription factors) compared to CSs, but no α-sarcomeric actinin (SA) nor cardiac troponin I (cTnI). CSs express high levels of both early and late cardiac genes.

CS structures exhibit greater proportions of c-kit$^+$ cells and upregulation of embryonic genes such as SOX2 and Nanog compared to cells cultured under traditional monolayer conditions or cells dissociated from CSs. Quantitative RT-PCR and immunostaining data show increased expression of stem cell-related factors and adhesion/extracellular-matrix (ECM) molecules in CSs, including insulin-like growth factor 1 (IGF-1), histone deacetylase 2 (HDAC2), telomerase (Tert), integrin-α2, laminin-β1, and matrix metalloproteinases (MMPs) compared to the above populations not assembled in CSs. Dissociation of CSs into single cells decreases the expression of ECM and adhesion molecules, reduces the resistance of cells to oxidative stress, and abrogates the advantages of CSs in terms of *in vivo* engraftment and functional improvement after MI. Thus, CSs mimic several features of cardiac stem cell niches, including the presence of both primitive and differentiating cells and expression of ECM and adhesion molecules, which are associated with enhanced *in vivo* cell survival and cardioprotection after MI.

6. Human CS and CDC Therapy in Animal Models

CDCs reduced scarring after MI, increased viable myocardium, and boosted cardiac function in preclinical animal models [25, 38, 45–49]. In the initial study by Messina et al. [25], human CSs were injected into the viable myocardium bordering a freshly infarcted area in SCID mice. Eighteen days after the intervention, infarct size did not significantly differ between the CS-treated group and the PBS-injected group. However, percent fractional shortening was higher in the former group (36.85% ± 16.43% versus 17.87% ± 5.95%; $P < 0.05$). Vigorous engraftment with bands of regenerating myocardium and newly formed blood vessels were observed in the CS-treated group.

Smith et al. [38] reported that percutaneous endomyocardial biopsy specimens grown in primary culture developed CSs (in 69 of 70 patients), from which CDCs were obtained. Human CDCs were injected into the border zone of acute MIs in immunodeficient mice. CSs and CDCs expressed antigenic characteristics of stem cells at each stage of processing, as well as proteins essential for cardiac contractile and electrical function. Human CDCs cocultured with neonatal rat ventricular myocytes exhibited biophysical signatures characteristic of myocytes, including calcium transients synchronous with those of neighboring myocytes. Human CDCs injected into the border zone of MIs engrafted and migrated into the infarct zone. After 20 days, both the percentage of viable myocardium within the MI zone and left ventricular ejection fraction were greater in the CDC-treated group compared with the fibroblast-treated control group.

Chimenti et al. [46] showed that human adult CSs and CDCs release many growth factors in culture media, which mediate both proangiogenic effects on human umbilical vein endothelial cells and antiapoptotic effects on neonatal rat ventricular myocytes *in vitro*. When transplanted into the peri-infarct zone in a SCID mouse MI model, human CDCs secreted vascular endothelial growth factor 1 (VEGF1), hepatocyte growth factor (HGF), and IGF1. These effects were associated with the upregulation of the prosurvival factor Akt, reduced the activation of caspase 3 and apoptosis, increased capillary density, and improved cardiac function. The relative contribution of the paracrine effects of the transplanted human CDCs versus their direct differentiation into cardiovascular cells was assessed by immunohistochemistry using two different antibodies raised against human-specific epitopes. The number of human-specific cells relative to overall increases in capillary density and myocardial viability indicated that direct differentiation of the transplanted cells accounted for 20% to 50% of the observed effects. These findings demonstrate that transplanted human CDCs act mainly by stimulating endogenous cardiac regeneration through paracrine mechanisms, while direct cardiac differentiation of CDCs *in situ* is also playing contributory roles.

Recently, Li et al. [47] conducted a direct comparison between different stem cell types *in vitro* for various assays of cell potency and *in vivo* for functional myocardial repair in the same mouse MI model. *In vitro*, human CDCs showed the greatest myogenic differentiation potency, the highest angiogenic potential, and relatively high production of several angiogenic and antiapoptotic factors compared with human BM-derived MSCs, adipose tissue-derived MSCs, and

BM-derived mononuclear cells. *In vivo*, injection of CDCs into infarcted mouse hearts resulted in superior improvement of cardiac function, the highest cell engraftment and myogenic differentiation rates, the lowest number of apoptotic cells, and the least-abnormal heart morphology 3 weeks after treatment. The c-kit$^+$ subpopulation purified from CDCs produced lower levels of paracrine factors and mediated lower functional benefits compared with unsorted CDCs. It should be noted, however, that these c-kit$^+$ cells were purified from CDCs and not directly from cardiac tissue specimens, which represents a methodological difference to the recent SCIPIO trial [17]. To validate the comparison of cells from various human donors, results were verified in cells of different types derived from individual rats. These data demonstrate that CDCs have greater regeneration potential compared to other cell types currently used for cardiac repair.

7. Autologous versus Allogeneic CDC Therapy in Animal Models

Malliaras et al. [49] compared between syngeneic, allogeneic, and xenogeneic CDCs for cardiac regeneration. *In vitro*, CDCs expressed major histocompatibility complex (MHC) class I but not class II antigens or B7 costimulatory molecules. In mixed-lymphocyte reactions, allogeneic CDCs elicited negligible lymphocyte proliferation and inflammatory cytokine secretion. *In vivo*, syngeneic and allogeneic CDCs survived at similar levels in rat hearts 1 week after cell delivery, but few syngeneic (and even fewer allogeneic) CDCs persisted at 3 weeks. Allogeneic CDCs induced a transient, mild, and local immune reaction in the heart, without histologically evident rejection or systemic immunogenicity. Improvements in cardiac structure and function were comparable with syngeneic and allogeneic CDCs up to 6 months after cell delivery. Allogeneic CDCs stimulated endogenous regenerative mechanisms (cell cycling, recruitment of c-kit$^+$ cells, and angiogenesis) and increased myocardial VEGF1, IGF1, and HGF equally with syngeneic CDCs. The persistence of benefit despite a transient survival of the transplanted cells suggested an indirect mechanism of action involving paracrine effects. These results indicated that allogeneic CDC therapy without immunosuppression was safe and improved heart function in a rat model of myocardial infarction. As such, allogeneic CDCs might obviate the limitations associated with patient-specific tissue harvesting and cell processing, suggesting that allogeneic human CDCs may represent a potential off-the-shelf product for cell heart therapy.

8. Clinical Testing of CDC Therapy in Patients after MI

The results of the prospective, randomised cardiosphere-derived aUtologous stem cells to reverse ventricular dySfunction (CADUCEUS) trial (registered with ClinicalTrials.gov, NCT00893360) were published recently [18]. Patients 2–4 weeks after MI (with depressed left ventricular ejection fraction of 25–45%) were enrolled at two medical centers in the USA and randomly allocated in a 2 : 1 ratio to receive CDCs ($n = 17$) or standard care ($n = 8$). For patients assigned to receive CDCs, autologous cells were grown from endomyocardial biopsy specimens. Prescribed cell doses were achieved within 36 ± 6 days (mean ± SD) and infused into the infarct-related artery 1.5–3 months after MI. The primary endpoint was proportion of patients at 6 months who died due to ventricular tachycardia, ventricular fibrillation, or sudden unexpected death or had MI after cell infusion, new cardiac tumor formation on MRI, or a major adverse cardiac event (composite of death and hospital admission for heart failure or nonfatal recurrent MI). Preliminary efficacy data were collected using cardiac magnetic resonance imaging (MRI) at 6 months. No complications were reported within 24 h of CDC infusion. By 6 months, no patients had died or developed cardiac tumors, or major adverse cardiac event in either group. Four patients (24%) in the CDC group had serious adverse events compared with one control (13%; $P = 1.00$). Compared with controls at 6 months, MRI analysis of patients treated with CDCs showed reductions in scar mass ($P = 0.001$) and increases in viable heart mass ($P = 0.01$) and regional contractility ($P = 0.02$) as well as regional systolic wall thickening ($P = 0.015$). However, changes in end-diastolic volume, end-systolic volume, and left ventricular ejection fraction did not differ between groups by 6 months. These results indicate that intracoronary infusion of autologous CDCs after MI is safe. The observed increase in viable myocardium suggests that therapeutic regeneration may have occurred.

9. Cell Therapy versus Secreted Factors

The demonstration of beneficial effects of cell therapy despite short-lived survival of the delivered cells [49], together with the observed trophic effects on culture media conditioned by progenitor cells [46], suggests that secreted factors may be the active component of cell therapy for cardiac regeneration. Cells communicate with each other via released molecules such as short peptides, proteins, nucleotides, and lipids that bind to surface receptors on neighboring cells. In addition, eukaryotic cells communicate with each other through the release of microparticles and exosomes in their extracellular environment. Exosomes are membrane vesicles (40–100 nm in diameter) formed by endocytosis. They are smaller than microparticles (100–1000 nm in diameter), which are released by budding of the plasma membrane (ectocytosis) [50]. Exosomes display a broad spectrum of bioactive substances on their surfaces and carry a concentrated set of proteins, lipids, and even nucleic acids that are taken up by other cells and regulate their function [51–53]. Sahoo et al. [54] reported angiogenic effects of exosomes derived from human CD34$^+$ BM stem cells in isolated endothelial cells and murine models of vessel growth. In some of the *in vitro* and *in vivo* assays, the exosomes from CD34$^+$ cells appeared more potent than the cells themselves. Vrijsen et al. [55] reported that exosomes mediated the angiogenic activity of media conditioned by human fetal cardiac progenitor cells *in vitro*. Timmers et al. [56] showed that injection of media conditioned by ESC-derived MSCs reduced infarct size and improved cardiac function in a pig model of ischemia/reperfusion injury,

and that exosomes within the conditioned medium contained the active component. Lai et al. [57] found that exosomes secreted by MSCs similarly reduced myocardial ischemia/reperfusion (I/R) injury in mice. Barile et al. [58] recently showed that exosomes isolated from mouse cardiac progenitor cells protected H9C2 from oxidative stress by inhibiting caspase 3/7 activation *in vitro*, while also reducing cardiomyocyte apoptosis in a mouse model of myocardial I/R *in vivo*. We have provided ultrastructural evidence of exosome secretion by adult human CSs [59]. Further studies are needed to assess whether exosomes isolated from CSs are as cardioprotective as the respective cells of origin. Of note, exosomes may offer major advantages over cell transplantation for therapeutic applications. First, it might be possible to use exosomes secreted by cells from young, healthy individuals for allogeneic applications, even though this hypothesis remains to be verified. This possibility would pave the way to "off-the shelf" exosome-based therapeutic products. Second, exosomes can be stored without potentially toxic cryopreservatives at −20°C for 6 months with no loss in their biochemical activities [60]. Third, exosomes protect their contents from degradation *in vivo* [61, 62], thereby potentially preventing some of the problems associated with small soluble molecules such as cytokines, growth factors, transcription factors, and RNAs, which are rapidly degraded.

Increasing evidence suggests that exosomes may act as a vector of genetic information. Indeed, mRNAs carried by exosomes can be translated into proteins in the target cell. Accordingly, ESC-derived microvesicles were shown to reprogram hematopoietic progenitors by mRNA transfer and protein delivery [63]. MicroRNA families can be selectively secreted into the extracellular environment through exosomes [64].

10. Cell versus Gene Therapy

Gene therapy may provide an alternative to cell transplantation for cardiac protection and repair. Clearly, the two approaches can be used in combination by transplanting genetically engineered cells. Gene therapy has a potential for circumventing some hurdles associated with cell therapy, such as the need for *in vitro* cell expansion; however, it also has peculiar limitations, such as the need for using either viral or nonviral gene transfer vectors. Fujii et al. [65] recently showed that ultrasound-targeted gene delivery of vascular endothelial growth factor (VEGF) or stem cell factor (SCF) induced angiogenesis and improved ventricular function after MI in mice. Yaniz-Galende et al. [66] reported cardiac repair by soluble SCF gene transfer after MI via *in situ* recruitment and expansion of c-kit$^+$ cells. This observation is in line with increased capillary density and reduced apoptosis in the peri-infarct area in a mouse model of tetracycline-inducible, cardiac-specific overexpression of membrane-associated SCF [67].

Exosomes carry microRNA molecules [58, 64], as mentioned above, which may play key regulatory roles in many processes such as cardiomyocyte proliferation [68], differentiation [69], hypertrophy [70], as well as aging and function [71]. Eulalio et al. [68] recently showed that exogenous administration of two microRNAs (hsa-miR-590 and hsa-miR-199a), which were identified by high-throughput functional screening for human microRNAs that promoted neonatal cardiomyocyte proliferation using a whole-genome microRNA library, markedly stimulated cardiomyocyte proliferation in both neonatal and adult rodents. After MI in mice, these microRNAs stimulated marked cardiac regeneration and almost complete recovery of cardiac functional parameters. Adenoassociated virus- (AAV-) based vectors were used to deliver microRNAs *in vivo*. Further studies are needed to evaluate whether these microRNAs likewise induce proliferation in human cardiomyocytes. Boon et al. [71] recently reported that miR-34a was induced in the aging heart and that *in vivo* silencing or genetic deletion of miR-34a reduced age-associated cardiomyocyte cell death. Moreover, miR-34a inhibition reduced cell death and fibrosis, while improving myocardial function after acute MI in mice. PNUTS, a novel direct miR-34a target, reduced telomere shortening, DNA damage responses, and cardiomyocyte apoptosis, thereby improving cardiac function after acute MI.

11. Conclusions

CSs have attracted great interest as an *in vitro* model of a stem cell niche-like microenvironment rich in both primitive and differentiating cells, and as a cell source for cell heart therapy. The cellular outgrowths from cultured tissue explants may enrich progenitor cells that migrate out of the explant. Moreover, both cell-cell and cell-matrix interactions within CSs may promote the specification of cardiac-resident progenitors towards cardiovascular fates. CDCs have proven safe in a phase-1 clinical trial in patients after MI, and initial results have been promising. Meanwhile, exosomes and microRNAs are emerging as alternate, cell-free strategies for cardiac protection and regeneration.

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Review Article

Evidence for Bone Marrow Adult Stem Cell Plasticity: Properties, Molecular Mechanisms, Negative Aspects, and Clinical Applications of Hematopoietic and Mesenchymal Stem Cells Transdifferentiation

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In contrast to the pluripotent *embryonic stem cells* (ESCs) which are able to give rise to all cell types of the body, mammalian *adult stem cells* (ASCs) appeared to be more limited in their differentiation potential and to be committed to their tissue of origin. Recently, surprising new findings have contradicted central dogmas of commitment of ASCs by showing their plasticity to differentiate across tissue lineage boundaries, irrespective of classical germ layer designations. The present paper supports the plasticity of the *bone marrow stem cells* (BMSCs), bringing the most striking and the latest evidences of the transdifferentiation properties of the *bone marrow hematopoietic and mesenchymal stem cells* (BMHSCs, and BMMSCs), the two BM populations of ASCs better characterized. In addition, we report the possible mechanisms that may explain these events, outlining the clinical importance of these phenomena and the relative problems.

1. Introduction

1.1. Evidence for BMSCs Plasticity. It has long been believed that the differentiation potential of ASCs is restricted to the production of the cell types normally found in the organ in which ASCs reside. Classical experiments showed that when fragments or cells dissociated from an organ or a tissue are transplanted to a new site or cultured, they tend to maintain their originalcharacter; although they may lose some of their properties, they usually do not acquire characteristics of a different cell lineage [1]. The first suggestion that ASCs, committed to a specific developmental lineage, switch into another cell type of an unrelated tissue (transdifferentiation) came from studies of whole BM transplantation in humans and animal models. In 1997 Eglitis and Mezey reported that transplanted mouse BM cells could give rise to brain astrocytes in adult mice [2]. The most striking suggestion of stem cell plasticity was published in 1998 by an Italian group, which found that mouse BM cells could give rise to skeletal muscle cells when transplanted into a mouse muscle that had been damaged by an injection of a muscle toxin [3]; thus mouse BMSCs could migrate to sites of muscle injury and participate in muscle regeneration, albeit at low efficiency. From 1999 up to date it was reported that transplanted BM cells could produce hepatocytes [4–7], endothelial [8] and myocardial cells [9–11], central nervous system (CNS) neurons, and glial cells [12–14]. The reason why these forms of plasticity were not been seen before is probably due to the methods used. In earlier experiments, organ or tissue fragments were usually transplanted, and so the donor cells continued to have neighbors of the same tissue type. In the subsequent experiments, cell suspensions were usually

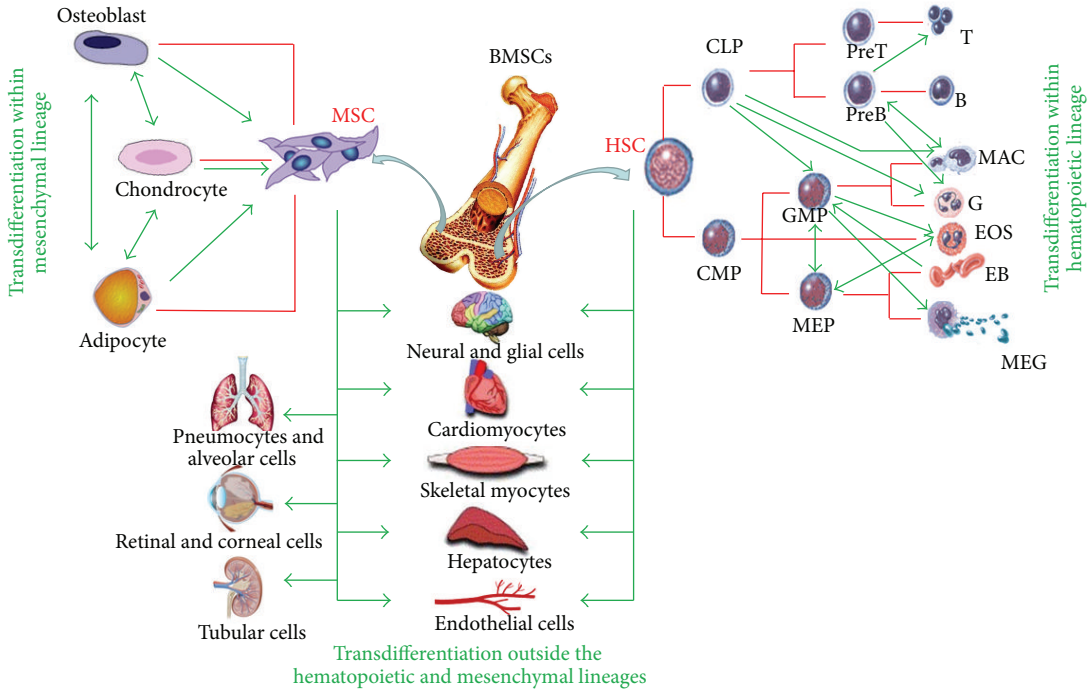

Figure 1: Plasticity of BM HSC and MSC.

transplanted so that individual donor cells could end up surrounded by cells of a different tissue type. Moreover, the donor cells were genetically marked so that even rare cells expressing donor cell genes could be identified in tissue sections. Sex chromosome markers (Y chromosome DNA sequences to detect male donor-derived cells in female hosts) have been used to detect plasticity in BM transplant patients, where BM or blood cells were reported to give rise to either hepatocytes [15, 16] or epithelial cells in skin and gut [16].

These and similar studies, performed with transplanted BM cells, suggested that BM is a source of different kinds of ASCs which, given the appropriate environmental signals, show pluripotent properties and transdifferentiate into cells of many different organs, including skeletal muscle, heart, liver, and endothelial and even brain cells.

Our focus is to critically evaluate the evidence in favor of HSCs and MSCs plasticity.

1.2. From Multipotent to Pluripotent BMHSC. HSCs are essential for the generation and homeostasis of the blood system. They give rise to all the blood cell types, including lymphocytes, erythrocytes, monocytes, granulocytes, and platelets, and they replenish these cells [17] (Figure 1). Contrary to ASCs from other tissues, HSCs are easy to obtain, as they can be either aspirated directly out of the BM or stimulated to move into the peripheral blood (PB) stream, where they can easily be collected. According to the hierarchy of hematopoietic development, an HSC would be positioned at a branch bifurcation with its potential restricted to generating *common lymphoid precursors* (CLPs) [18] and *common myeloid precursors* (CMPs) [19].

1.2.1. Transdifferentiation of BMHSCs into Nonhematopoietic Cells. To support the hypothesis that HSCs are able to transdifferentiate into nonhematopoietic cells (Figure 1), several groups transplanted purified BMHSCs in a variety of settings. Gussoni et al. transplanted HSCs from male mice into female mdx mice, a model of Duchenne muscular dystrophy [20]. They were able to track the fate of the transplanted cells by detecting the Y chromosome with fluorescent in situ hybridization. The donor cells efficiently replenished the BM of the recipients as expected, and cells from the males expressing dystrophin were found at low levels in host muscle fibres, indicating differentiation of the transplanted cells into muscle. Analogous studies have shown that HSCs can also contribute to the repair of capillaries and cardiomyocytes in a mouse model of coronary artery infarction [21]. Orlic et al. observed that when a population enriched in HSCs was injected directly into injured hearts, it could participate in the regeneration of cardiac muscle, leading to an apparent improvement of cardiac function [9]. Lagasse et al. also supported the concept of transdifferentiation at functional level. They showed that HSCs injected into mice with an inducible lethal liver disease, tyrosinemia type 1, could repopulate the haematopoietic system as well as differentiate into hepatocytes and rescue the animals from hepatic failure and death [22].

1.2.2. Transdifferentiation of BM HSCs within the Hematopoietic System. Over the past two decades, results from *in vitro* studies have challenged the notion of a strictly hierarchical branching model of hematopoiesis. Numerous investigations have shown that both nontransformed and malignant

hematopoietic precursors can switch cell types within the hematopoietic lineage [23] (Figure 1). Many distinct lymphoid-to-myeloid and myeloid-to-erythroid switches were shown by inducing transcription factor expression, cytokine or drug treatments, and changes in environmental conditions [24, 25] (Figure 1). The first experiment demonstrating switch between lymphoid and myeloid cells was conducted by Boyd and Schrader, who tested the effects of 5-azacytidine on Abelson virus-transformed pre-B lymphoma cell lines. They found that a subset of these cells acquired properties of macrophages [26]. A similar effect was seen in pre-B and B-cell lines immortalized with *Eu-myc*, in which Klinken et al. overexpressed the *v-raf* oncogene. The *v-raf*-transfected cells not only expressed myelomonocytic markers (such as the *colony-stimulating factor* (CSF)-1 receptor and lysozyme), but also retained immunoglobulin rearrangements characteristic of the original cells [27]. Similarly, a proportion of early B-cell lines ectopically expressing the *v-fms* oncogene (encoding a constitutively active form of the CSF-1 receptor) switched into macrophages [28]. Moreover a study reported a switch of B-lymphoid cells to neutrophil granulocytes [29]. A surprising degree of plasticity was discovered in B-lineage cells derived from Pax5 knockout mice: in the absence of Pax5, commitment of lymphoid progenitor to the B-lymphoid lineage was blocked and pre-B cells from mice, carrying a deletion in the Pax5 gene, could generate multilineage hematopoietic cells [30]. It appears that the main role of Pax5 in the establishment of B-cell commitment is the repression of lineage inappropriate genes, such as the CSF-1 receptor gene (*c-fms*), which is expressed in the pre-B Pax5 knockout cells. Another series of experiments demonstrated that CLPs can be reprogrammed to become myelomonocytic and that lineage plasticity has been observed also within the myeloid/erythroid compartment [23].

1.3. From Multipotent to Pluripotent BMMSC. MSCs isolated from the BM of adult organisms were initially characterized as plastic adherent, fibroblastoid cells with the capacity to differentiate into osteoblasts, adipocytes, and chondrocytes *in vitro* (Figure 1) and in to heterotopic osseous tissue when transplanted *in vivo* [31]. In addition to BM, MSCs have also been elaborated from skeletal muscle, adipose tissue, umbilical cord, synovium, the circulatory system, dental pulp, and amniotic fluid as well as fetal blood, BM, liver, and lung [32]. Therefore, it appears that MSCs reside within the connective tissue of most organs as predicted by early studies with chick embryos [32]. However, it should be noted that these populations are not functionally equivalent with respect to their *in vivo* differentiation potential [33]. Despite their functional heterogeneity, MSCs populations obtained from most tissues commonly express a number of surface receptors including CD29, CD44, CD49a-f, CD51, CD73, CD105, CD106, CD166, and Stro-1 and lack expression of definitive hematopoietic lineage markers including CD11b, CD14, and CD45 [33]. However, it is important to realize that no single isolation method is regarded as a standard in the field. Therefore, the varied approaches used to culture, expand, and select MSCs make it difficult to directly compare experimental results.

1.3.1. Transdifferentiation of BMMSCs in Non-Mesenchymal Cells. Kopen et al. first demonstrated that MSCs injected into the CNS of newborn mice migrated throughout the brain and adopted morphological and phenotypic characteristics of astrocytes and neurons [33]. These findings were confirmed by other laboratories [34, 35], which tried to identify the conditions that induced neural differentiation of MSCs *in vitro*. Several groups reported that exposure to reducing agents and antioxidants or chemicals, that increase intracellular cyclic AMP levels, induced MSCs to adopt a neuron-like morphology and express various neural specific proteins including nestin, *glial fibrillary acidic protein* (GFAP), *neurofilament heavy chain* (NF-HC), and β-III tubulin [36, 37]. Studies by Neuhuber et al. [38] showed that these agents promoted retraction of the cell cytoplasm due to disruption of the actin network in MSCs and not neurite outgrowth as seen in neurons. Microarray [39] and proteomic studies [40] further demonstrated that the set of genes modulated in MSCs after neural differentiation was distinct from the set differentially expressed between untreated MSCs and neural tissue. Therefore, cytoskeletal alterations induced by these agents rather then transdifferentiation accounted for the neuron-like morphology of MSCs. Moreover BM is also innervated by nervous tissue, which explains the finding that MSCs from BM also express various neuroregulatory proteins including neurotrophins, neurite-inducing factors, and neuropeptides. Surprisingly in 2008 Tondreau et al. reported that BM MSCs have the potential to differentiate in to neuronal cells with specific gene expression and functional properties [41]. More recently it has been reported that BMMSCs possess a great potential to differentiate into functional neurons because they not only expressed neuron phenotype and membrane channel protein, but also exhibited functional ion currents [42]. Thus evidence for transdifferentiation of BMMSCs into neurons is contradictory.

BMMSCs have also been reported to differentiate into various epithelial cell types after systemic administration *in vivo*. It was shown that BMMSCs engraftment in lung of mice was enhanced in response to bleomycin exposure and that a small percentage of MSCs, localized to areas of lung injury, resembled epithelial cells and copurified with type II pneumocytes [32]. Moreover BMMSCs engrafted in lung differentiated into type I pneumocytes or assumed phenotypic characteristics of all major cell types in lung including fibroblasts, type I and type II epithelial cells, and myofibroblasts [32]. Recently it has been reported that BMMSCs can differentiate into type II alveolar epithelial cells *in vitro* [43]. BMMSCs can also differentiate into skin epithelial cells, sebaceous duct cells [44], retinal pigment epithelial cells [45], corneal keratocytes phenotype [46], and tubular epithelial cells [47].

MSCs can also differentiate and integrate into muscle cells [48]. A recent report supports the myogenic potential of BMMSCs *in vitro* and *in vivo* for the treatment of urinary incontinence [49]. MSCs appear also to be involved in the

generation of myocardial cell types [50]. Not only expression of some genes indicative of myocardial differentiation like troponin T, *β-myosin heavy chain* (*β*MHC), *myosin regulatory ligh chain 2* (Myl 2) but also detailed analysis of contractility, excitation-contraction coupling and signalling pathways demonstrated that MSCs can generate functional cardiomyocytes *in vitro* [51].

Transdifferentiation of cultured naïve MSCs into hepatocyte-like cells has been claimed to occur by adding specific differentiation media [52]. Recently Zhang et al. have resumed the factors and the methods used to differentiate MSCs, from BM and other tissues, into hepatocyte-like cells underlying their liver regenerative potential [53]. However in many transplantation experiments naïve or differentiated murine or human MSCs were not able to generate liver tissue and to rescue the liver phenotype in an *albumin-urokinase promoter* (Alb-uPA) transgenic mice or in *fumarylacetoacetate-hydrolase*-(FAH-)-deficient mice ($FAH^{(-/-)}$). Transplantation of BMMSCs-derived hepatocyte-like cells into a patient with homozygous familial hypercholesterolemia failed to affect the cholesterol levels [54]. Evidence from the literature also points towards protective and trophic effects of MSCs when injected into the injured liver but the exact therapeutic mechanisms are unknown [55].

1.3.2. Transdifferentiation of BMMSCs within Mesenchymal System. Similar to transdifferentiation observed in the hematopoietic system (lymphoid-to-myeloid and myeloid-to-erythroid switches), transdifferentiation examples have also been reported in mesenchymal system (Figure 1). Song and Tuan reported that fully differentiated osteoblasts, from BMMSCs, were able to change their differentiation program and became lipid-producing adipocytes and chondrocytes that produced proteoglycan, collagen type II, and link protein [56]. They also demonstrated that human MSCs that had differentiated into adipocytes transdifferentiated into osteoblasts or chondrocytes by replacing the inducing culture media. Similarly, chondrocytes derived from MSCs in the presence of TGF-*β*3 could be induced to differentiate into osteoblasts and adipocytes [56]. In the same report the authors showed that without the pressure of inducing factors, fully differentiated MSC-derived cells could resume cell proliferation, modify their gene expression profile, and return to a more primitive stem cell-like stage. Accompanying the phenotypic changes observed, was a fluctuation in the expression of lineage-specific transcription factors: Cbfa 1 for osteogenesis, Sox 9 for chondrogenesis, and PPAR*γ*2 for adipogenesis. As expected, expression of Cbfa 1 was upregulated during osteogenesis, whereas both Sox 9 and PPAR*γ*2 were downregulated compared with undifferentiated human MSCs. On the other hand, expression levels of all three transcription factors decreased during osteoblast dedifferentiation, which suggested that cells might return to an uncommitted developmental stage from a fully determined cell type (dedifferentiation) [56]. Thus differentiation processes are not unidirectional as regarded for a long time; dedifferentiation of committed progenitors and successive differentiation in other cell types are possible, at least for mesenchymal and hematopoietic system. It remains to be determined whether dedifferentiation of committed progenitors is only an experimentally induced effect or whether this is also taking place normally under physiological conditions.

The red lines indicate normal lineage relationships, and the thick green lines represent transdifferentiation within and outside the hematopoietic and mesenchymal lineages. (These switches do not necessary imply direct transitions.)

1.4. Mechanisms Underlying BM HSC and MSC Plasticity

1.4.1. Microenvironment-Dependent Reprogramming of Gene Expression Profile Underlying Transdifferentiation in HSCs and MSCs. In order to undergo transdifferentiation and fate changes compared to their own lineage commitment, ASCs need to change or modify their gene expression programs. Therefore, temporary inactivation of cellular memory of transcriptional state is required. It is well known that BM microenvironment, in which HSCs and MSCs reside, provides signals for survival and external control of stem cell activity. In this regard it can be assumed that new microenvironment signals should be able to modulate the cellular memory of transcriptional state and to lead to a switch in stem cell gene expression and in its cellular identity. Transplanted ASCs may recognise heterotopic environments through cell surface receptors, which stimulate signaling transduction pathways connecting the outside of the stem cell with inside responsive transcription factors and regulatory molecules [17]. At the molecular level the process of transdifferentiation for HSCs could be based on the finding that multipotent hematopoietic progenitors are primed for low-level transcription of nonhematopoietic loci, and that new microenvironment signals and the transcription factors balances could initiate gene expression of primed loci [17]. It can assume the same also for MSCs or other kinds of ASCs. In fact as BMHSCs, BMMSC are usually present in the BM stem cell niches under hypoxic conditions. Hypoxic conditions therefore influence MSCs proliferation and cell fate commitment, meaning that gradients of oxygen tensions influence the prolonged maintenance of a stem cell phenotype and pluripotency [57]. It has also been demonstrated that the culture of MSC under hypoxic conditions is accompanied by increased Oct4 expression and telomerase activity [57] which are involved in the maintenance of stemness. Hypoxic conditions induce the transcription factor hypoxia-inducing factor-*α* which can promote certain differentiation phenotypes in MSCs. Other lines of evidence, of microenvironment-dependant reprogramming of MSCs gene expression profile, come from studies on MSCs isolated from adipose tissue (ATMSCs). Thus, chondrogenic differentiation of ATMSC has been observed at enhanced levels under hypoxic conditions where osteogenesis is inhibited. In contrast, enhanced osteogenic differentiation of ATMSC can be induced under normoxia.

Functional changes of MSCs under hypoxia also include increased secretory activity, that is, of vascular endothelial growth factor and interleukin-6 as well as mobilization and homing by the induction of stromal cell-derived factor-1

expression and the corresponding receptor CXCR4 [57]. In this context, MSC subpopulations displaying a high aldehyde-dehydrogenase activity have been reported with increased responsiveness to hypoxia, including an upregulation of Flt-1, CXCR4, and angiopoietin-2 [57]. Together, these findings further substantiate that BM hypoxic microenvironment and the chance in the microenvironment oxygen tension (from hypoxia to normoxia) contribute to the regulation of MSC function and fate [57].

Among the transcription factors known having essential roles in hematopoietic lineage decisions, there are GATA-1, Friend of GATA-1 (FOG-1), PU.1, *CCAAT/enhancer-binding protein* beta (C/EBP-β) and Pax. The zinc-finger transcription factor GATA-1 and its cofactor FOG-1 have been found to be essential for erythroid and megakaryocytic differentiation; the physical interaction between GATA-1 and FOG-1 is required for terminal erythroid and megakaryocyte maturation both *in vivo* and *in vitro* [58]. PU.1 is essential in the development of cells of the monocytic, granulocytic, and lymphoid lineage [58]. The cross-antagonism observed between GATA-1 and PU.1 and the relative abundance of each factor predict the lineage decision of a multipotent HSC. Moreover FOG expression is downregulated at the transcriptional level by C/EBP-β. This downregulation is a prerequisite for commitment to the eosinophil lineage [58]. Pax5 is another transcription factor whose expression in the hematopoietic system is restricted to cells of the B-cell lymphoid lineage. It was reported that pro-B cells derived from $Pax5^{-/-}$ mice gave rise to several distinct lineages including macrophages, osteoclasts, and dendritic cells [58]; thus Pax5 normally represses alternative lineage programs. Another group of proteins which play a role in the regulation of cell identity, transcriptional memory, and plasticity is that encoded by the *Polycomb* (PcG) and *Trithorax* (trx-G) *Genes*. These proteins which regulate *Hox* genes expression pattern and determine segment identity in *Drosophila* are strongly involved in the regulation of hematopoiesis [17].

Less is known about genes and molecules involved in MSCs commitment and transdifferentiation. Among the transcription factors upregulated we note Cbfa 1 for induction of osteogenesis, Sox 9 for chondrogenesis, and PPARγ2 for adipogenesis [56]. Satija et al. reported two other transcription factors governing osteogenic differentiation of MSCs: Osterix and Runx2 [59]. Several signalling pathways modulated by specific chemical compounds appear to be involved in the generation of myocardial cell types from MSCs, including the *bone morphogenic protein 4* (BMP4), *Wingless+ Int-1* (Wnt), and *fibroblast growth factor 2* (FGF2) signalling, as well as inhibition of Wnt signalling by the factor *Dickkopf1* (Dkk1) and the treatment with DNA-demethylating agent *5-azacytidin,* (5-aza) [50]. Recently it has been published that the transcription factor GATA-4 increases MSCs transdifferentiation into cardiac phenotype and enhances the MSCs secretome, promoting postinfarction cardiac angiogenesis [60]. What permits or restricts the access of transcription factors, coactivators, or constituents of transcriptional memory to genome regions and to particular genes within those regions? We know that nuclear programs consist of specific temporal, spatial, and geometric chromatin configurations. Epigenetic modifications (histone modifications, DNA methylation/demethylation, and ATP-dependent chromatin remodeling complex activation) that are generated in response to changing microenvironments, regulate these features of chromatin structure, support or not the opening of the chromatin, and are critical for the required nuclear reprogramming and thus transdifferentiation [61].

1.4.2. MicroRNAs (miRNAs). MicroRNAs (miRNA) are a class of noncoding RNAs which bind the 3′UTR of target mRNAs to mediate translational repression in cells. Many miRNAs are specifically expressed during hematopoietic lineage commitments [62]. miR-181, miR-223, and miR-142s were differentially or preferentially expressed in hematopoietic tissues; miR-142s expression was lowest in the erythroid and T-lymphoid lineages and highest in B-lymphoid and myeloid lineages; miR-223 expression was confined to myeloid lineages, with barely detectable expression in T- and B-lymphoid and in erythroid lineages [62]. Expression of miR-181, miR-223, and miR-142s was low in HSCs, suggesting that these miRNAs are also induced during lineage differentiation [62]. Moreover their differential expression in specific hematopoietic lineages suggested that they might influence hematopoietic lineage commitment and differentiation. In BMMSCs, miR-130 and miR-206 have been shown to regulate the synthesis of neurotransmitter substance P in human MSCs-derived neuronal cells [63].

1.4.3. Cell Fusion rather than Transdifferentiation. It has been largely assumed that the nuclear reprogramming and transdifferentiation in response to environmental changes are the mechanism by which committed HSCs give rise to multiple cell types. However Terada et al. first reported the surprising results of an *in vitro* spontaneous cellular fusion between HSCs and totipotent ESCs in coculture [64]. Later, other reports contradicted the transdifferentiation phenomena of HSCs, showing that Purkinje neurons can fuse with BM-derived cells in both mice and humans [65]. The question is whether this *in vitro* fusion results in denying the transdifferentiation for *in vivo* HSCs switching. However, we must not forget that HSCs isolation protocols require manipulation that could expose highly enriched HSCs to concentrated pluripotent precursors types that might mediate cell fusion. In addition, one transplantation study in mice showed the 30%–50% efficiency of HSCs reconstitution of hepatocytes, which is far greater than the frequency ($1 \sim 500.000$) of HSC-ESC fusion observed [61]. This means that the lineage switching is not due to cell fusion but to transdifferentiation.

1.4.4. BM Is a Source of Different Tissue-Specific Stem Cells. There is always the possibility that the BM hosts a variety of dedicated tissue-specific stem cells, such as muscle stem cells, neuronal stem cells, and hepatic progenitors, although there is, as yet, no evidence for the presence of these progenitors cells in the BM. It has also been postulated that a universal BMSC exists [23]. In this extreme view the

various types of stem cells residing in the BM are considered to represent different states of a universal adult progenitor whose phenotype is defined by its local environment. These stem cells may move from one tissue into another via the circulation and may be more plastic in early than in more differentiated stages [23].

1.5. Negative Aspects of HSC and MSC Transdifferentiation. The transdifferentiation potential of HSCs and MSCs and their capacity for tissue renewal and damage repair have attracted much attention among biotechnologists and clinicians [66]. However some negative aspects must be considered. As Anderson has pointed out [67], there is a big difference between what cells normally do and what they can do if put in culture or if transplanted to a new location. From the perspective of cell therapy, however, it is what cells can do that may matter the most. In most reported cases, the phenotype of the donor-derived cells, that apparently switches their normal fate, was assessed by morphology and antibody staining, but rarely by function. Thus the cells may have acquired only a few of the characteristics of the new cell type but not any new functions. Cho et al. [68] reported that MSC-derived neurons exhibited synaptic transmission, but no evidence was provided that currents measured in cells were modulated by neurotransmitters. Similarly, Wislet-Gendebien et al. reported that MSC-derived neurons exhibit an evoked action potential, but a voltage spike induced only modest membrane depolarization [69]. Moreover in MSC-derived cardiomyocytes, the expression of cardiac markers such as cardiac α-actin, the *Desmosomal Type Junction Proteins Desmoglein 2* (Dsg2), *Desmocollin 2* (Dsc2), desmoplakin and plakophilin 2, and the junction protein myozap has not been found [50]. This is a major problem as all these molecules are known to be important for the formation of the composite junctions in the intercalated disk [50]. Even if they are the stimuli of the microenvironment to direct transdifferentiation, it is also possible that the differentiation is directed towards unwanted tissues. Recently BMMSCs injected into rat hearts were shown to differentiate into bone tissue and to drive its calcification [70].

A negative aspect of HSCs transdifferentiation is their contribution to BM neovascularization which represent a problem in those cancers which home and expand in the BM. Ria et al. demonstrated that in patients with multiple myeloma (MM), but not in those with *Monoclonal gammopathy of undetermined significance* (MGUS), *hematopoietic stem and progenitor cells* (HSPCs) differentiate into cells with endothelial features, contributing to the neovessels wall building together with MM endothelial cells (MMECs) [71]. Moreover in patients with MM, BM macrophages and mast cells transdifferentiate in to endothelial cells thus contributing to vasculogenic mimicry [72, 73]. We know that BM neovascularization contributes to MM progression [74]. Finally, we assume that the mechanism of transdifferentiation could be congenial not only to ASCs or to their precursor cells, but also to their tumor staminal counterparts: the *cancer stem cells* (CSCs). In gliomas transdifferentiation of CSCs into vascular mural cells contributes to tumor neovascularization [75]. Assuming the existence of hematopoietic and mesenchymal cancer stem cells residing in the BM, it can equally suppose their ability to migrate through the bloodstream and reach new districts where, in response to new microenvironment stimuli, they could transdifferentiate in to several tumor cell types generating metastasis and new tumors.

1.6. Clinical Applications of BM HSC and MSC. BMSCs are an attractive source of cells for therapy, especially in view of the recent claims that they are remarkably plastic in their differentiation potential when exposed to new environments.

Transplantation of BMSCs is traditionally used for haematological diseases, but there are increasing numbers of clinical trials using BMSCs for the treatment of non-hematological disorders. Xu and Liu resumed the studies carried out in animal models and in humans underlying the therapeutic potential of BMSCs in liver diseases [7]. This potential consisted in the restoration of liver function and liver mass, supply of growth factors, antifibrosis, and gene therapy. Recently the clinical trials involving BMSCs transplantation for the therapy of myocardial infarction [11] and *spinal cord injury* (SCI) [76] have been resumed. Another clinical application of BMSCs could be the treatment of xerostomia due to head and neck irradiation for cancer therapies and in Sjogren's syndrome and reestablishing of the salivary gland functions [77].

Among BMSCs, HSCs are the only stem cells being routinely used in the clinics [78]. They constitute only a small fraction of BM population (1 in 10^4 to 1 in 10^8 of BM nucleated cells), but the stimulation with mobilizing agents, including cytokines such as *Granulocyte Colony-Stimulating Factor* (G-CSF) alone or in combination with *granulocyte-macrophage colony-stimulating factor* (GM-CSF) and/or other agents, dramatically increases the release of HSCs from BM to PB [78]. HSCs are primarily used in the treatment of patients with haematological malignancies. During the course of treatment, patients' cancerous cells are first destroyed by chemo/radiotherapy and subsequentally replaced with autologous PB/G-CSF HSCs collected prior to the treatment, and reinfused into the patients, or with BM or PB/G-CSF transplant from a human-leukocyte-antigen-(HLA)-matched donor [78]. Allogenic BM transplant have also been used in the treatment of hereditary blood disorder including aplastic anemia, β-thalassemia, Wiskott-Aldrich syndrome, and *severe combined immunodeficiency* (SCID) as well as in metabolism errors as Hunter's syndrome and Hurler's syndrome [78]. HSCs transplants are also used as a therapeutic strategy against various types of solid tumors [78].

MSCs have become a recent focus of interest for cellular therapy in tissue regeneration. Wound healing studies have focused on MSCs as the cell population within the BM that can contribute to cutaneous regeneration [79]. Experiments with diabetic murine models have been particularly useful in assessing the clinical utility of MSCs in wound repair. Promising findings in animal models have led to a very limited number of human trials examining the effects of autologous MSCs on chronic wounds. Injection of primary BM cells into

the wound edge followed by topical application of cultured MSCs, resulted in the complete closure of three chronic wounds which had failed traditional therapy including autologous skin grafting [79]. Dash et al. conducted a randomized trial investigating the use of autologous MSCs expanded in culture and injected intramuscularly into the wounds edges of 24 patients with nonhealing ulcers secondary to diabetes or vasculitis. Ulcer size in the MSC-treated group decreased 73% [80]. MSCs enhace wound healing not only by differentiating into epidermal cells, but also into vessel forming endothelial cells contributing to neovascularization, necessary to supply oxygen and nutrients to the damaged tissue [80]. Another clinical application of MSCs would be to exploit their osteoblastic potential for treating bone disorders as in osteogenic imperfecta (OI). After a first demonstration of the potency of MSCs to differentiate into functional osteoblasts in a mouse model of OI, following a first BM transplantation, MSCs were used in children with type III OI [81]. These children showed improved growth and even low osteopoietic engraftment of MSCs was evident [81]. Recently the clinical trials have been also resumed involving BMMSCs in the treatment of neurological diseases such as traumatic brain injury, *spinal cord injury* (SCI), parkinson's disease, multiple sclerosis and amyotrophic lateral sclerosis, but these current data do not support the possibility that most of the reported effects occur as a result of direct transdifferentiation and cell replacement [82]. Some clinical trials have been performed with MSCs to treat heart damage. A Chinese group performed intracoronary short injection of autologous cultured BM cells after acute myocardial infarction and for the treatment of chronic ischemic cardiomyopathy [83]. The authors found improved cardiac function in patients the receiving cells [83]. A group from Greece performed a similar study and found the procedure to be safe and contributing to regional regeneration of myocardium [84].

1.6.1. Advantages and Disadvantages of BM HSCs and MSCs Therapy. BMSCs have a major advantage over stem cells from other organs: they are well defined, easy to isolate, and can be injected systemically reaching other tissues through the bloodstream. Thus they are more suitable than other kinds of stem cells for the therapeutic use. The advantage of BMSCs isolation compared to other types of ASCs (neuronal, heart, and kidney stem cells) resides in the properties of mobilization and homing. In fact BMSCs migrate from their BM niche to PB and then return to a new site in the BM. Presumably, some of the mechanisms that regulate stem cell trafficking are the same that regulate homing and lodging of BMHSCs during transplantations. The comparison between the clinical use of BMHSCs and BMMSCs shows a greater number of applications for the latter than the former. Although preclinical studies have demonstrated the plasticity of both BMHSCs and BMMSCs, the majority of clinical trials see the use of BMHSCs for the treatment of hematological malignancies in which the capacity of HSCs to reconstitute the hematopoietic system of the patient rather than the transdifferentiation potential is exploited. On the contrary BMMSCs applicability in therapy exploits their potential to differentiate into different cell types and this explains their increased use in the clinical trials of various diseases. However in the treatment of haematological diseases BMHSCs show many advantages compared to other sources of HSCs such as *Cord Blood Stem Cells* (CBSCs). It was learned that one umbilical cord contains an adequate number of HSCs for a successful engraftment only in low body weight patients (up to 40 kg) to reconstitute their immune system. The total number of cells, comprising hematopoietic progenitors, collected from one umbilical cord is significantly lower (roughly 5×10^6) than from BMSCs or from PB after BMSCs mobilization (roughly 1×10^8) [85]. Furthermore obese patients can be treated by BM transplants, as multiple units of cord blood are required [85]. BMSCs and BMSCs mobilized into the PB show also some disadvantages: they are mostly nondividing cells and have, respectively, 3 times and 6 times less repopulating cells than CBHSCs [85]. Another disadvantage of using autologous BMHSCs in cancer therapy is that cancer cells are sometimes inadvertently collected and reinfused back into the patients with the HSCs. One team of investigators finds that they can prevent reintroducing cancers cells by purifying cells and preserving only cells are CD34+, Thy-1^+ [86]. As BMHSCs, BMMSCs show minor proliferative capacity, life span, and differentiation potential compared to MSCs from birth-associated tissues such as placental andumbilical cord MSCs [57]. BMMSCs show an important advantage compared to BMHSCs: immunosuppressive properties. MSCs infusions in autologous or allogenic HSC transplantation could reduce the risk of graft failure and the incidence of acute *graft-versus-host disease* (GvHD) [81]. In fact MSCs have been shown to interact with many cell types of the immune system affecting both innate and adaptive immunity by inhibiting proliferation, differentiation as well as the function of monocytes, dendritic cells, NK cells, T cells and B cells. However it has been reported that MSCs may also act as non-professional antigen-presenting cells and that they express toll-like receptors and thus can respond to pathogen-associated molecules that stimulate immunoresponse. Thus the exact mechanisms how MSCs regulate the immune system are still not completely understood. However for their immunosoppressive properties, MSCs were found to help with tumor development *in vivo* promoting the development of a permissive stroma for the tumor, as was demonstrated in MM [81]. Another advantage of BMMSCs therapy is that they secrete many growth factors stimulating hematopoiesis, provide a scaffold for hematopoiesis, and support primitive progenitors cells *in vitro* [81]. Thus MSCs improve HSCs engraftment [81].

1.6.2. Potential Bottlenecks in BM HSC and MSCs Therapeutics. Although BMHSCs and BMMSCs belong to the most intensely studied stem cell types in cell therapy, comparison of existing preclinical and clinical data is hampered by a poor standardisation and harmonisation concerning protocols for isolation, expansion, and delivery.

As with BMHSCs, BMHSCs mobilized into PB contain a mixture of hematopoietic stem cells, progenitor cells, and

other kinds of cells. Consequently, the resulting cell preparation that is infused back into patients is not a pure HSCs preparation, but a mixture of HSCs, progenitors, and various contaminants, including T cells and in the case of autologous graft from cancer patients quite possible tumor cells as described previous. BMHSCs normally passed through a device that enriches cells that express CD34+, a marker of both stem and progenitor cells. The use of highly purified HSCs as graft is rare [87]. The main problem associated with clinical use of highly purified HSCs is the additional labor and costs involved in obtaining highly purified cells in sufficient quantities.

Efficient expansion of HSCs in culture remains one of the major goals despite their ability to self-renew. Attempts to expand HSCs in tissue culture with known stem-cell stimulators (growth factors and cytokines) have never resulted in a significant expansion of HSCs. Rather, these compounds induce many HSCs into cell division that are always accompanied by cellular differentiation [88]. Compared to HSCs, MSCs are strictly anchorage dependent and therefore need a surface to attach and proliferate. Simple ways for the cultivation of adherent cells in larger quantities are monolayer culture flasks such as roller bottles or multiple plate vessels. It has been shown that in static monolayer cultures MSCs proliferate slower and the differentiation potential is affected as well [89]. The use of a bioreactor is an alternative to the expansion in flasks. Bioreactors provide conditions similar to the *in vivo* situation of the cells, including advantages such as efficient nutrient supply, waste removal, minimal shear stress, and the possibility to control the cultivation via online measreuments of critical values [89].

Another bottleneck of stem cells therapeutics is the way of administration and the cells delivery. In transplants HSCs are generally infused intravenously. For MSCs the researchers have tried to optimize the delivery. In the treatment of cutaneous wounds, most studies have utilized the technically simple method of injecting a cell suspension intradermally into or around the wound defect; however the true therapeutic potential of MSCs appears to be limited due to poor engraftment efficiency and cell retention at the wound site. A fibrin spray system, to topically administer autologous MSCs to nonhealing lower extremity wounds, has been used in human subjects. Stem cells were found to survive within the fibrin layer and migrate into the wound tissue [79]. Hydrogels are synthetic biomaterials that emulate the hygroscopic nature of extracellular matrix making them an ideal vehicle for MSCs delivery [79]. A novel collagen-pullulan hydrogel that is noncytotoxic and provides protection from oxidative stress was recently described. MSCs seeded and cultured in this hydrogel significantly accelerated wound closure and improved quality of cutaneous regeneration when compared to intradermal injection strategies [79].

Previous examples demonstrate that questions needs to be answered in applying BMSCs in therapeutics, such as: (i) when to use pure BMSCs preparation in transplants; (ii) whether markers of HSCs, currently known, can distinguish them from their tumor counterparts; (iii) how to improve BMSCs expansion in culture without altering their stemness and differentiation potential; (iv) how to determine the most efficient method of administration of BMMSCs and how to optimize their delivery. Answering these questions will lead to a better standardization of methods and protocols used in the manipulation of BMSCs and to the overcoming of the most common bottlenecks in BM HSCs and MSCs therapeutics.

1.7. Conclusions and Future Perspectives. The therapeutic potential of BMSCs as powerful tools in tissue regeneration and engineering has been recognised, and intense efforts are ongoing to harness and direct HSCs and MSCs plasticity. However before HSCs and MSCs are currently used therapeutically in patients with degenerative disorders of the liver, heart, or brain, the properties of such cells must be well characterized, the functionality proved, and the potential risks of their use well defined. Understanding the molecular mechanisms underlying cell fate switching of BMSCs will be an essential contribution to ensuring their safe use in regenerative medicine. Moreover, even if the transdifferentiation events described in most of these studies were rare under physiological conditions, in the future, it will most likely be possible to transplant genetically modified stem cells carrying genes critical for transdifferentiation into desired cell populations. Finally pharmacologic molecules would also be used to directly influence the trans- or redifferentiation potential of ASCs, both prior and after their administration into patients.

Abbreviations

BMSCs: Bone marrow stem cells
HSC: Hematopoietic stem cell
MSC: Mesenchymal stem cell
CLP: Common lymphoid progenitor
CMP: Common myeloid progenitor
GMP: Granulocyte/macrophage progenitor
MEP: Megakaryocyte erythrocyte progenitor
T: T-lymphocyte
B: B-lymphocyte
MAC: Macrophage
G: Neutrophil granulocyte
EOS: Eosinophil
EB: Erythroblast
MEG: Megakaryocytes.

Authors' Contribution

I. Catacchio and S. Berardi contributed equally to this paper.

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