Stem Cells International

Advances and New Technologies Towards Clinical Application of Oral Stem Cells and Their Secretome

Guest Editors: Imad About, Athina Bakopoulou, George T.-J. Huang, Werner Geurtsen, and Mahmoud Rouabhia

Advances and New Technologies Towards Clinical Application of Oral Stem Cells and Their Secretome

Stem Cells International

Advances and New Technologies Towards Clinical Application of Oral Stem Cells and Their Secretome

Guest Editors: Imad About, Athina Bakopoulou, George T.-J. Huang, Werner Geurtsen, and Mahmoud Rouabhia

Editorial Board

Contents

Hindawi Publishing Corporation
Stem Cells International
Volume 2017, Article ID 6367375, 3 pages
http://dx.doi.org/10.1155/2017/6367375

Editorial

Advances and New Technologies towards Clinical Application of Oral Stem Cells and Their Secretome

Athina Bakopoulou,[1] George T.-J. Huang,[2] Mahmoud Rouabhia,[3] Werner Geurtsen,[4] and Imad About[5]

[1]*Department of Fixed Prosthesis and Implant Prosthodontics, School of Dentistry, Aristotle University of Thessaloniki, Thessaloniki, Greece*
[2]*University of Tennessee Health Science Center, Memphis, TN, USA*
[3]*Dental Faculty, University of Laval, Quebec, QC, Canada*
[4]*Hannover Medical School, Hannover, Germany*
[5]*Aix-Marseille University, CNRS, Institute of Movement Science (ISM), Marseille, France*

Correspondence should be addressed to Imad About; imad.about@univ-amu.fr

Received 19 December 2016; Accepted 22 December 2016; Published 24 January 2017

Oral and dental diseases are major public health problems worldwide with profound effects on quality of life. Recent advances in tissue engineering have raised significant hopes for innovative alternative therapies based on regeneration strategies for tissues in the whole craniofacial area. The discovery and subsequent characterization of stem cells (SCs) from various oral sources, in conjunction with the cutting edge basic research of recent years towards understanding their biology, have expanded and deepened our knowledge of the complex role of SCs in developmental and repair processes. The application of this knowledge in translational studies has been a decisive milestone in bringing these technologies closer to clinical applications. Amazing technological advances, such as bioprinting for fabricating customized biomimetic scaffolds and tissue-specific organoids, development of bioreactors for manipulating the stem cell niche, use of microfluidics for single-cell analysis, and many others, have provided valuable tools for translating scientific advances into clinical settings. The establishment of Good Manufacturing Practice (GMP) protocols according to European regulations has facilitated feasibility and "proof-of-concept" clinical trials on the application of oral SCs in orofacial tissue regeneration. Most recently, the possibility of using the plethora of secreted trophic and immunomodulatory cytokines produced by SCs (secretome) as a therapeutic surrogate module for cells has been proposed as a safer alternative to stem cell transplantation, raising the therapeutic dilemma: stem cell or secretome therapy?

In this special issue, 9 papers focusing on advances and new technologies towards understanding the activation, recruitment, and clinical application of oral SCs and their secretome have been selected after being peer-reviewed from a number of submissions. These articles encompass multiple components of craniofacial tissue engineering, from teeth to salivary gland regeneration and neurovascular engineering.

Some of the papers present novel aspects of the biological characteristics of different oral stem cell populations and their potential to regenerate specific tissues. In particular, K. M. F. El-Sayed and C. E. Dörfer have coauthored a review article about gingival mesenchymal stem/progenitor cells (G-MSCs) as a promising tool to regenerate oral tissues. Using pertinent selections from the literature, the authors have provided a succinct overview of the isolation of G-MSCs, their characterization, and regenerative capacity. Although the available experimental data suggest potential therapeutic applications of G-MSCs, further studies are essential before the clinical use of these specific SCs could become a reality. Recent data also indicate that MSCs isolated from gingival tissue and dental follicles of children may have a multilineage differentiation potential, making them of increasing interest

as an alternative source for oral SCs once the relevant gene expression pattern has been clarified. This led C.-M. Kang et al. to address this aspect in their study using 9 gingiva and 9 dental follicle biopsies from children. Their findings indicate that gingival tissue expressed more genes associated with keratinization, ectodermal development, and chemotaxis, while tissues from dental follicles showed a higher expression of genes involved in tooth and embryonic development. These results suggest that gingival tissue might be a good source for SCs to be used in regenerative dental procedures. In another innovative study, J. Xiong et al. investigated the cell surface proteome of human periodontal ligament stem cells (PDLSC) in comparison with other cell populations, such as human gingival fibroblasts and epithelial cells. In addition to the expression of well recognized MSC-associated cell surface antigens, such as CD73 and CD90, PDLSCs were also found to express two novel cell surface proteins: Annexin A2 and sphingosine kinase 1, which are not expressed in mature cells such as skin keratinocytes and play an important role in maintaining "stemness." These proteomic findings provide a basis for further defining the cell surface protein expression profiles of PDLSCs. This, in turn, would enable further characterization of this cell population to facilitate novel isolation and purification strategies allowing their application in oral tissue engineering.

Other papers in this special edition focus on the neurogenic and/or neurovascular properties of dental stem cells, highlighting their importance in dental/orofacial tissue engineering. The neurogenic potential of dental pulp stem cells (DPSCs) has attracted intense interest as these cells spontaneously express neural markers in cultures. They have shown ability to differentiate into glial cells and functional neurons under specific conditions, even though different reports have been inconsistent indicating the need for further investigation and characterization of DPSC neurogenic potential. This special issue also included two articles investigating DPSCs for their neurogenic potential: one by F. I. Young et al. demonstrating for the first time the ability of single cell-derived clonal cultures of murine DPSCs (high nestin-expressing clone) to differentiate into immature neuron-like and oligodendrocyte-like cells in vitro. The membrane capacitance of the DPSC-derived neuron-like cells resembled that of cultured primary striatal neurons. The other article by J. Jung et al. demonstrated the use of xeno/serum-free condition to culture human DPSCs at early stage after DPSC isolation. The xeno/serum-free cultured DPSCs showed expression of PAX6, an important marker for cells committing to neural lineages. These cells also survived after transplantation into normal rat brain and injured spinal cord. The two articles indicate the importance of clonal selection and culture conditions on the neurogenic potential of DPSCs. In addition to these research articles, a review paper by J. Ratajczak et al. focuses on the neurovascular properties of dental stem cells (DPSCs, SHEDs, SCAPs, FSCs, and PDLSCs) and their importance in the vascularization and innervation that secures the viability of regenerated tissues in dental/orofacial tissue engineering. These authors provide a systematic analysis of the numerous extant studies on the ability of dental SCs to differentiate into endothelial cells and neural cell types, as well as on the angiogenic, neuroprotective, and neurotrophic effects of their secretome.

Innovative three-dimensional bioprinting nanotechnologies for SCs and their secretome have opened up previously unimaginable vistas for clinical application. J. N. Ferreira et al. have reviewed these exciting advances by focusing on salivary gland (SG) regeneration. This alleviates a wide range of medical conditions, from autoimmune to metabolic disorders, as well as damage after radiotherapy to treat specific head and neck cancers which lead to a poor quality of life due to xerostomia (dry mouth). These technologies are based on an ex vivo generation of organotypic cultures and SG organoids/miniglands using coculture systems to integrate the different SG cellular/tissue components, such as epithelial acinar and ductal cells, myoepithelial cells, and the networks of parasympathetic nerves, as well as ducts and vessels. This research improves our understanding of the properties of SC secretome and its potential use for SG regeneration.

Current research leading to the development of clinical-grade oral SCs and the application of GMP conditions, an essential step in the clinical application of dental SCs and their secretome, are concisely reviewed in another article coauthored by A. Bakopoulou and I. About. The salient biological properties of dental MSCs, critical for the translational pathway "from bench to clinic," are presented. The capacity of these cells to reconstitute various dental and nondental tissues and the inherent osteo-/odontogenic, angiogenic, neurogenic, and immunomodulatory properties of their secretome are systematically described. Finally, key milestone achievements exemplifying their clinical utility in regenerative dentistry and initial data on well-designed clinical trials are discussed for the first time.

An original long-term clinical trial by S. Baba et al. provides novel data of phase I/II clinical trial on transplantation of autologous bone marrow SCs embedded into a 3D woven-fabric scaffold made of biodegradable poly-L-lactic acid resin fibers for treatment of chronic periodontitis. After SCs implantation into periodontal intrabony defects, a 36-month follow-up showed significant improvement of attachment level, pocket depth, and linear bone growth, providing solid clinical evidence for the application of SCs into the regeneration of oral tissues.

To conclude, this special issue highlights and encapsulates the amazing advances in knowledge and technologies regarding oral stem cell biology and tissue engineering. The aim has been to summarize the "state of the art" and consolidate the clinical utility of oral MSCs and their secretome in oral/craniofacial tissue regeneration. These innovative scientific data significantly improve our understanding of the potential risks involved in the use of these technologies, thus spurring efforts to surmount emerging problems and create a viable therapeutic option for the patient, the putative ultimate beneficiary of this groundbreaking technology.

Acknowledgments

We would like to thank all researchers, scientists, and clinicians who have contributed their important scientific work

to this issue, which we hope will serve as a springboard for further scientific developments in the promising field of regenerative dentistry.

Athina Bakopoulou
George T.-J. Huang
Mahmoud Rouabhia
Werner Geurtsen
Imad About

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 7564689, 9 pages
http://dx.doi.org/10.1155/2016/7564689

Review Article

Three-Dimensional Bioprinting Nanotechnologies towards Clinical Application of Stem Cells and Their Secretome in Salivary Gland Regeneration

Joao N. Ferreira,[1,2] Sasitorn Rungarunlert,[3] Ganokon Urkasemsin,[3] Christabella Adine,[1] and Glauco R. Souza[4,5]

[1] *Department of Oral & Maxillofacial Surgery, Faculty of Dentistry, National University of Singapore, Singapore*
[2] *National Institute of Dental and Craniofacial Research, National Institutes of Health, Bethesda, MD, USA*
[3] *Department of Preclinical and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, Nakhon Pathom, Thailand*
[4] *The University of Texas Health Science Center at Houston, Houston, TX, USA*
[5] *Nano3D Biosciences (n3D), Houston, TX, USA*

Correspondence should be addressed to Joao N. Ferreira; denjnarf@nus.edu.sg

Received 1 October 2016; Accepted 23 November 2016

Academic Editor: Athina Bakopoulou

Salivary gland (SG) functional damage and severe dry mouth (or xerostomia) are commonly observed in a wide range of medical conditions from autoimmune to metabolic disorders as well as after radiotherapy to treat specific head and neck cancers. No effective therapy has been developed to completely restore the SG functional damage on the long-term and reverse the poor quality of life of xerostomia patients. Cell- and secretome-based strategies are currently being tested in vitro and in vivo for the repair and/or regeneration of the damaged SG using (1) epithelial SG stem/progenitor cells from salispheres or explant cultures as well as (2) nonepithelial stem cell types and/or their bioactive secretome. These strategies will be the focus of our review. Herein, innovative 3D bioprinting nanotechnologies for the generation of organotypic cultures and SG organoids/mini-glands will also be discussed. These bioprinting technologies will allow researchers to analyze the secretome components and extracellular matrix production, as well as their biofunctional effects in 3D mini-glands ex vivo. Improving our understanding of the SG secretome is critical to develop effective secretome-based therapies towards the regeneration and/or repair of all SG compartments for proper restoration of saliva secretion and flow into the oral cavity.

1. Introduction

Irreversible salivary gland (SG) damage and dry mouth (or xerostomia) are commonly present in a vast range of systemic conditions (e.g., Sjögren's syndrome, uncontrolled diabetes, and thyroid disease), and it is particularly severe after radiotherapy (RT) for head and neck cancers (HNC) [1]. On an annual basis, about 500,000 new cases of HNC develop worldwide for whom xerostomia-induced RT is the main treatment modality. Saliva secretions are essential for digestion, lubrication, oral homeostasis, and protection against a variety of environmental hazards. Hence, xerostomia can cause various life disrupting side effects such as oral infections, pain, and tooth loss. These side effects will impair daily activities related to taste perception, speech, mastication, and swallowing [2]. Salivary secretion has partial improvements after novel modalities, such as SG sparing or intensity-modulated radiation therapy, are utilized [2–4]. Despite these recent efforts, about 40% of dry mouth cases are still irreversible. When the radiation field (during RT) lays on the SG, radiation damage is elicited on the secretory epithelial cell compartment, blood vessels, and adjacent nerves [5, 6]. Following RT, patients lose the majority of acinar epithelial cells (about 80% of total epithelial cells) with the surviving secretory cells being primarily ductal; consequently, RT will irreversibly impact salivary secretion and cause inflammatory damage and fibrosis on the long-term. This radiation damage further depletes the SG stem/progenitor cell niche

deterring healing and natural gland regeneration [5, 7–9]. Yet, no effective therapy has been devised to treat RT-induced xerostomia, and current treatment strategies are confined to the minimization of SG radiation damage or to the administration of artificial saliva substitutes and stimulators of saliva secretion (e.g., pilocarpine) [2, 5].

Radiation-induced xerostomia can be an irreversible lifelong condition that can significantly affect the quality of life of HNC patients. Thus, novel and effective therapeutical strategies for SG hypofunction are required [10]. Due to the depletion of the self-renewable progenitor/stem cell pool during RT damage, cell-based therapies are essential not only to generate new saliva-secreting tissues [10–13] but also to potentially repair the damaged SG via the production and extracellular release of bioactive secretory proteins by transplanted cells [14–17]. This group of non-membrane-bound secretory proteins has been named the salivary secretome [18]. According to the human secretome atlas, salivary glands produce the most abundant proteins found in the human body [18]. Important cellular differences exist within the three major salivary glands (parotid, submandibular, and sublingual), mostly in the ratio of serous to mucous epithelial acinar cells and potentially in their pool of progenitor/stem cells. Despite these differences, researchers mainly focused their secretome-based and SG regenerative studies with 3D systems on either the submandibular or the parotid glands. The salivary secretome produced by different stem/progenitor cells will be discussed in the next sections since it could transform the way we restore the salivary flow in patients with xerostomia in the near future.

2. Salivary Stem/Progenitor Cells and Their Secretome

The first proof of concept study on transplantation of autologous SG cells to rescue salivary hypofunction using in vitro floating spheroid-like cultures of mouse SG progenitor cells, named salispheres. In vitro salisphere cultures have been shown to enrich SG stem/progenitor cell populations that include KIT (C-KIT, CD117), Sca-1, and Mushashi-1 [11]. KIT-expressing (KIT+) progenitors are also found in other epithelial organs beside the SG, such as the prostate gland and lungs, where KIT+ progenitors have remarkable regeneration capabilities [20, 21]. In a salisphere study in mice, 100–300 KIT+ donor-derived cells isolated from the salisphere cultures were sufficient to form both new acini and saliva-transporting ductal structures, restoring the morphology and function of irradiated SG. Since human salispheres do contain KIT+ cells, there is a potential for future clinical use of KIT+ cell subpopulations [22]. Recently, Pringle and others [13] have successfully transplanted human salispheres into irradiated mice restoring the salivary flow, particularly when these salispheres were positively selected for KIT. However, the subpopulation of KIT+ cells in human SGs is very limited being less than 0.4% of the total population in younger adults, and this number substantially decreases with aging [13]. Moreover, these salispheres have a restricted in vitro self-renewal and proliferative capacities that confines their growth to 2-3 population doublings at earlier passages (P1–P4) [13].

Table 1: List of secretome components (matrix peptides, cytokines, growth factors, and enzymes) from SG cell lines that can be potentially used in SG regeneration strategies. More details about each secretome component can be found in [18, 23]. ALDH3: aldehyde dehydrogenase 3; EDA: ectodysplasin A; EGF: epidermal growth factor; FGF: fibroblast growth factor; IGF: insulin growth factor; IL: interleukin; SHH: sonic hedgehog; SCF: stem cell factor.

Secretome components	References
ALDH3 activator	[24]
EDA	[25]
EGF	[26]
FGF2	[27]
FGF7	[28, 29]
FGF10	[29–31]
Heparan sulfate	[31, 32]
IGF1	[33]
IL-6	[34]
SHH	[35]
SCF	[32]
Wnt	[36–38]

Thus, it is crucial to understand how progenitors proliferate and expand particularly during organogenesis. Several researcher groups have demonstrated that KIT and fibroblast growth factor receptor 2b (FGFR2b) signaling are essential for progenitor survival and expansion in the fetal submandibular gland, lung, pancreas, tooth, and skin [39–41]. Moreover, other putative markers can be used to isolate SG stem/progenitor cells including KRT5 (Cytokeratin 5), CD49f, CD29 (Itga1), CD133 (Prom1), Sca1, CD44, CD34, CD90 (Thy1), CD105, CD9, and CD81, but only few populations were proven to actively restore damaged glands [11, 42–45]. Yet, the KIT+ cell population still appears to have the highest stem/progenitor-like potential.

Research efforts have been made to increase the number of KIT+ cells ex vivo using growth factors [32] or to administer secretome factors to reverse SG damage in vivo [60]. Several secretome components have been studied including specific heparan sulfate peptides [32] and several growth factors and cytokines (see Table 1 for a complete list). The majority of these secretome components (EGF, IGF1, FGF2 [26, 27, 33], FGF7 (or KGF) [28], IL-6 [34], ALDH3 [24], or EDA activators [25]) have similar cellular downstream effects such as the reduction in cell apoptosis and/or the promotion of epithelial proliferation. These secretome-based strategies could be advantageous, although the absolute cell number required for functional regeneration of the human SG is still unknown. Instead, non-SG cells may be considered to curb this constraint.

Taken together, multiple research groups have shown that rodent SG-specific epithelial cell transplantation is a feasible approach to repair irradiated SGs. Future studies will determine whether human SG cells behave in a similar manner in ex vivo and in vivo assays [13]. While success has been achieved with epithelial KIT+ cells in rodents,

Table 2: In vivo and in vitro tested oral stem cell lines for salivary gland regeneration. SG: salivary gland, BM: bone marrow, MSC: mesenchymal stem cells, ESC: embryonic stem cells, and iPSC: induced-pluripotent stem cells.

Tested cell sources	Origin (species)	References
Major SG progenitor/stem cells	Mouse, rat, human	[46–48]
Minor SG epithelial cells	Human	[49]
BM-derived stem cells	Human	[50, 51]
BM-derived MSC	Human	[14, 52]
Adipose-derived MSC	Human	[53, 54]
Minor SG-derived MSC-like cells	Human	[55]
Amniotic epithelial cells	Human	[56, 57]
ESC	Mouse	[58]
iPSC	Mouse	[59]

currently, other more multipotent stem/progenitor cell candidates and/or compartment reservoir cells can be investigated (e.g., cytokeratin 14) [61]. Despite this, in clinical scenarios where autologous SG cell numbers are reduced, we may need to take advantage of the regenerative capacity of non-SG stem cells, nonepithelial cells (e.g., bone marrow-derived), or simply their secretome. These potential therapeutical options are reviewed in the following section.

3. Nonsalivary Gland Cells and Their Secretome

There are a vast number of reports on the advantageous effects of non-SG stem cells and their secretome to regenerate irradiated SGs (see Tables 2 and 3). These reports include several types of stem cells such as bone marrow- (BM-) derived cells [63, 64], BM-derived mesenchymal stem cells (MSCs) [14, 52], human adipose-derived MSCs [53, 54], SG-derived MSC-like cells [55], amniotic cells [56, 57], embryonic stem cells (ESC) [58], and induced-pluripotent stem cells (iPSC) [59].

Recently, BM-derived transplants using either mesenchymal stem cells (MSC) or BM secretome (also named "soup" or "bioactive lysates") have been shown to induce paracrine prosurvival effects on remaining SG tissues towards a more functional SG tissue architecture [14, 15]. When intraglandular transplantation of BM cells and their secretome was implemented, the outcomes in irradiated mouse SG were promising; and those included an improvement in saliva production, reduction in apoptosis, and changes in microvessel density [15]. Earlier studies in mouse irradiated SG had similar functional outcomes, when BM-derived cells were mobilized by G-CSF/FLT3/SCF [50, 62]. The clinical translation of these cellular paracrine effects led investigators to identify such bioactive secretome components secreted by BM-derived cells [15, 16]. Protein microarrays detected several angiogenesis-related factors (CD26, FGF1, HGF, MMP-8, MMP-9, OPN, PF4, and SDF-1) and cytokines (IL-1ra, IL-16) in the BM secretome (Table 3) [16]; thereby, several signaling pathways may be involved and the contribution of each secretome component towards epithelial repair and SG regeneration requires further investigation.

Table 3: List of secretome components (cytokines, growth factors, and proteinases) from adult stem cells (e.g., bone marrow-derived stem cells and adipose mesenchymal stem cells) that can be potentially used in SG regeneration strategies. More details about each secretome component can be found in [18, 23]. FGF: fibroblast growth factor; FLT3: Fms related tyrosine kinase 3; G-CSF: granulocyte-colony stimulating factor; GM-CSF: granulocyte macrophage-colony stimulating factor; HGF: hepatocyte growth factor; IGF: insulin growth factor; IL: interleukin; MMP: matrix metalloproteinase; OPN: osteopontin; PF4: platelet factor 4; SCF: stem cell factor; SDF1: stromal cell derived factor-1; VEGF: vascular endothelial growth factor.

Secretome components	References
CD26	[16]
FGF1	[16]
FLT-3	[62]
G-CSF	[62]
GM-CSF	[17]
HGF	[16]
IGF-1	[17]
IL-1ra	[16]
IL-6	[17]
IL-16	[16]
MMP8	[16]
MMP9	[16]
OPN	[16]
PF4	[16]
SCF	[62]
SDF1	[16]
VEGF	[17]

Despite tentative differentiation of BM-derived cells and MSCs into SG acinar cells in vitro, their actual contribution to epithelial differentiation in vitro and in vivo is puzzling. Highly homogenous BM clonal MSC (BM-cMSC) has recently shown potential to regenerate SGs, although the current mechanisms of regeneration are not well understood [14]. In addition, an in vitro study using BM stem cells (BMSCs) cocultured with neonatal rat parotid acinar cells

showed an increase in the induction of acinar-specific α-amylase expression in BMSCs [51]. This coculture scenario with mesenchymal and epithelial stem/progenitor cells can be an interesting therapeutical approach when used in combination with relevant secretome factors. Further studies are still needed to test the secretory function of these acinar-like cells from bone marrow sources. As somewhat expected, both BM-MSC and mesenchymal-like cells derived from SG can suppress the immune system [65].

Interestingly, researchers have also looked at adipose sources of stem cells. Human adipose-derived mesenchymal stem cells (hAdMSCs) via systemic administration exhibit improved salivary flow rates 4 months after radiation therapy [54]. Glands with hAdMSC transplants showed lesser epithelial acinar apoptosis and tissue fibrosis and higher secretory mucin and amylase levels. At 4 weeks, a large number of infused hAdMSCs were detected in vivo and were found to have differentiated [54]. Moreover, the secretome from hypoxia-preconditioned hAdMSC comprised high levels of GM-CSF, VEGF, IL-6, and IGF-1 (Table 3) [17]. This hAdMSC secretome strongly induced epithelial proliferation and exerted antiapoptotic effects in the SG in vivo. A common finding across these adult stem cell secretome studies is the presence of secretome-based paracrine effects to reduce radiation-induced epithelial apoptosis, proliferate the host SG progenitor cells, and induce angiogenesis.

The known components of the secretome derived from adult stem cells are summarized in Table 3 since they are multiple. The antiapoptotic, proproliferative, and proangiogenesis cues found in the secretome can support not only the repair of the epithelial cells but also the microenvironment [17]. However, the following question can be posed: could the secretome strategy be a successful therapy in every patient, particularly for the patients without any remaining SG cells left after radiotherapy? The secretome strategy like the current ones involving salivary stimulation (e.g., stimulation with oral pilocarpine tablets) relies on the amount of remaining SG cells; thus, clinical outcomes will depend on the remaining cells that need paracrine stimulation.

While proangiogenesis factors have been reported in certain secretomes, it is not known yet whether neurotrophic factors are present [66]. Parasympathetic neurons are known to support epithelial regeneration after RT [43, 60]. Neurotrophic factors such as neurturin (NRTN) or glial cell-derived neurotrophic factor (GDNF) are currently being tested to revert the hypofunctional status of irradiated SGs [43, 60].

Other pluripotent cell types such as ESC and iPSC have recently been investigated as new cell sources to generate mature salivary gland cells [58, 59]. A study with mouse ESCs cocultured with human SG-derived fibroblast has provided (to ESCs) the cues to express SG-specific markers and to reconstitute SG structures; however, it is still unclear whether SG function can be restored [58]. Both ESC- [58] and iPS-derived SG cells [59] have the potential to be an adjuvant cell-based therapy as long as properties such as genomic stability and lack of tumorigenesis are secured at transplantation.

Nonetheless, in clinical scenarios where whole new SG organs or mini-glands are necessary for in vivo transplantation, three-dimensional (3D) SG in vitro culture systems (with or without bioscaffolds) are required to integrate multiple cell lines (under specific growth factor conditions) for the generation of all gland compartments (acinar and ductal epithelial, myoepithelial, endothelial/vascular, and neuronal).

4. Generating Salivary Gland Organoids/Organs and the Role of 3D Bioprinting

A recent breakthrough in the field of SG whole organ regeneration showed that a bioengineered gland made from fetal epithelium and mesenchyme can be transplanted into an adult mouse to form a new whole functional gland in the adult microenvironment [67]. This bioengineered gland contained a variety of embryonic cells, including progenitors of epithelial, mesenchymal, endothelial, and neuronal cells. Importantly, the gland reconnected with the existing ductal system and was functional in terms of saliva secretion, protection of the oral cavity from bacteria, and restoration of normal swallowing. Thus, this concept may lead to the creation of new surgical techniques for the prompt implantation of ex vivo SG organs to integrate with the existing circulatory and nervous system structures and align endogenous salivary ductal structures. However, this mouse model system may not fully translate into clinics due to the use of fetal glands. Thus, this major advance prompted researchers to develop 3D organotypic cultures to produce SG organoids or mini-glands that can recapitulate the in vivo native environment and SG morphology and architecture [10].

As a result, novel 3D bioprinting nanotechnologies have been recently developed using magnetic patterning or levitation, in which cells bind with a magnetic nanoparticle assembly overnight to render them magnetic [19]. These bioprinting systems are time efficient as they require less than 24 hours of working time to assemble cells in 3D, depending on the cell type and number of magnetic nanoparticles used (Figures 1 and 2(a)) [68, 69]. Their magnetic nanoparticle assembly includes gold, iron oxide, and poly-L-lysine, which can easily tag different cell types at the plasma membrane level. When resuspended in medium, an external magnetic field levitates and can concentrate different SG cells at the air-liquid interface, where they aggregate to form larger 3D organoids (Figures 1 and 2(a)). The resulting dense cultures can synthesize extracellular matrix and can be analyzed similarly to other 2D/3D culture systems, using assays/techniques such as cytotoxicity assays, immunohistochemical analysis, western blotting, and other biochemical assays [70]. These 3D bioprinted systems have been previously found to recapitulate the native extracellular matrix from several tissues such as fat, lung, aortic valve, blood vessels, and breast and glioblastoma tumors [19, 68, 69, 71–74].

These magnetic-based bioprinting strategies are an avenue that we are currently exploring since their biocompatibility is comparable to conventional 3D systems using

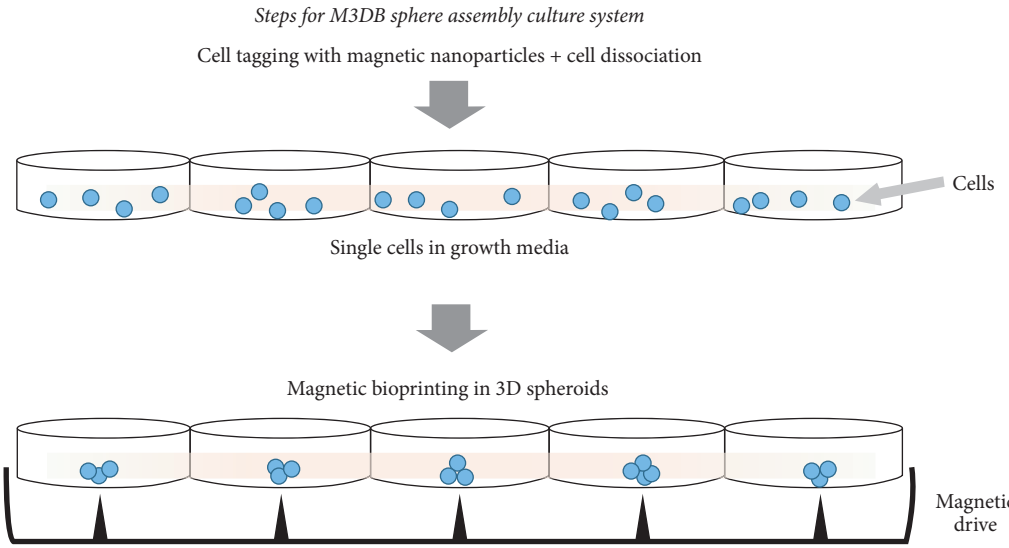


Figure 1: Diagram showing magnetic 3D bioprinting (M3DB) sphere assembly culture system by magnetic force driven patterning of tagged cells [19].

centrifugation-based force aggregation (Figure 2(b)). These bioprinting cell assembly systems can integrate all human SG cellular compartments (acinar/ductal epithelial, myoepithelial, endothelial, and neuronal) into organotypic cultures. More interestingly, these 3D bioprinting systems have been tested in cultures with oral stem cells such as human dental pulp stem cells (hDPSC) in combination with secretome components (e.g. FGF-10) and have shown to produce α-amylase-secreting cells (Figure 2(c)). However, the polarity in these secretory epithelial cells still needs to be evaluated.

During the development of the SG organoid, the creation of the apicobasal polarity in epithelial cells and of branched lumenized ducts is paramount to achieve a proper directionality for the salivary flow and production of saliva. These epithelial polarity properties of the SG organoids or mini-glands have been difficult to achieve [75]. However, these bioprinting strategies have shown promise when applied in in vivo rodent models using magnets [76]. In this particular in vivo study, the magnetized stem cells were biocompatible and successfully targeted a locally damaged neuronal tissue restoring its function.

Taken together, these innovative magnetic-based 3D bioprinting strategies are relevant in the SG regeneration field because they may (1) first generate scaled-up xeno-free biocompatible 3D tissue compartments that provide an architecture with environmental cues to support cell growth, differentiation, and biointegration in the remaining tissues (after damage) to restore homeostasis and functionality; (2) secondly they may establish coculture methods to generate SG cell-derived secretome, matrices, and tissue compartments on a scaled-up manner. These cocultures will allow researchers to integrate, in a 3D architecture, the complexity of different human SG component; and (3) lastly test new surgical techniques using magnetic fields in vivo to promptly implant and hold/stabilize magnetized SG organoids/mini-glands onto the injury site [76].

5. Future Directions

There has been a research trend towards the development of secretome-based therapeutical strategies to repair and/or restore salivary glands (SG) damaged by radiotherapy. These strategies have been relatively successful in rodent models for the clinical scenarios where the majority of SG cells and tissue compartments still remain. Nonetheless, when a patient needs a whole new SG, organotypic 3D cell culture systems are required to generate robust 3D organoids or mini-glands ex vivo for proper acinar epithelial stimulation, saliva production, and release into the oral cavity. These 3D mini-glands can be established using coculture systems to integrate in 3D the complexity of the different SG cellular/tissue components, such as epithelial acinar and ductal cells, myoepithelial cells, the networks of parasympathetic nerves, and lumenized ducts and vessels. For this purpose, novel 3D bioprinting approaches have been developed to assemble all the above SG cells in coculture and produce 3D tissue compartments and ductal structures that resemble mini-SG.

In summary, secretome-based and 3D organotypic cell-based strategies will certainly become the next generation of biomedical therapies to either repair a damaged SG or to develop an in vitro SG organoid/mini-gland for transplantation in humans suffering from xerostomia.

Competing Interests

All authors have nothing to disclose and have no financial interests, except for Dr. Glauco Souza. Dr. Glauco Souza is the

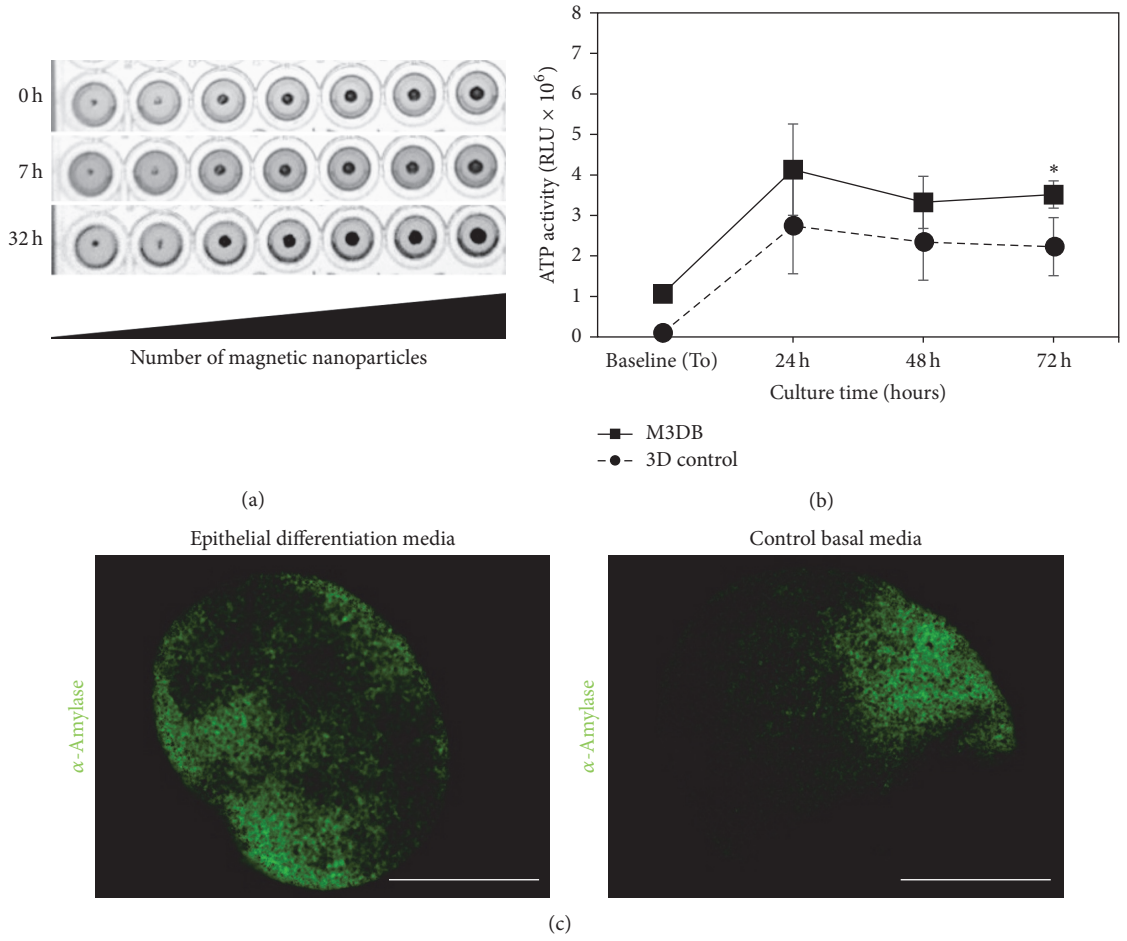


Figure 2: Morphology and viability of the M3DB spheroid-like organoids after 3D bioprinting of human dental pulp stem cell (hDPSC) cultures in a 96-well plate. (a) Morphology of the M3DB spheroids after 7 h and 32 h of culture of 3×10^5 hDPSC using increased concentration of magnetic nanoparticles for cellular tagging and magnetization. (b) ATP activity of M3DB compared to a conventional 3D system (3D control) from baseline to 72 hours after seeding 1×10^5 hDPSC at baseline (time 0 h). ATP activity was measured by a luciferase ATP-based 3D assay (CellTiter-Glo 3D Cell Viability Assay, Promega, USA) with a Glomax luminometer (RLU: raw luminescent units); significant difference found between the two culture systems (M3DB and 3D control) at 72 h ($^* p = 0.0286$); $N = 4$-5; Two-tailed t-test. (c) Organoids expressing α-amylase salivary protein after epithelial differentiation (GlutaMAX basal media with FGF-10 40 ng, Gibco) of hDPSC for 14 days. Organoids were processed for whole mount immunofluorescence staining with α-amylase primary antibody and Alexa Fluor® 488 (green) followed by confocal fluorescence microscopy. Images are a maximum intensity projection of a z-stack of images taken through the entire organoid thickness (magnification: 10x; scale bar: 250 μm).

President (CEO) and Chief Scientific Officer of the company "Nano3D Biosciences Inc.," Houston, TX, USA; and he is affiliated as an Adjunct Assistant Professor at The University of Texas Health Science Center at Houston, Texas, USA.

Acknowledgments

Our research studies are currently funded by the National University of Singapore ODPRT start up grant (R-221-000-072-133), by the Singapore's National Medical Research Council grant (CNIG14NOV008), and by Mahidol University.

References

[1] I. von Bültzingslöwen, T. P. Sollecito, P. C. Fox et al., "Salivary dysfunction associated with systemic diseases: systematic review and clinical management recommendations," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology and Endodontology*, vol. 103, supplement, pp. S57.e1–S57.e15, 2007.

[2] O. B. Wijers, P. C. Levendag, M. M. J. Braaksma, M. Boonzaaijer, L. L. Visch, and P. I. M. Schmitz, "Patients with head and neck cancer cured by radiation therapy: a survey of the dry mouth syndrome in long-term survivors," *Head & Neck*, vol. 24, no. 8, pp. 737–747, 2002.

[3] S. B. Jensen, A. M. L. Pedersen, A. Vissink et al., "A systematic review of salivary gland hypofunction and xerostomia induced

by cancer therapies: management strategies and economic impact," *Supportive Care in Cancer*, vol. 18, no. 8, pp. 1061–1079, 2010.

[4] C. M. Nutting, J. P. Morden, K. J. Harrington et al., "Parotid-sparing intensity modulated versus conventional radiotherapy in head and neck cancer (PARSPORT): a phase 3 multicentre randomised controlled trial," *The Lancet Oncology*, vol. 12, no. 2, pp. 127–136, 2011.

[5] A. Vissink, J. B. Mitchell, B. J. Baum et al., "Clinical management of salivary gland hypofunction and xerostomia in head-and-neck cancer patients: successes and barriers," *International Journal of Radiation Oncology Biology Physics*, vol. 78, no. 4, pp. 983–991, 2010.

[6] O. Grundmann, G. C. Mitchell, and K. H. Limesand, "Sensitivity of salivary glands to radiation: from animal models to therapies," *Journal of Dental Research*, vol. 88, no. 10, pp. 894–903, 2009.

[7] B. J. Baum, "Principles of saliva secretion," *Annals of the New York Academy of Sciences*, vol. 694, pp. 17–23, 1993.

[8] B. J. Baum, C. Zheng, I. Alevizos et al., "Development of a gene transfer-based treatment for radiation-induced salivary hypofunction," *Oral Oncology*, vol. 46, no. 1, pp. 4–8, 2010.

[9] I. M. Lombaert and M. P. Hoffman, "Stem cells in salivary gland development and regeneration," in *Stem Cells in Craniofacial Development and Regeneration*, G.T.-J. Huang and I. Thesleff, Eds., pp. 271–284, John Wiley & Sons, New York, NY, USA, 2013.

[10] I. Lombaert, M. M. Movahednia, C. Adine, and J. N. Ferreira, "Salivary gland regeneration: therapeutic approaches from stem cells to tissue organoids," *Stem Cells*, 2016.

[11] I. M. A. Lombaert, J. F. Brunsting, P. K. Weirenga et al., "Rescue of salivary gland function after stem cell transplantation in irradiated glands," *PLoS ONE*, vol. 3, no. 4, Article ID e2063, 2008.

[12] T. Sugito, H. Kagami, K. Hata, H. Nishiguchi, and M. Ueda, "Transplantation of cultured salivary gland cells into an atrophic salivary gland," *Cell Transplantation*, vol. 13, no. 6, pp. 691–699, 2004.

[13] S. Pringle, M. Maimets, M. van der Zwaag et al., "Human salivary gland stem cells functionally restore radiation damaged salivary glands," *Stem Cells*, vol. 34, no. 3, pp. 640–652, 2016.

[14] J.-Y. Lim, T. Yi, J.-S. Choi et al., "Intraglandular transplantation of bone marrow-derived clonal mesenchymal stem cells for amelioration of post-irradiation salivary gland damage," *Oral Oncology*, vol. 49, no. 2, pp. 136–143, 2013.

[15] S. D. Tran, Y. Liu, D. Xia et al., "Paracrine effects of bone marrow soup restore organ function, regeneration, and repair in salivary glands damaged by irradiation," *PLoS ONE*, vol. 8, no. 4, Article ID e61632, 2013.

[16] D. Fang, S. Hu, Y. Liu, V.-H. Quan, J. Seuntjens, and S. D. Tran, "Identification of the active components in Bone Marrow Soup: a mitigator against irradiation-injury to salivary glands," *Scientific Reports*, vol. 5, Article ID 16017, 2015.

[17] H.-Y. An, H.-S. Shin, J.-S. Choi, H. J. Kim, J.-Y. Lim, and Y.-M. Kim, "Adipose mesenchymal stem cell secretome modulated in hypoxia for remodeling of radiation-induced salivary gland damage," *PLoS ONE*, vol. 10, no. 11, Article ID e0141862, 2015.

[18] M. Uhlén, "Proteomics. Tissue-based map of the human proteome," *Science*, vol. 347, no. 6220, Article ID 1260419, 2015.

[19] W. L. Haisler, D. M. Timm, J. A. Gage, H. Tseng, T. C. Killian, and G. R. Souza, "Three-dimensional cell culturing by magnetic levitation," *Nature Protocols*, vol. 8, no. 10, pp. 1940–1949, 2013.

[20] K. G. Leong, B.-E. Wang, L. Johnson, and W.-Q. Gao, "Generation of a prostate from a single adult stem cell," *Nature*, vol. 456, no. 7223, pp. 804–810, 2008.

[21] J. Kajstura, M. Rota, S. R. Hall et al., "Evidence for human lung stem cells," *The New England Journal of Medicine*, vol. 364, no. 19, pp. 1795–1806, 2011.

[22] J. Feng, M. van der Zwaag, M. A. Stokman, R. van Os, and R. P. Coppes, "Isolation and characterization of human salivary gland cells for stem cell transplantation to reduce radiation-induced hyposalivation," *Radiotherapy and Oncology*, vol. 92, no. 3, pp. 466–471, 2009.

[23] K. Musselmann, J. A. Green, K. Sone et al., "Salivary gland gene expression atlas identifies a new regulator of branching morphogenesis," *Journal of Dental Research*, vol. 90, no. 9, pp. 1078–1084, 2011.

[24] N. Xiao, H. Cao, C.-H. Chen et al., "A novel aldehyde dehydrogenase-3 activator (Alda-89) protects submandibular gland function from irradiation without accelerating tumor growth," *Clinical Cancer Research*, vol. 19, no. 16, pp. 4455–4464, 2013.

[25] G. Hill, D. Headon, Z. I. Harris, K. Huttner, and K. H. Limesand, "Pharmacological activation of the EDA/EDAR signaling pathway restores salivary gland function following radiation-induced damage," *PLoS ONE*, vol. 9, no. 11, Article ID 112840, 2014.

[26] B. Ohlsson, C. Jansen, I. Ihse, and J. Axelson, "Epidermal growth factor induces cell proliferation in mouse pancreas and salivary glands," *Pancreas*, vol. 14, no. 1, pp. 94–98, 1997.

[27] T. Kojima, S.-I. Kanemaru, S. Hirano et al., "The protective efficacy of basic fibroblast growth factor in radiation-induced salivary gland dysfunction in mice," *The Laryngoscope*, vol. 121, no. 9, pp. 1870–1875, 2011.

[28] I. M. A. Lombaert, J. F. Brunsting, P. K. Wierenga, H. H. Kampinga, G. De Haan, and R. P. Coppes, "Keratinocyte growth factor prevents radiation damage to salivary glands by expansion of the stem/progenitor pool," *STEM CELLS*, vol. 26, no. 10, pp. 2595–2601, 2008.

[29] H. P. Makarenkova, M. P. Hoffman, A. Beenken et al., "Differential interactions of FGFs with heparan sulfate control gradient formation and branching morphogenesis," *Science Signaling*, vol. 2, no. 88, p. ra55, 2009.

[30] V. N. Patel, S. M. Knox, K. M. Likar et al., "Heparanase cleavage of perlecan heparan sulfate modulates FGF10 activity during ex vivo submandibular gland branching morphogenesis," *Development*, vol. 134, no. 23, pp. 4177–4186, 2007.

[31] V. N. Patel, K. M. Likar, S. Zisman-Rozen et al., "Specific heparan sulfate structures modulate FGF10-mediated submandibular gland epithelial morphogenesis and differentiation," *Journal of Biological Chemistry*, vol. 283, no. 14, pp. 9308–9317, 2008.

[32] V. N. Patel, I. M. A. Lombaert, S. N. Cowherd et al., "Hs3st3-modified heparan sulfate controls KIT+ progenitor expansion by regulating 3-O-sulfotransferases," *Developmental Cell*, vol. 29, no. 6, pp. 662–673, 2014.

[33] K. H. Limesand, S. Said, and S. M. Anderson, "Suppression of radiation-induced salivary gland dysfunction by IGF-1," *PLoS ONE*, vol. 4, no. 3, Article ID e4663, 2009.

[34] Y. Marmary, R. Adar, S. Gaska et al., "Radiation-induced loss of salivary gland function is driven by cellular senescence and prevented by IL-6 modulation," *Cancer Research*, vol. 76, no. 5, pp. 1170–1180, 2016.

[35] B. Hai, L. Qin, Z. Yang et al., "Transient activation of hedgehog pathway rescued irradiation-induced hyposalivation by

preserving salivary stem/progenitor cells and parasympathetic innervation," *Clinical Cancer Research*, vol. 20, no. 1, pp. 140–150, 2014.

[36] B. Hai, Z. Yang, L. Shangguan, Y. Zhao, A. Boyer, and F. Liu, "Concurrent transient activation of Wnt/β-catenin pathway prevents radiation damage to salivary glands," *International Journal of Radiation Oncology, Biology, Physics*, vol. 83, no. 1, pp. e109–e116, 2012.

[37] B. Hai, Z. Yang, S. E. Millar et al., "Wnt/β-catenin signaling regulates postnatal development and regeneration of the salivary gland," *Stem Cells and Development*, vol. 19, no. 11, pp. 1793–1801, 2010.

[38] M. Maimets, C. Rocchi, R. Bron et al., "Long-term in vitro expansion of salivary gland stem cells driven by wnt signals," *Stem Cell Reports*, vol. 6, no. 1, pp. 150–162, 2016.

[39] I. M. A. Lombaert, S. R. Abrams, L. Li et al., "Combined KIT and FGFR2b signaling regulates epithelial progenitor expansion during organogenesis," *Stem Cell Reports*, vol. 1, no. 6, pp. 604–619, 2013.

[40] A. Petiot, F. J. A. Conti, R. Grose, J.-M. Revest, K. M. Hodivala-Dilke, and C. Dickson, "A crucial role for Fgfr2-IIIb signalling in epidermal development and hair follicle patterning," *Development*, vol. 130, no. 22, pp. 5493–5501, 2003.

[41] L. De Moerlooze, B. Spencer-Dene, J.-M. Revest, M. Hajihosseini, I. Rosewell, and C. Dickson, "An important role for the IIIb isoform of fibroblast growth factor receptor 2 (FGFR2) in mesenchymal-epithelial signalling during mouse organogenesis," *Development*, vol. 127, no. 3, pp. 483–492, 2000.

[42] L. S. Y. Nanduri, M. Maimets, S. A. Pringle, M. Van Der Zwaag, R. P. Van Os, and R. P. Coppes, "Regeneration of irradiated salivary glands with stem cell marker expressing cells," *Radiotherapy and Oncology*, vol. 99, no. 3, pp. 367–372, 2011.

[43] S. M. Knox, I. M. A. Lombaert, C. L. Haddox et al., "Parasympathetic stimulation improves epithelial organ regeneration," *Nature Communications*, vol. 4, article 1494, 2013.

[44] S. Pradhan-Bhatt, D. A. Harrington, R. L. Duncan, M. C. Farach-Carson, X. Jia, and R. L. Witt, "A novel in vivo model for evaluating functional restoration of a tissue-engineered salivary gland," *Laryngoscope*, vol. 124, no. 2, pp. 456–461, 2014.

[45] N. Rotter, J. Oder, P. Schlenke et al., "Isolation and characterization of adult stem cells from human salivary glands," *Stem Cells and Development*, vol. 17, no. 3, pp. 509–518, 2008.

[46] J. Jeong, H. Baek, Y.-J. Kim et al., "Human salivary gland stem cells ameliorate hyposalivation of radiation-damaged rat salivary glands," *Experimental and Molecular Medicine*, vol. 45, no. 11, article no. e58, 2013.

[47] R. P. Coppes and M. A. Stokman, "Stem cells and the repair of radiation-induced salivary gland damage," *Oral Diseases*, vol. 17, no. 2, pp. 143–153, 2011.

[48] L. S. Y. Nanduri, I. M. A. Lombaert, M. Van Der Zwaag et al., "Salisphere derived c-Kit+ cell transplantation restores tissue homeostasis in irradiated salivary gland," *Radiotherapy and Oncology*, vol. 108, no. 3, pp. 458–463, 2013.

[49] S. I. Jang, H. L. Ong, A. Gallo, X. Liu, G. Illei, and I. Alevizos, "Establishment of functional acinar-like cultures from human salivary glands," *Journal of Dental Research*, vol. 94, no. 2, pp. 304–311, 2015.

[50] I. M. A. Lombaert, P. K. Wierenga, T. Kok, H. H. Kampinga, G. DeHaan, and R. P. Coppes, "Mobilization of bone marrow stem cells by granulocyte colony-stimulating factor ameliorates radiation-induced damage to salivary glands," *Clinical Cancer Research*, vol. 12, no. 6, pp. 1804–1812, 2006.

[51] C.-Y. Lin, B.-S. Lee, C.-C. Liao, W.-J. Cheng, F.-M. Chang, and M.-H. Chen, "Transdifferentiation of bone marrow stem cells into acinar cells using a double chamber system," *Journal of the Formosan Medical Association*, vol. 106, no. 1, pp. 1–7, 2007.

[52] J. Xu, D. Wang, D. Liu et al., "Allogeneic mesenchymal stem cell treatment alleviates experimental and clinical Sjögren syndrome," *Blood*, vol. 120, no. 15, pp. 3142–3151, 2012.

[53] T. Kojima, S.-I. Kanemaru, S. Hirano et al., "Regeneration of radiation damaged salivary glands with adipose-derived stromal cells," *Laryngoscope*, vol. 121, no. 9, pp. 1864–1869, 2011.

[54] J.-Y. Lim, J. C. Ra, I. S. Shin et al., "Systemic transplantation of human adipose tissue-derived mesenchymal stem cells for the regeneration of irradiation-induced salivary gland damage," *PLoS ONE*, vol. 8, no. 8, Article ID e71167, 2013.

[55] L. Lu, Y. Li, M.-J. Du et al., "Characterization of a self-renewing and multi-potent cell population isolated from human minor salivary glands," *Scientific Reports*, vol. 5, Article ID 10106, 2015.

[56] G.-L. Huang, N.-N. Zhang, J.-S. Wang, L. Yao, Y.-J. Zhao, and Y.-Y. Wang, "Transdifferentiation of human amniotic epithelial cells into acinar cells using a double-chamber system," *Cellular Reprogramming*, vol. 14, no. 4, pp. 377–383, 2012.

[57] N.-N. Zhang, G.-L. Huang, Q.-B. Han et al., "Functional regeneration of irradiated salivary glands with human amniotic epithelial cells transplantation," *International Journal of Clinical and Experimental Pathology*, vol. 6, no. 10, pp. 2039–2047, 2013.

[58] M. Kawakami, H. Ishikawa, T. Tachibana, A. Tanaka, and I. Mataga, "Functional transplantation of salivary gland cells differentiated from mouse early ES cells in vitro," *Human Cell*, vol. 26, no. 2, pp. 80–90, 2013.

[59] H. Ono, A. Obana, Y. Usami et al., "Regenerating salivary glands in the microenvironment of induced pluripotent stem cells," *BioMed Research International*, vol. 2015, Article ID 293570, 11 pages, 2015.

[60] N. Xiao, Y. Lin, H. Cao et al., "Neurotrophic factor GDNF promotes survival of salivary stem cells," *The Journal of Clinical Investigation*, vol. 124, no. 8, pp. 3364–3377, 2014.

[61] M. H. Aure, S. F. Konieczny, and C. E. Ovitt, "Salivary gland homeostasis is maintained through acinar cell self-duplication," *Developmental Cell*, vol. 33, no. 2, pp. 231–237, 2015.

[62] I. M. A. Lombaert, J. F. Brunsting, P. K. Wierenga, H. H. Kampinga, G. De Haan, and R. P. Coppes, "Cytokine treatment improves parenchymal and vascular damage of salivary glands after irradiation," *Clinical Cancer Research*, vol. 14, no. 23, pp. 7741–7750, 2008.

[63] Y. Sumita, Y. Liu, S. Khalili et al., "Bone marrow-derived cells rescue salivary gland function in mice with head and neck irradiation," *International Journal of Biochemistry and Cell Biology*, vol. 43, no. 1, pp. 80–87, 2011.

[64] S. D. Tran, Y. Sumita, and S. Khalili, "Bone marrow-derived cells: a potential approach for the treatment of xerostomia," *International Journal of Biochemistry and Cell Biology*, vol. 43, no. 1, pp. 5–9, 2011.

[65] J.-Y. Lim, T. Yi, S. Lee et al., "Establishment and characterization of mesenchymal stem cell-like clonal stem cells from mouse salivary glands," *Tissue Engineering—Part C: Methods*, vol. 21, no. 5, pp. 447–457, 2015.

[66] J. N. Ferreira and M. P. Hoffman, "Interactions between developing nerves and salivary glands," *Organogenesis*, vol. 9, no. 3, pp. 199–205, 2013.

[67] M. Ogawa, M. Oshima, A. Imamura et al., "Functional salivary gland regeneration by transplantation of a bioengineered organ germ," *Nature Communications*, vol. 4, article no. 2498, 2013.

[68] H. Tseng, J. A. Gage, R. M. Raphael et al., "Assembly of a three-dimensional multitype bronchiole coculture model using magnetic levitation," *Tissue Engineering Part C: Methods*, vol. 19, no. 9, pp. 665–675, 2013.

[69] H. Jaganathan, J. Gage, F. Leonard et al., "Three-dimensional in vitro co-culture model of breast tumor using magnetic levitation," *Scientific Reports*, vol. 4, article 6468, 2014.

[70] H. Tseng, J. A. Gage, T. Shen et al., "A spheroid toxicity assay using magnetic 3D bioprinting and real-time mobile device-based imaging," *Scientific Reports*, vol. 5, Article ID 13987, 2015.

[71] H. Tseng, L. R. Balaoing, B. Grigoryan et al., "A three-dimensional co-culture model of the aortic valve using magnetic levitation," *Acta Biomaterialia*, vol. 10, no. 1, pp. 173–182, 2014.

[72] J. S. Lee, J. D. Morrisett, and C.-H. Tung, "Detection of hydroxyapatite in calcified cardiovascular tissues," *Atherosclerosis*, vol. 224, no. 2, pp. 340–347, 2012.

[73] A. C. Daquinag, G. R. Souza, and M. G. Kolonin, "Adipose tissue engineering in three-dimensional levitation tissue culture system based on magnetic nanoparticles," *Tissue Engineering Part C: Methods*, vol. 19, no. 5, pp. 336–344, 2013.

[74] G. R. Souza, J. R. Molina, R. M. Raphael et al., "Three-dimensional tissue culture based on magnetic cell levitation," *Nature Nanotechnology*, vol. 5, no. 4, pp. 291–296, 2010.

[75] S. Pradhan, C. Liu, C. Zhang, X. Jia, M. C. Farach-Carson, and R. L. Witt, "Lumen formation in three-dimensional cultures of salivary acinar cells," *Otolaryngology—Head and Neck Surgery*, vol. 142, no. 2, pp. 191–195, 2010.

[76] H. Lin, N. Dhanani, H. Tseng et al., "Nanoparticle improved stem cell therapy for erectile dysfunction in a rat model of cavernous nerve injury," *Journal of Urology*, vol. 195, no. 3, pp. 788–795, 2016.

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 6205910, 7 pages
http://dx.doi.org/10.1155/2016/6205910

Clinical Study

Phase I/II Trial of Autologous Bone Marrow Stem Cell Transplantation with a Three-Dimensional Woven-Fabric Scaffold for Periodontitis

Shunsuke Baba,[1] Yoichi Yamada,[1,2] Akira Komuro,[1] Yoritaka Yotsui,[3] Makoto Umeda,[4] Kimishige Shimuzutani,[3] and Sayaka Nakamura[2,5]

[1]*Department of Oral Implantology, Osaka Dental University, 1-5-17 Otemae Chuo-ku, Osaka 540-0008, Japan*
[2]*Department of Oral and Maxillofacial Surgery, Aichi Medical University School of Medicine, 1-1 Yazakokarimata, Nagakute, Aichi 480-1195, Japan*
[3]*Department of Oral Radiology, Osaka Dental University, 1-5-17 Otemae Chuo-ku, Osaka 540-0008, Japan*
[4]*Department of Periodontology, Osaka Dental University, 8-1 Kuzuhahanazono-cho Hirakata, Osaka 573-1121, Japan*
[5]*Department of Biochemistry, School of Dentistry, Aichi Gakuin University, 1-100 Kusumoto-cyo, Chikusa-ku, Nagoya 464-8650, Japan*

Correspondence should be addressed to Yoichi Yamada; yyamada@aichi-med-u.ac.jp

Received 20 August 2016; Revised 2 October 2016; Accepted 17 October 2016

Academic Editor: Imad About

Regenerative medicine is emerging as a promising option, but the potential of autologous stem cells has not been investigated well in clinical settings of periodontal treatment. In this clinical study, we evaluated the safety and efficacy of a new regenerative therapy based on the surgical implantation of autologous mesenchymal stem cells (MSCs) with a biodegradable three-dimensional (3D) woven-fabric composite scaffold and platelet-rich plasma (PRP). Ten patients with periodontitis, who required a surgical procedure for intrabony defects, were enrolled in phase I/II trial. Once MSCs were implanted in each periodontal intrabony defect, the patients were monitored during 36 months for a medical exam including laboratory tests of blood and urine samples, changes in clinical attachment level, pocket depth, and linear bone growth (LBG). All three parameters improved significantly during the entire follow-up period ($p < 0.0001$), leading to an average LBG of 4.7 mm after 36 months. Clinical mobility measured by Periotest showed a decreasing trend after the surgery. No clinical safety problems attributable to the investigational MSCs were identified. This clinical trial suggests that the stem cell therapy using MSCs-PRP/3D woven-fabric composite scaffold may constitute a novel safe and effective regenerative treatment option for periodontitis.

1. Introduction

Periodontitis is a highly prevalent disease well known to reduce the quality of life of middle-aged and older people [1]. This common chronic inflammatory disease is caused by the formation of a bacterial biofilm on the tissues supporting the mouth and teeth, leading to the progressive destruction of the tissues and the loss of the affected teeth. The formation of intrabony defects is a frequent complication of periodontitis, which is generally treated by functional periodontal regeneration involving restoration of the alveolar bone and new cementum [2]. Conventional nonsurgical periodontal treatment and/or open flap debridement can reduce pocket depth and inflammation. However, the functional regeneration of the lost periodontal tissue and the normal structure are insufficient [3].

Historically, β-tricalcium phosphate (β-TCP) and ceramics were used for regenerative approach, but these grafting procedures resulted in the formation of long junctional epithelium [4]. Systematic reviews revealed that bone replacement grafts (including autogenous bones and demineralized freeze-dried bone allografts) and guided tissue regeneration (GTR) [5] are effective methods for periodontal regeneration [4]. On the other hand, ongoing trials are exploring new treatments to accelerate the regeneration of periodontal tissue. The consensus is that biologics, such as

enamel matrix derivatives (EMD) and recombinant human platelet-derived growth factor-BB (rhPDGF-BB) with β-TCP, enhance the effectiveness of periodontal regenerative therapies [2, 4]. Although these applications generally improve bone filling and the clinical parameters, complete regeneration is not achieved, particularly in cases with advanced periodontal defects [3]. Moreover, the cellular cementum regenerated by GTR is apparently different from the natural cementum formed during tooth development, whereas the cementum regenerated by EMD is acellular [6]. There are also concerns about the fact that EMD preparations are poorly characterized [4]. Accordingly, further theoretical and technical developments are required in the field of periodontal regenerative therapies.

Regenerative medicine is more attractive optional treatment for the regeneration of periodontal tissues and function. The main concept involves three elements: stem cells, scaffolds, and growth factors. The mesenchymal stem cells (MSCs) found within the periodontal tissues are multipotent cells that can replicate as undifferentiated cells and possess the capacity of multilineage differentiation [7–9]. They are more recent and highly regarded because of their potential for use in cell-based therapy for systemic diseases. However, very few studies reported clinical applications of regenerative medicine using stem cells for periodontal regeneration [10].

Our team performed several clinical studies using isolated autogenous MSCs with platelet-rich plasma (PRP) as a source of signal molecules and scaffolds for bone regeneration and succeeded in achieving bone formation in the grafted area [9, 11, 12]. PRP contains a variety of growth factors and is autologously modified fibrin glue. It is reported to promote early consolidation and graft mineralization. The growth factors include fibroblast growth factor (FGF), platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and transforming growth factor-β (TGF-β), and they also stimulate wound healing through their effects on the proliferation and differentiation of various cell types [13].

A preclinical animal study indicated that MSCs also play an important role in the cementification process and the structure of the regenerated cementum was more similar to natural cementum of roots than to the one produced by GTR. A study using cell tracking assays with green fluorescent protein (GFP) demonstrated that implanted MSCs would survive and participate in cementum regeneration [9]. Thus, we conducted a small-scale pilot study using bone marrow-derived MSCs (BMMSCs) for the treatment of periodontal disease [11]. Although the results support the potential of BMMSCs for successful periodontal regeneration, more effective scaffolds are needed for cases of advanced periodontal disease.

Our team developed a three-dimensional (3D) woven-fabric scaffold composed of biodegradable poly-L-lactic acid resin fibers [14]. The porous basket-shaped material was designed to enhance bone regeneration by securing spaces in which implanted cells proliferate and differentiate as well as internal spaces retaining the gel-like graft material. Our previous preclinical study using the canine mandibles model demonstrated that this scaffold considerably accelerates regeneration of the bone and periodontal tissue in defects created on the tooth root aspect [14]. Therefore, here we present the results of a long-term clinical trial conducted to evaluate the safety, efficacy, and stability of a novel periodontal regeneration therapy using MSCs, PRP, and our woven-fabric scaffold.

2. Materials and Methods

2.1. Study Participants. Adult patients who visited the Institute of Biomedical Research and Innovation hospital (Kobe, Japan) for the treatment of chronic periodontitis were enrolled in this phase I/II clinical trial. The inclusion criteria were as follows: (1) age 35–60 years, (2) a probing pocket depth (PPD) $\geq$ 4 mm, (3) at least 10 teeth in the mandibular, (4) teeth for which recovery by an existing periodontal surgery is not to be expected, and (5) good general health without any sign of systemic disease. The exclusion criteria were as follows: smoking within the last 6 months before study enrollment and pregnancy. All patients included in this study received standard nonsurgical periodontal therapy. Afterward, teeth exhibiting significant vertical bone resorption on dental X-ray and periodontal pockets $\geq$ 4 mm were selected. Two healthy teeth per patient were used as the control. Verbal and written informed consents were obtained from the patients. This study conformed to STROBE Guidelines and was approved by the institutional review board of the Foundation for Biomedical Research and Innovation registered in ClinicalTrails.gov (NCT00221130, Supplemental Data available online at http://dx.doi.org/10.1155/2016/6205910).

2.2. Preparation of Bone Marrow-Derived Mesenchymal Stem Cells. BMMSCs were prepared as previously described [9, 11, 12] and the cells were prepared in cell processing center of Translational Research Informatics Center in Foundation for Biomedical Research and Innovation that was compliant with Good Manufacturing Practice guidelines. In brief, 1 month before the periodontal surgery, autogenous MSCs were harvested by aspirating the iliac bone marrow from each subject. Base media and low-glucose Dulbecco's modified Eagle medium containing growth supplements (50 mL serum, 10 mL 200 mM L-glutamine, and 0.5 mL penicillin-streptomycin mixture containing 25 IU penicillin and 25 μg streptomycin) were purchased from Cambrex (Walkersville, MD). The supplements used to induce osteogenesis (dexamethasone, sodium β-glycerophosphate, and L-ascorbic acid 2-phosphate) were bought from Sigma Chemical (St Louis, MO). The presence of osteoblast-like cells was confirmed by measuring alkaline phosphatase (ALP) activity.

2.3. Preparation of Platelet-Rich Plasma (PRP). Total hemocyte count was determined during the preoperative hematologic assessment, whereas a blood sample was obtained 1 day before the surgery. The blood was collected under sterile conditions in a bag containing anticoagulant citrate. The blood was centrifuged to separate the yellow plasma containing the platelets and the buffy coat containing the leukocytes. The plasma was centrifuged to consolidate the

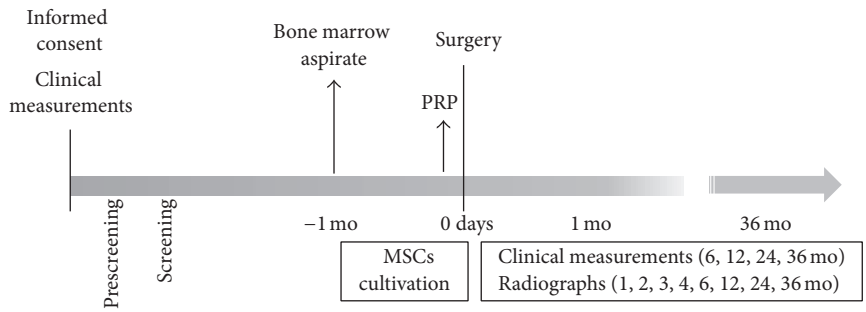


Figure 1: Treatment schedule.

platelets into a pellet. The platelet-poor plasma and the plasma supernatant were discarded. The pellet of platelets and buffy coat/plasma fraction were resuspended into the remaining plasma used to prepare a platelet gel.

2.4. Cell Transplantation. The clinical trial was conducted according to the schedule shown in Figure 1. Before the surgery, each subject rinsed their mouth with 0.2% chlorhexidine solution. Then, the surgery was initiated under local anesthesia with xylocaine adrenaline 2% (Astra). To avoid interactions from the xylocaine, the injection was given at about a tooth's width. After making the pocket and releasing incisions, the full-thickness flap was elevated on both the buccal and lingual aspects, and the inner epithelium of the flap was removed. The granulation tissue was thoroughly removed in the area of the bone defect, and the tooth root was planned to smoothen the surface. No bone recontouring was performed. The mixture of BMMSCs and PRP, combined with human thrombin (5,000 units) dissolved in 10% calcium chloride, was perfused in a 3D woven-fabric composed of poly-L-lactic acid resin fibers [14], which was then placed around the tooth root. The flap was repositioned and the wound was closed by a suture. The suture was removed after 2 weeks. The subjects were instructed to rinse their mouth using solution of chlorhexidine digluconate three times a day. No mechanical cleaning was allowed on the surgical site during the first 4 weeks after surgery. After this period, professional mechanical tooth cleaning was performed once every 2 months.

2.5. Clinical Evaluation. The primary endpoints of this study were the clinical attachment level (CAL) and pocket depth (PD). CAL or PD were measured at each follow-up visit (postoperative 6-, 12-, 24-, and 36-month time-points), and the changes were calculated relative to the pretreatment measurements. The median of five measurements on each tooth was used for the efficacy analysis. Clinical mobility was based on the Periotest value, which was measured three times at the center of the buccal aspect of the tooth, and the mean was used in the analysis. Postoperative changes in vertical bone defects were assessed by X-rays at each follow-up visit (postoperative 1-, 2-, 3-, 4-, 6-, 12-, 24-, and 36-month time-points) and defined as the linear bone growth (LBG). The postoperative depth of the intrabony defect was calculated from the distance between the apex and cementoenamel junction on the preoperative and postoperative radiographs at the same magnification.

2.6. Safety Evaluation. Each follow-up visit included a medical exam of the entire body and oral cavity (postoperative 1- and 2-week and 6-, 12-, 24-, and 36-month time-points) as well as laboratory tests of blood and urine samples (postoperative 6-, 24-, and 36-month time-points). Adverse events were documented and analyzed for any temporal relationship with the surgical procedure and/or scaffold.

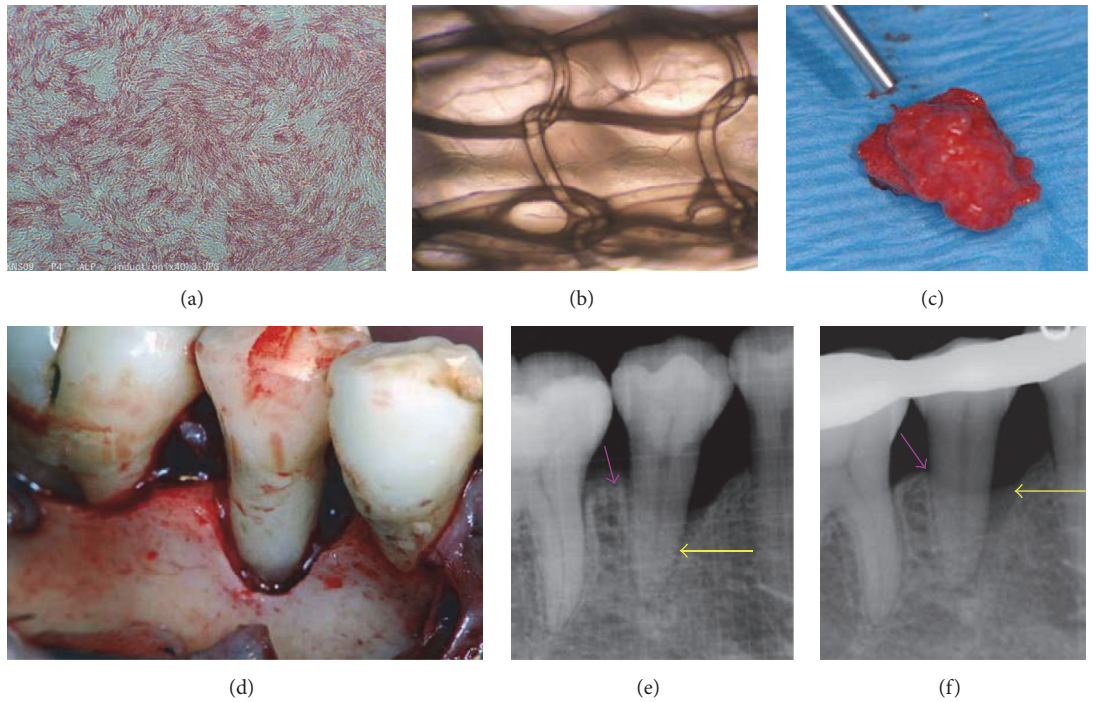
2.7. Statistical Analysis. The changes in CAL, PD, the depth of the intrabony defect, and clinical mobility before and after implantation were analyzed using a linear mixed model assuming a compound symmetry covariance structure. Multiple comparisons by the Dunnett-Hsu method were performed at each follow-up time-point using the preoperative value as the control.

3. Results

3.1. Transplantation of the BMMSCs-PRP/3D Woven-Fabric Composite Scaffold Gel. A total of 10 patients (three males and seven females; mean age: 48.4 years) completed the standard periodontal therapy and were enrolled in the study. The intrabony defects included one-wall defects at three sites, two-wall defects at six sites, and three-wall defects at one site.

The characteristics of BMMSCs were assessed based on ALP staining and enzyme activity. After the induction of osteogenesis, the cells were positive for ALP staining (Figure 2(a)) and exhibited high ALP activity (BMMSCs: 2.01 units/well and induced osteoblast: 18.05 units/well), thereby establishing the osteogenic capacity of BMMSCs. The *in vitro* experiment demonstrated that BMMSCs could proliferate on the basket-shaped 3D woven-fabric composite scaffold used in this clinical study (Figure 2(b)).

When BMMSCs-PRP solution was combined with thrombin dissolved in a calcium chloride solution, the mixture adopted a gel-like texture (Figure 2(c)) in the 3D woven-fabric composite scaffold fitted in the periodontal bony defects. The mean cell number transplanted to each patient was 3.5×10^7 cells. In PRP, the mean platelet count



(a) (b) (c)

(d) (e) (f)

Figure 2: (a) Positive alkaline phosphatase staining of osteoinduced BMMSCs. (b) BMMSCs proliferate on the basket-shaped scaffold. (c) The combination of BMMSCs/PRP and thrombin in calcium chloride generates a gel-like structure. (d) Representative image of the periodontal intrabony defect (preoperative). (e, f) X-ray images taken before and 8 weeks after the surgery.

Table 1: Clinical outcome.

	Mean change (SD)				p value
	6 months	12 months	24 months	36 months	
CAL regained (mm)					
Test	2.72 (0.92)	2.68 (1.017)	3.09 (1.073)	3.24 (0.857)	<0.0001
Control	−0.52 (0.766)	−0.95 (0.852)	−0.84 (0.94)	−1.06 (1.125)	0.0004
Pocket depth changed (mm)					
Test	−2.46 (1.052)	−2.48 (0.998)	−3.02 (0.854)	−3.16 (0.583)	<0.0001
Control	0.33 (0.789)	0.73 (0.880)	0.52 (1.041)	0.68 (1.127)	0.0512
Mobility (Periotest value)					
Test	−1.36 (4.856)	−1.5 (3.095)	−1.87 (9.351)	−4.62 (7.567)	0.4802
Control	−0.34 (4.461)	−0.44 (5.623)	2.34 (9.152)	−0.01 (6.193)	0.8104

(1,352,600/μL) was 490% higher than that at the baseline, which confirmed the sequestration ability of the scaffold.

3.2. Periodontal Inspections. The mean changes in CAL, PD, and clinical mobility are shown in Table 1. CAL decreased over time, and therapy efficacy was significant at each follow-up visit, based on the linear mixed model (each time-point: $p < 0.0001$). The multiple comparisons by the Dunnett-Hsu method revealed significantly different CAL at each follow-up time-point ($p < 0.0001$ at the 6-, 12-, 24-, and 36-month time-points), compared with the preoperative level. On the other hand, CAL of the control group increased significantly from the preoperative time-point to the postoperative 12- ($p = 0.0076$), 24- ($p = 0.0019$), and 36-month ($p = 0.0001$) time-points. In addition, PD of the test group was significantly lower during the entire follow-up period compared with the preoperative level (each time-point: $p < 0.0001$). In contrast, PD of the control group showed an increasing trend at each follow-up period.

The clinical mobility of the test group showed a decreasing trend after the surgery, but there was no significant efficacy for the control or test groups at any of the follow-up time-points, based on multiple comparisons by the Dunnett-Hsu method or Wilcoxon's signed-rank test. The control group showed no significant difference in clinical mobility at all time-points.

The test group showed a significant decrease in the depth of the intrabony defect during the follow-up period ($p < 0.0001$) (Figures 2(d)–2(f) and 3). The multiple comparisons

Table 2: Adverse event.

Extraoral reactions	Number of events	Outcome		
		remain	resission	healing
Pain				
Lumbar	1	0	0	1
Tenderness				
Cheek	1	0	0	1
Swelling				
Face	5	0	0	5
Submandibular lymph nodes	1	0	0	1
Lumbar	1	0	0	1
Abnormal perception	1	0	0	1
Intraoral reactions				
Pain	0	0	0	0
Bleeding	1	0	0	1
Gingival swelling	4	0	1	3
Aesthetic problem of gingiva	1	0	0	1
Redness of gingiva	2	0	0	2
Hematoma	1	0	0	1
Hyperaesthesia	2	0	0	2
Angular cheilosis	3	0	0	3
Canker sore	0	0	0	0
Gingival abscess	0	0	0	0
Tooth mobility	0	0	0	0
Discomfort	0	0	0	0

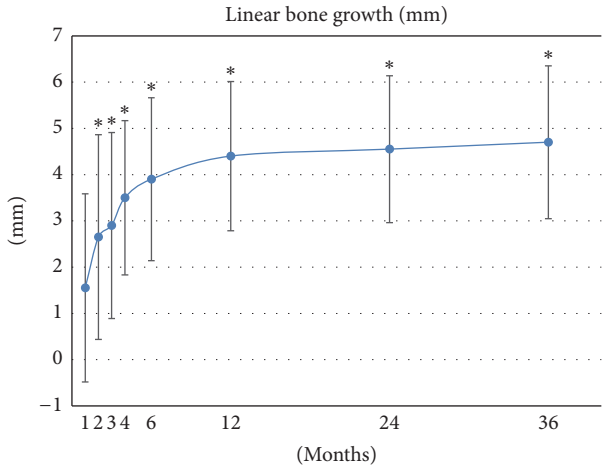


Figure 3: Mean change in the depth of the intrabony defect after operation. Linear bone growth (LBG) significantly increased during the follow-up period. $^{*}p < 0.01$.

revealed significant improvement of the intrabony defect at the 2- ($p = 0.0078$), 3- ($p = 0.002$), 4- ($p = 0.002$), 6- ($p = 0.002$), 12- ($p = 0.002$), 24- ($p = 0.0039$), and 36-month ($p = 0.0039$) time-points, compared with the preoperative level.

3.3. Safety Evaluation. During the follow-up period, the overall plaque control record of the subjects remained <30%, indicating favorable oral hygiene practice (data not shown). The frequency of each adverse event observed throughout the follow-up period is shown in Table 2. The most frequent adverse event shown to have a causative relationship with the therapy was facial swelling (five cases), followed by gingival swelling (four cases) and angular cheilosis (three cases). All patients recovered spontaneously and completely from all adverse events. No patients have pain nor discomfort that had causative relationship with the therapy in intraoral region. In addition, no tooth loss was found after surgery during follow-up period. None of the abnormal laboratory test values obtained from blood and urine analyses were related to the therapy (data not shown).

4. Discussion

Conventional treatments of periodontitis may reduce the progression of periodontitis, but they generally fail to restore the normal periodontal supporting structures that were damaged by the disease [15]. Tissue engineering involves three strategies adopted for the creation of new tissue, isolated stem cells, signal molecules, and biocompatible scaffolds [16]. Regenerative medicine by autogenous cell transplantation is considered among the most promising therapeutic concepts currently developed because it solves several issues, namely, the morbidity at the donor site due to autologous grafts, immunogenicity of the allogenic grafts, and loosening of the alloplastic implants. Scaffolds and growth factors have been applied to the engineering concept of periodontal treatments, but few studies tested the potential of their combined effects

in clinical trials [11]. Furthermore, successful periodontal regeneration requires the addition of progenitor cells that can proliferate and differentiate into specialized cells of regenerative capacity [17]. Many animal studies provided evidence that MSCs can be safe and effectively used to support periodontal regeneration [3]. The present study proposes a treatment combining all three elements: BMMSCs, a 3D polylactic-acid-based synthetic material, and a PRP solution of autologously modified fibrin glue and growth factors.

A study assessing the outcome of conventional periodontal surgery for periodontitis reported gains in CAL of 0.2–1.5 mm, reductions in PD of 1.5–2.7 mm, and LBG of 0.3–1.1 mm, measured 12 months after the procedure [18]. In contrast, the unique composition of the mixed transplants that we developed led to >twofold higher gains in CAL (2.68 mm) as well as higher reductions in PD (2.48 mm) and fourfold higher LBG (4.4 mm) 12 months after the procedure. Furthermore, our study was designed to monitor these three parameters over a period of 36 months to determine the linearity and persistence of these improvements. The analysis showed a gradual improvement in all three parameters over at least 36 months.

Previous clinical studies incorporated growth factors into their scaffold to stimulate periodontal regeneration, but they consistently yielded lower LBG values compared to those observed in the present study: rhPDGF-BB/β-TCP (3.3 mm at 24 months) [19], rh-FGF-2/β-TCP (3.7 mm at 6 months) [20], rh-FGF2/hydroxypropyl cellulose (1.9 mm at 9 months), or EMD (1.3 mm at 9 months) [21]. Altogether, these data demonstrate the importance of stem cell therapy for an effective periodontal regeneration. This finding is supported by animal studies that showed BMMSCs differentiating into periodontal cells to regenerate periodontal tissues [9, 22]. Furthermore, the signaling molecules contained in PRP may stimulate cell migration, proliferation, and differentiation.

Our clinical trial included an interview, whole-body physical examinations, extraoral and intraoral reaction tests, and clinical monitoring for potential adverse events related to this regenerative medicine therapy. There was no relationship between the stem cell therapy and frequency of the adverse events, which were all normal reactions to periodontal surgery. In addition, all patients spontaneously and completely recovered from all adverse events, without any specific treatment. These results are comparable to the previous studies that indicated no adverse reactions after local cell administration [9–12, 22]. Moreover, none of the abnormal laboratory test results, obtained from blood or urine samples, were related to the therapy. The scaffold materials were autogenous in origin basically and/or biodegradable. Previously, we performed the safety assessment of the cultured BMMSCs and no mycoplasma infection was detected and no tumorigenesis was found. In addition, flow cytometry analysis showed that the BMMSCs were positive for MSC markers and negative for hematopoietic lineage and monocytic markers [9]. These data would indicate that cultured BMMSCs used in this study were safe and possessed characteristics of MSCs. Collectively, these data would support the safety of this new effective treatment of periodontitis.

Since the main limitation of this study is the small sample size, multicenter, well-designed randomized controlled trials are needed to confirm the efficacy and safety of this treatment. Further investigation is required to develop more suitable scaffolding materials in an attempt to treat more severe periodontal defects. Also, the outcome of periodontal regeneration is negatively affected by poor oral hygiene [2]. In the present study, the overall plaque control record of the subjects remained <30% throughout the follow-up, indicating favorable oral hygiene practice. This factor probably contributed to the successful periodontal regeneration and should be closely monitored during future large-scale studies assessing the potential of this new therapy.

5. Conclusion

Long-term clinical trials, including a follow-up period of at least 3 years, are preferable to monitor the regeneration of periodontal tissues based on all key factors, namely, CAL, PD, and LBG. However, very few long-term clinical trials have been reported. The present 3-year trial showed that our new BMMSCs-PRP/3D woven-fabric composite scaffold gel was safe and provides significant long-term improvements in all clinical endpoints and efficient regeneration of the true periodontal tissue. Therefore, this new stem cell periodontal regenerative therapy may constitute a new promising regenerative treatment option for periodontal diseases. Future more rigorous clinical trials are recommended to determine safety and efficacy of the stem cell-based periodontal therapy.

Competing Interests

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

Authors' Contributions

Shunsuke Baba and Yoichi Yamada contributed equally to this work.

Acknowledgments

The authors wish to pay their respects to Kenji Ito and express their deepest regret as this kind and generous man died last year. They wish to thank him and Masanori Fukushima at Translational Research Informatics Center in Foundation for Biomedical Research and Innovation for their encouragements and important contribution to the completion of this study.

References

[1] D. F. Kinane, "Causation and pathogenesis of periodontal disease," *Periodontology 2000*, vol. 32, pp. 24–35, 2001.

[2] M. A. Reynolds, R. T. Kao, P. M. Camargo et al., "Periodontal regeneration—intrabony defects: a consensus report from the AAP Regeneration Workshop," *Journal of Periodontology*, vol. 86, no. 2, supplement, pp. S105–S107, 2015.

[3] F.-M. Chen, H.-H. Sun, H. Lu, and Q. Yu, "Stem cell-delivery therapeutics for periodontal tissue regeneration," *Biomaterials*, vol. 33, no. 27, pp. 6320–6344, 2012.

[4] R. T. Kao, S. Nares, and M. A. Reynolds, "Periodontal regeneration—intrabony defects: a systematic review from the AAP Regeneration Workshop," *Journal of Periodontology*, vol. 86, no. 2, pp. S77–S104, 2015.

[5] S. Nyman, J. Lindhe, T. Karring, and H. Rylander, "New attachment following surgical treatment of human periodontal disease," *Journal of Clinical Periodontology*, vol. 9, no. 4, pp. 290–296, 1982.

[6] L. Heijl, G. Heden, G. Svardstrom et al., "Enamel matrix derivative (Emdogain) in the treatment of intrabony periodontal defects," *Journal of Clinical Periodontology*, vol. 24, no. 9, pp. 705–714, 1997.

[7] M. F. Pittenger, A. M. Mackay, S. C. Beck et al., "Multilineage potential of adult human mesenchymal stem cells," *Science*, vol. 284, no. 5411, pp. 143–147, 1999.

[8] B. M. Seo, M. Miura, S. Gronthos et al., "Investigation of multipotent postnatal stem cells from human periodontal ligament," *The Lancet*, vol. 364, no. 9429, pp. 149–155, 2004.

[9] Y. Yamada, S. Nakamura, K. Ito et al., "Injectable bone tissue engineering using expanded mesenchymal stem cells," *STEM CELLS*, vol. 31, no. 3, pp. 572–580, 2013.

[10] F.-M. Chen, L.-N. Gao, B.-M. Tian et al., "Treatment of periodontal intrabony defects using autologous periodontal ligament stem cells: a randomized clinical trial," *Stem Cell Research & Therapy*, vol. 7, no. 1, article 33, 2016.

[11] Y. Yamada, M. Ueda, H. Hibi, and S. Baba, "A novel approach to periodontal tissue regeneration with mesenchymal stem cells and platelet-rich plasma using tissue engineering technology: a clinical case report," *International Journal of Periodontics and Restorative Dentistry*, vol. 26, no. 4, pp. 363–369, 2006.

[12] Y. Yamada, S. Nakamura, K. Ito et al., "Injectable Tissue-engineered bone using autogenous bone marrow derived stromal cells for maxillary sinus augmentation: clinical application report-from 2 to 6 years follow up," *Tissue Engineering. Part A*, vol. 14, no. 10, pp. 1699–1707, 2008.

[13] R. E. Marx, E. R. Carlson, R. M. Eichstaedt, S. R. Schimmele, J. E. Strauss, and K. R. Georgeff, "Platelet-rich plasma: growth factor enhancement for bone grafts," *Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontics*, vol. 85, no. 6, pp. 638–646, 1998.

[14] S. Baba, Y. Hashimoto, T. Inoue et al., "Evaluation of a 3-D, woven-fabric, composite scaffold using experimental canine models of bone defects in mandibles," *Journal of Oral Tissue Engineering*, vol. 8, no. 3, pp. 212–221, 2011.

[15] K. Hynes, D. Menicanin, S. Gronthos, and P. M. Bartold, "Clinical utility of stem cells for periodontal regeneration," *Periodontology 2000*, vol. 59, no. 1, pp. 203–227, 2012.

[16] R. Langer and J. P. Vacanti, "Tissue engineering," *Science*, vol. 260, no. 5110, pp. 920–926, 1993.

[17] P. M. Bartold, S. Shi, and S. Gronthos, "Stem cells and periodontal regeneration," *Periodontology 2000*, vol. 40, no. 1, pp. 164–172, 2006.

[18] L. J. Heitz-Mayfield, L. Trombelli, F. Heitz et al., "A systematic review of the effect of surgical debridement vs non-surgical debridement for the treatment of chronic periodontitis," *Journal of Clinical Periodontology*, vol. 29, no. 3, pp. 92–102, 2002.

[19] M. Nevins, R. T. Kao, M. K. McGuire et al., "Platelet-derived growth factor promotes periodontal regeneration in localized osseous defects: 36-month extension results from a randomized, controlled, double-masked clinical trial," *Journal of Periodontology*, vol. 84, no. 4, pp. 456–464, 2013.

[20] D. L. Cochran, T. J. Oh, M. P. Mills et al., "A randomized clinical trial evaluating rh-FGF-2/β-TCP in periodontal defects," *Journal of Dental Research*, vol. 95, no. 5, pp. 523–530, 2016.

[21] M. Kitamura, M. Akamatsu, M. Kawanami et al., "Randomized placebo-controlled and controlled non-inferiority phase III trials comparing trafermin, a recombinant human fibroblast growth factor 2, and enamel matrix derivative in periodontal regeneration in intrabony defects," *Journal of Bone and Mineral Research*, vol. 31, no. 4, pp. 806–814, 2015.

[22] H. Kawaguchi, A. Hirachi, N. Hasegawa et al., "Enhancement of periodontal tissue regeneration by transplantation of bone marrow mesenchymal stem cells," *Journal of Periodontology*, vol. 75, no. 9, pp. 1281–1287, 2004.

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 4209891, 20 pages
http://dx.doi.org/10.1155/2016/4209891

Review Article

Stem Cells of Dental Origin: Current Research Trends and Key Milestones towards Clinical Application

Athina Bakopoulou[1] and Imad About[2]

[1]*Department of Fixed Prosthesis and Implant Prosthodontics, School of Dentistry, Aristotle University of Thessaloniki, Thessaloniki, Greece*
[2]*Aix-Marseille University, CNRS, Institute of Movement Science (ISM), Marseille, France*

Correspondence should be addressed to Athina Bakopoulou; abakopoulou@dent.auth.gr

Received 25 May 2016; Accepted 14 September 2016

Academic Editor: Armand Keating

Dental Mesenchymal Stem Cells (MSCs), including Dental Pulp Stem Cells (DPSCs), Stem Cells from Human Exfoliated Deciduous teeth (SHED), and Stem Cells From Apical Papilla (SCAP), have been extensively studied using highly sophisticated *in vitro* and *in vivo* systems, yielding substantially improved understanding of their intriguing biological properties. Their capacity to reconstitute various dental and nondental tissues and the inherent angiogenic, neurogenic, and immunomodulatory properties of their secretome have been a subject of meticulous and costly research by various groups over the past decade. Key milestone achievements have exemplified their clinical utility in Regenerative Dentistry, as surrogate therapeutic modules for conventional biomaterial-based approaches, offering regeneration of damaged oral tissues instead of simply "filling the gaps." Thus, the essential next step to validate these immense advances is the implementation of well-designed clinical trials paving the way for exploiting these fascinating research achievements for patient well-being: the ultimate aim of this ground breaking technology. This review paper presents a concise overview of the major biological properties of the human dental MSCs, critical for the translational pathway "from bench to clinic."

1. Introduction

A disparate variety of multipotent postnatal or Adult Stem Cells (ASCs) has been identified over the last decade within the oral cavity, raising the intriguing prospect of several alternative therapies in the burgeoning field of Regenerative Dentistry. Oral ASCs can be classified into dental stem cells, encompassing Dental Pulp Stem Cells (DPSCs) [1], Stem Cells from Human Exfoliated Deciduous teeth (SHED) [2], and Stem Cells From Apical Papilla (SCAP) [3, 4], as well as nondental oral SCs, including Dental Follicle Stem Cells (DFSCs) [5], Periodontal Ligament Stem Cells (PDLSCs) [6], Gingival Mesenchymal Stem Cells (GMSCs) [7], Oral Mucosa Stem Cells (OMSCs) found in the lamina propria of adult human gingiva [8], Bone Marrow Mesenchymal Stem Cells (BMMSCs) from orofacial bones [9], Periosteum-Derived Stem Cells (PSCs) [10], and Salivary Gland-Derived Stem Cells (SGSCs) [11]. All these cells are considered as resident in "stem cell niches" of the respective mesenchymal oral tissues and are referred to as mesenchymal stem cells or multipotent mesenchymal stromal cells (MSCs) [12]. In addition to cells derived from healthy tissues, MSCs can also be isolated from damaged oral tissues, such as inflamed pulp [13, 14] or periapical cysts [15].

There is substantial evidence suggesting that dental MSCs reside in a quiescent, slow-cycling state in the perivascular niches of human pulp or apical papilla [16]. It has been further shown by means of genetic lineage tracing in rodent incisors that MSCs residing in the dental pulp may be of dual origin, consisting of not only NG2+ pericyte cells, whose presence is closely dependent on tissue vascularity, but also MSCs of non-pericyte origin, contributing to tissue growth and repair [17]. Dental MSCs are thought to originate from the cranial neural crest, expressing both MSC and neuroectodermal SC markers. These cells comply with the minimal criteria stipulated by the International Society of Cellular Therapy (ISCT) in 2006

[18], including (1) ability to adhere rapidly to plastic culture surfaces, (2) potential for trilineage differentiation towards osteogenic, adipogenic, and chondrogenic phenotypes under the appropriate inductive conditions, and (3) expression of common MSC markers, such as CD105, CD73, and CD90, in conjunction with lack of expression of CD45, CD34, CD14, CD11b, CD79a, CD19, and HLA-DR. Additionally, dental MSCs are characterized by significant population heterogeneity [19], most probably connected to different stages of developmental commitment, reinforced by epigenetic modifications occurring during their *ex vivo* expansion [20, 21]. Importantly, recent studies have shown the pivotal role of not only stem/progenitor cells but also nonprogenitor supportive cells, such as injured fibroblasts occurring via secretion of multiple growth factors and complement bioactive fragments in dentin/pulp regeneration processes, revealing the significance of all different cellular components of the heterogeneous population [22–25].

Among the important advantages of dental MSCs compared to other SC sources, such as bone marrow and adipose tissues, are their higher proliferative capacity, facilitating *ex vivo* expansion in sufficient cell numbers [26, 27]; easy isolation by noninvasive routine clinical procedures (e.g., extraction of impacted third molars or premolars for orthodontic reasons); and the absence, as reported so far, of major adverse reactions, concerning, for example, teratoma formation following *in vivo* application [28]. Previous studies have shown that DPSCs have the ability to produce single-cell derived Colony Forming Units (CFUs), survive for longer periods without undergoing senescence, and exhibit higher (80–100 times) proliferation rates than BMMSCs [1].

The vast majority of published studies provides evidence on the *in vitro* multilineage differentiation potential of dental MSCs towards osteo/odontogenic, adipogenic, chondrogenic, neurogenic, angiogenic, and myogenic lineages when grown under defined culture conditions [19, 28]. *In vivo* studies, mostly in ectopic but less often in orthotopic animal models, have supported their potential to reconstitute functional dentin/pulp complexes when mixed with ceramic substrates (such as, Hydroxyapatite Or Tricalcium Phosphate) [29, 30], as well as other tissues, such as bone [31], cementum [32], blood vessels [33–35], and neural tissues [36, 37]. Most recently, attention has been focused on the biological properties of the plethora of soluble trophic and immunomodulatory cytokines produced by dental MSCs (MSC secretome) because of their angiogenic, neurogenic, and tissue repair properties [38]. Additionally, a growing number of preclinical and few clinical "proof-of-concept" studies that have been initiated provide substantial evidence that dental MSCs and/or their secretome can be successfully utilized for dental [39, 40] and nondental biomedical applications [41].

Taking all the above into consideration, this review paper aims to provide a concise overview of the major biological properties of the adult dental MSCs (including DPSCs, SHED, and SCAP) which are critical for Tissue Engineering (TE) applications; among these properties being of major interest to the dental community is their inherent potential to regenerate highly vascularized (angiogenesis) and innervated (neurogenesis) soft and hard dental tissues (dentin/pulp complex, alveolar bone). Current research trends and key milestone achievements that exemplify their clinical utility in Regenerative Dentistry will be also highlighted.

2. Localization and Immunophenotypic Characterization of Dental MSCs

Dental MSCs abundantly express (>95% of the cell population) MSC markers, such as, CD90, CD73, and several Cell Adhesion Molecules (CAMs), mainly integrins but also cadherins [42], with the former being responsible for mediating SC adhesion to Extracellular Matrix (ECM) proteins and the latter for cell-cell interactions [43]. Among these, CD29/b1-integrin, CD49 (subunits b/a_2-integrin, d/a_4-integrin, e/a_5-integrin, and f/a_6-integrin), CD51/a_v-integrin, CD61/b_3-integrin, and CD166/ALCAM have been found to be variably expressed in different types of dental MSCs, including DPSCs, SHED, and SCAP, further indicating the heterogeneity of these cell populations [19, 28, 42]. Other MSC markers, such as CD146, CD105, CD106, and STRO-1, may show variable expression, dependent on the type and maturity of the dental MSC population and on interindividual variations among various cell donors [44]. In particular, STRO-1, a marker that recognizes a trypsin insensitive epitope on perivascular cells [45], has been used in isolating MSCs populations from human dental pulp [46] and apical papilla [47] with enhanced "stemness" properties and osteo/odontogenic differentiation potential. Immunolocalization studies have, in addition, demonstrated that a subpopulation of SCs coexpressing STRO-1, the perivascular marker CD146 [48] and the pericyte antigen 3G5, reside in this niche within the adult pulp [16]. Cells expressing another group of markers (STRO-1, CD90, CD105, and CD146) were also identified together with the vascular and nerve fibers of the pulp tissue [13]. Most recently, [49] it was shown that ALDH1-, CD90-, and STRO-1-positive cells are located in both perivascular areas and nerve fibers of dental pulps, indicating the possibility of the existence of more than one SC niche. Finally, a recent report [50] identified a rare (1.5% by flow cytometry) subpopulation of SCAP, coexpressing NOTCH-3, STRO-1, and CD146, which, according to *in situ* immunostaining, were associated with blood vessels.

All types of dental MSCs also abundantly express nestin (neural stem cells), while the positive presence of other neural crest SC markers (musashi-1, p75, snail-1, -2, slug, Sox-9, etc.) has been also reported and linked to their embryonic origin [51, 52]. Sakai et al. [53] have also shown that the majority of DPSCs and SHED expressed several neural lineage markers, including nestin, Doublecortin (DCX; neuronal progenitor cells), βIII-tubulin (early neuronal cells), NeuN (mature neurons), GFAP (neural stem cells and astrocytes), S-100 (Schwann cells), A2B5, and CNPase (oligodendrocyte progenitor cells). Other, less commonly found markers, such as CD44, CD9, CD10, CD13, CD59, and MSCA-1, have also been reported as expressed in DPSCs [54], while CD44 and CD13 are also expressed in SHED [55]. Dental MSCs, including DPSCs [56], SHED [57], and SCAP [58], also show variable but increased expression of embryonic SC markers,

such as Nanog, Oct3/4, SSEAs (-1, -3, -4, and -5), and to a less extent TRA-1-60 and TRA-1-81 [56], as compared to other MSC types [19], such as BMMSCs. Other pluripotency markers, such as SOX-2 and MYC, not normally found in other ASCs, have been reported in tooth germ-derived MSCs [59]. Finally, dental MSCs lack expression of CD45, CD31, HLA-DR, and, in most studies, CD14, while most but not all [60–62] studies have reported absence of expression of CD117 (c-kit) and CD34. Although the ISCT minimal criteria suggest that the absence of CD34 expression is a prerequisite for defining MSCs, more recent studies indicate that CD34 may be expressed in primitive pluripotent stromal stem cells but is progressively eliminated during cell culturing [63]. It has been previously shown that CD34/c-Kit and STRO-1 coexpression confirm a neural crest-derived DPSC niche [56], while, in more recent studies [61] two different (STRO-1+/c-Kit+/CD34− and STRO-1+/c-Kit+/CD34+) DPSC subpopulations with noticeable differences in their stem cell characteristics have been characterized.

Finally, in a recent study [64], the importance of CD271/NGFR in defining a subpopulation of DPSCs with enhanced odontogenic differentiation potential, as compared to other (CD51/CD140a and STRO-1/CD146) subpopulations also showing odontogenic differentiation capacity, has been emphasized. This is in accordance with studies on BMMSCs showing that CD271/NGFR defines an infrequent but very primitive subset (<1%) of the cell population showing enhanced stem cell characteristics [65].

The immunophenotypic characteristics of dental MSCs are summarized in Table 1.

3. Differentiation Potential and Paracrine Activity of Dental MSCs *In Vitro* and *In Vivo*

3.1. Osteo/Odontogenic Differentiation Potential of Dental MSCs and Regeneration of Dentin/Pulp- and Bone-Like Tissues. One of the most salient characteristics of dental MSCs concerning dental TE applications rests on their odontogenic differentiation potential. Previous studies have shown that dental MSCs, including DPSCs, SHED, and SCAP, have the capacity to differentiate into odontoblastic lineages *in vitro* and of regenerating dentin/pulp-like complexes or bone-like tissues ectopically and around teeth and implants [29, 31, 66] (the literature summarized in Table 2).

Specifically, *DPSCs* have demonstrated the capacity to differentiate into odontoblastic-like cells with characteristic cell polarity [67]. When seeded onto dentin, DPSCs may convert into odontoblast-like cells with polarized cell bodies and cellular processes extending into the dentinal tubules [68]. In addition, in recently published work using transcriptome analysis of odontoblasts at different stages of maturity, the p38/MAPK signaling has been identified as the crucial pathway to controlling odontoblast secretory activity and therefore a key molecular target for the therapeutic application of DPSCs [40].

Early reports showed that DPSCs mixed with Hydroxyapatite/Tricalcium Phosphate (HA/TCP) led to the formation of ectopic pulp-dentin-like tissue complexes in immunocompromised mice [1, 6, 69]. Iohara et al. [70] combined 3-dimensional cell pellets and Bone Morphogenetic Protein 2 (BMP-2) to induce reparative dentin formation in a dog amputated pulp model. The same group also detailed the possibility of using a subfraction of CD31−/CD146− and CD105+ cells for pulp regeneration [71, 72] and in later studies described the effects of Granulocyte-Colony Stimulating Factor (G-CSF) and host age on pulp regeneration [73, 74]. In another study, DPSCs seeded onto collagen scaffolds in presence of Dentin Matrix Protein 1 (DMP-1) induced the formation of a pulpal-like tissue [75]. Similarly, when implanted in enlarged root canals of immunocompromised mice, DPSCs showed the ability to synthesize newly formed dentin and vascularized pulp-like tissue [76], thus providing prospects for utilization of DPSC transplantation for dentin-pulp regeneration.

Other *in vivo* studies have shown the capacity of DPSCs in bone regeneration in a variety of animal models, including repair of critical-size calvarial defects [77–79] and segmental alveolar defects in a New Zealand rabbit model [80], as well as the capacity for enhancement of implant osteointegration in sites of experimental canine mandibular bone defects [81]. Swine Dental Pulp Stem Cells seeded on TCP scaffolds were also able to regenerate mandibular bone defects in the symphyseal regions of a minipig model [82].

Notably, various scaffolding materials with differing chemical, physical, and mechanical characteristics have been selected for use in pulp/dentin and bone regeneration protocols using dental MSCs, including long-lasting porous bioceramics (e.g., HA, β-TCP, or bioactive glasses), natural molecules of medium duration (e.g., collagen, chitosan, hyaluronic acid-based hydrogels, and silk fibroin), and short-life polymers, such as Polyglycolic Acid (PGA), Polylactic Acid (PLA), or their combinations [39, 83]. In addition, injectable hydrogels (including self-assembling multidomain peptides [84] and a commercial blend Puramatrix™) [85] have been suggested for pulp regeneration in the light of their ability to form nanofibrous matrices under physiological conditions. Recent studies have also proposed demineralized/chemically Treated Dentin Matrices (TDMs) [86] or Cryopreserved Treated Dentine Matrices (CTDM) [87], as ideal biologic scaffolds, because of their combination of favorable mechanical properties and ability to act as a reservoir of dentinogenesis-related growth/morphogenetic factors [88]; this is also validated by *in vivo* studies [89, 90]. Finally, strategies to improve stem cell/scaffold interfaces also include incorporation of various bioactive molecules [29], as the third component of the TE triad (cells/scaffolds/growth factors). The application of such growth factors without stem cells, in a cell homing versus cell transplantation strategy, has also been suggested as a more clinically translational approach for dentin-pulp regeneration. Based on this concept, ectopic regeneration of dental pulp-like tissues using basic Fibroblast Growth Factor (b-FGF), Vascular Endothelial Growth Factor (VEGF), or Platelet-Derived Growth Factor (PDGF) with a basal set of Nerve Growth Factor (NGF) and Bone Morphogenetic Protein 7 (BMP-7) has been reported [91], while other researches achieved complete pulp regeneration

Table 1: Marker expression in dental MSCs (SHED, DPSCs, and SCAP) and factors identified in their secretomes.

Dental MSCs	Positive markers	Negative markers	Factors in secretome involved in angiogenesis	Factors in secretome involved in neurogenesis
Stem cells from Human Exfoliatd Deciduous teeth (SHED)	CD90, CD73, CD105, CD146, STRO-1, CD44, CD13 Nestin, DCX, βIII-tubulin, NeuN, GFAP, S-100, A2B5, CNPase Nanog, Oct3/4, SSEAs (-3, -4)	CD11b CD45 CD34 CD14 CD19 CD43	VEGF-A, VEGF-C, EG-VEGF (PK-1), HGF, IGF-1, FGF-2, SDF-1, SCF, EGF, TIMP-1, -2, MMP-2, -3, -9, MCP-1, ANG, TGF-b	BDNF, GDNF, MCP-1, ED-Siglec-9, IL-6, NRCAM, GDF-15, NCAM-1, TACE, Nidogen-1 NRG-1, TIMP-1, -2, HGF, SCF, MMP-2, -3, -9, decorin, IL-22, IL28A, IL-29, osteopontin, SCF, ANG, VEGF-A, EG-VEGF, VEGF-C, growth hormone, insulin, PIGF, TGF-b
Dental Pulp Stem Cells (DPSCs)	CD90, CD73, CD105, CD146, STRO-1, CD106, CD29, CD49, CD51, CD61, CD166, ALDH1, 3G5, CD44, CD9, CD10 CD13, CD59, MSCA-1, CD81, CD24 CD271/NGFR, Nestin, DCX, βIII-tubulin, NeuN, GFAP, S-100, A2B5, CNPase, musashi-1, p75, snail-1, -2, slug, Sox-9 Nanog, Oct3/4, SSEAs (-1, -3, -4, -5), Notch-1, -2, -3	CD45 CD34 CD14, CD19 CD31, CD117, CD133 HLA-DR	VEGF, uPA, IL-8, TSP-1, IGFBP-3, TIMP-1, -4, MMP-9, PAI-1 (serpin E1), endostatin, ANGPT-1, ANG, DPPIV, EDN-1, PTX-3, PEDF (serpin F1), PDGF-AA and PDGF-AB/BB, MCP-1	NGF, BDNF, NT-3, CNTF, GDNF, MCP-1, VEGF, FGF-2, PDGF-AA and PDGF-AB/BB, MMP-9, ANG, TIMP-1, -4
Stem Cells from Apical Papilla (SCAP)	CD90, CD73, CD105, CD146, STRO-1, CD106, CD29, CD49, CD51, CD61, CD166, ALDH1, 3G5, CD44, CD9, CD10 CD13, CD59, MSCA-1, CD34, CD81, CD24, c-Kit CD271/NGFR, Nestin, NSE, CNPase, musashi-1, p75, snail-1, -2, slug, Sox-9 Nanog, Oct3/4, SSEAs (-1, -3, -4, -5), TRA-1-60-, TRA-1-81, Notch -2, -3	CD14 CD18 CD34 CD45 CD117 CD150	ANGPT-1, ANG, DPPIV, EDN-1, PTX-3, PEDF (serpin F1), IGFBP-1, -2, -3, TIMP-1, -4, TSP-1, VEGF, uPA, Activin A, HGF, FGF-7, PIGF (serpin E1), TGFb, CXCL-16, persephin, NRG1-b1, MCP-1	MDK, NEGF-1 (PTN), NEGF-2, CXCR4, MANF, AHNAK, NRP2, ANG, TIMP-1, -4, CXCL-16, NRG1-b1, MCP-1

Table 2: Key references on the osteo/odontogenic, angiogenic, and neurogenic differentiation potential of dental MSCs *in vitro* and *in vivo*.

Dental MSCs	Osteo/odontogenic differentiation		Angiogenic differentiation		Neurogenic differentiation	
	In vitro	*In vitro* and/or *in vivo* (bone or dentin/pulp)	*In vitro*	*In vitro* and/or *in vivo*	*In vitro*	*In vitro* and/or *in vivo*
Stem cells from Human Exfoliated Deciduous teeth (SHED)	[26, 98]	[2, 53, 55, 60, 67, 82, 85, 89, 90, 93–95]	[67]	[119, 120]	[57, 67, 142, 143]	[36, 53, 148, 150]
Dental Pulp Stem Cells (DPSCs)	[14, 21, 26, 49, 51, 54, 59, 61, 88, 97, 123]	[1, 13, 53, 55, 56, 62, 67, 69–81, 84, 113, 114]	[51, 59, 67, 115, 123]	[62, 116–118, 121, 122, 126–128]	[51, 54, 59, 61, 67, 135–139, 143, 144, 147]	[53, 56, 116, 140, 141, 151, 152]
Stem Cells from Apical Papilla (SCAP)	[4, 44, 47, 50, 97, 98, 102, 104–106, 108, 110–112]	[52, 76, 99–101, 103, 106, 109]	[58, 124, 125]	[121]	[47, 52, 144]	[145, 146]

in pulpectomized mature dog teeth by using a stromal cell-derived factor-1a- (SDF-1a-) loaded silk fibroin scaffold without DPSC transplantation [92].

Significant similarities, but also differences in osteo/odontogenic differentiation potential, have been reported for *SHED*. Pivotal studies by Miura et al. [2] showed that SHED are characterized by osteoinductive capacity *in vivo*, but only a quarter of the clones showed potential to generate ectopic dentin-like tissue. SHED were also able to form an osteoinductive template in immunocompromised mice,

inducing the recruitment of host murine osteogenic cells to repair critically sized calvarial defects [93]. Recently, it was shown that both DPSCs and SHED combined with Platelet-Rich Plasma (PRP) were able to regenerate vascularized bone tissue around dental implants in dog and puppy models, respectively [55]. Recent reports have also shown that 5-year cryopreserved SHED were still able to proliferate and undergo osteogenesis without immune reaction in a 9 mm mandibular defect in dogs [94] and to enhance mandibular distraction osteogenesis in a rabbit model [95].

Despite those studies showing the preferential osteogenic versus odontogenic differentiation potential of SHED, other studies also report that SHED are capable of differentiating into functional odontoblasts *in vitro* [2] and of regenerating a tissue with architecture and cellularity resembling the physiologic dental pulp when seeded in biodegradable scaffolds prepared within human tooth slices and transplanted into immunodeficient mice [89]. It has been recently shown that SHED can generate functional dental pulp when injected with scaffolds (Puramatrix or rhCollagen) into full-length root canals [85].

A very recent and interesting study mapping potential molecular differences between SHED and DPSCs identified several differentially regulated genes [96]. Among these high-mobility group AT-hook 2 (HMGA-2) protein, a stem cell-associated marker, together with several proliferation-related genes showed a robust expression in SHED, while ECM genes, such as collagen I, fibronectin, and signaling molecules, such as VEGF, Fibroblast Growth Factor Receptor 1 (FGFR-1), and Insulin Growth Factor Receptor 1 (IGFR-1) were upregulated in DPSCs, suggesting that SHED are more competent in self-renewal and proliferation and DPSCs in signaling and matrix synthesis.

Finally, *SCAP* appear as a cell population similar to, but significantly different from, DPSCs [97]. Although the apical papilla is the precursor tissue of the radicular pulp [18], it is an anatomically distinct area separated by a cell-rich zone. SCAP have been reported to display a higher proliferation rate, number of population doublings, dental tissue regeneration capacity, and STRO-1 expression in comparison with DPSCs [68]. In addition, SCAP have shown a higher expression of survivin and telomerase, two proteins critical for cell proliferation [4]. In contrast, SCAP have been shown to express lower levels of markers, such as Dentin Sialoprotein (DSP), Matrix Extracellular Phosphoglycoprotein (MEPE), transforming growth factor receptor II (TGFbRII), and Vascular Endothelial Growth Factor receptor I (VEGFR1) compared to DPSCs [19]. A recent study demonstrated significant variations in the mineral composition of mineralized tissues produced *in vitro* by various types of dental MSCs [98]. SCAP and SHED produced a more highly mineralized matrix in comparison with DPSCs but with lower crystallinity and carbonate substitution.

Studies have indicated that SCAP are capable of differentiating into odontoblastic-like cells [97] and osteogenic cells [99] *in vitro* and into vascularized dentin/pulp-like complexes, after transplantation into immunodeficient mice, in an appropriate carrier substrate [4, 68]. Additionally, transplantation of SCAP inside a root-shaped HA/TCP block coated with PDLSCs into the extraction socket of a minipig lower incisor demonstrated the successful regeneration of the root/periodontal structure over which a porcelain crown has been placed [100]. Furthermore, SCAP could generate cement/woven bone-like tissue with embedded cementocytes/bone-like cells; however, the precise nature of the mineralized tissue produced was not identified [101].

Although SCAP have not been so closely investigated as DPSCs, several later reports provide significant insight into the particular molecular mechanisms responsible for SCAP biological responses to various microenvironments, providing data pivotally useful for the design of future regenerative strategies for targeted dental TE. Among key inductive factors demonstrated to exhibit a major role in SCAP osteo/odontogenic differentiation are BMP-2 [102], BMP-9 [103], and the conjunction of BMP-2 and VEGF [104]. Other studies have highlighted the importance of Nuclear Factor I-C (NFIC) known to be involved in the regulation of root development [105] and its regulatory interaction with transforming growth factor-β1 (TGF-β1) in inducing odontogenic transformation of SCAP [106]. In a recent study, Plasminogen activator Inhibitor 1 (PAI-1), has been suggested as pivotal factor in inducing odontogenic differentiation of SCAP [107]. Finally, a number of studies have also closely studied the signaling pathways regulating odontogenic differentiation of SCAP; among these, differential activation of ALK5/Smad2 and MEK/ERK [108], canonical Wnt synergistic with BMP-9 [109], Notch [110], canonical NF-κB [111], and ERK and JNK signaling pathways in combination with a mechanical stress stimulus have been indicated as having a paramount role in the committed differentiation of SCAP [112].

It must be noted that a major problem concerning *in vivo* studies aiming at regenerating functional dentin-pulp complexes or bone around teeth and implants is the fact that the majority have been conducted in ectopic implantation models, mostly subcutaneously into immunocompromised mice [13, 29, 67, 76], and to a less extent in renal capsules of rats [113]. In contrast, only few attempts in orthotropic large-animal models (dogs or mini pigs) have been performed by a sole research group [70–73], probably in view of the considerable economic costs involved together with the ethical issues associated with animal welfare. Most recently, a root implant model in minipigs involving the middle sections of roots from freshly extracted swine incisors filled with scaffolds containing DPSCs and then implanted into the fresh postextraction sockets has been designed. This provided a valuable animal (although not really orthotopic) model simulating clinical situations [114].

Current pulp regeneration protocols have also been recently systematically reviewed by Fawzy El-Sayed et al. [30]. From 1364 screened articles the authors selected five studies for the quantitative analysis complying to specific inclusion/exclusion criteria. They revealed that stem cell transplantation was linked with significantly greater regenerated pulp and dentin per root canal total area when compared with controls. A solitary study reported on capillaries/nerves per unit surface area and found that the density of both nerves and capillaries was noticeably greater in stem/progenitor cell-transplanted pulps compared with controls [72]. The authors

emphasized the paucity of quantitative evaluations of the amount of regenerated tissue and the lack of consensus about defining the primary outcomes of the regenerative procedures, including neural, vascular, soft, or hard tissue/dentinal regeneration as the primary limitation of the majority of *in vivo* studies. It was also mentioned that conclusions were drawn on the basis of histological assessments without additional functional innervation and vascularization tests to provide a more comprehensive assessment of functional pulp/dentin regeneration. Interestingly, the majority of studies showed a high risk of selection, performance, detection, and reporting bias. The main causes of this bias were attributed to the fact that none of the studies had performed sample size calculations to enhance statistical power, while lack of standardization of the animal models and type of experimental defects was a cause of significant heterogeneity. In addition, no split-mouth designs were applied, while clustering of statistical units within the same animal was a common practice. Finally, randomization of treatments and blinding of examiners were reported in very few studies.

3.2. Angiogenic Properties of Dental MSCs

3.2.1. Endothelial Transdifferentiation Potential of Dental MSCs. Encouraged by the exceptional "plasticity" of dental MSCs, a limited number of studies have attempted to investigate the endothelial transdifferentiation potential of DPSCs [51, 62, 115–118], SHED [119, 120], and SCAP [58, 121] in the presence of specialized angiogenesis-inductive media (summarized in Table 2). The endothelial shift of MSCs in these studies is mainly indicated by the upregulation of typical endothelial cell (EC) markers, such as PECAM-1, VEGFR-2, vWF, and VE-cadherin and further evidenced by functional assays, such as ability to form capillary-like structures on Matrigel or other matrices or by uptake of Acetylated-Low Density LipoProtein Lipase (Ac-LPL), but also by various *in vivo* assays, including mouse Matrigel assays and Chicken Chorioallantoic Membrane (CAM) assays [33, 34, 122].

According to the *in vitro* studies, coculture of DPSCs [123] or SCAP [124] with ECs significantly improved the angiogenic potential of ECs, especially under hypoxic conditions [124, 125]. SHED differentiation into VEGFR-2/CD31 positive EC-like cells has been shown through a VEGF/MEK-1/ERK signaling pathway [120]. Moreover, a VEGFR-2-dependent function of murine DPSCs as pericyte-like cells has been substantiated, since a shRNA knockdown of VEGFR-2 produced a decreased expression of VEGFA, VEGF receptors, and Ephrin B-2 and reduced vascular density of Matrigel plugs *in vivo* [118]. Finally, short-term exposure of SCAP to serum, glucose, and oxygen deprivation (SGOD) conditions has been shown to be potent in eliciting a proangiogenesis program, as evidenced by activation of VEGF/VEGFR and Angiopoietins/Tie pathways [58]. These results confirm that dental MSCs can actually show considerable adaptability to severely adverse microenvironmental conditions, by undergoing a rapid endothelial shift rather than activating apoptosis.

Despite encouraging data, most of the above-mentioned studies actually indicate but fail to prove a functional and homogenous *in vitro* differentiation of MSCs into ECs, suggesting that it might be inaccurate to designate EC-switched MSCs as mature ECs, but rather as an intermediate EC-like population, primarily supporting typical functions of mature ECs or mainly acting in a paracrine way (as analyzed below). Thus, identification of additional microenvironmental cues as well a more detailed understanding of the molecular mechanisms responsible for skewing dental MSCs into mature ECs could constitute a critical step for utilizing them as neoangiogenesis sources in TE.

In addition to *in vitro* data, additional evidence from *in vivo* studies could show that SHED differentiate into ECs when seeded in biodegradable scaffolds and transplanted into immunodeficient mice [89]. DPSCs alone or primarily in coculture with Human Umbilical Vein Endothelial Cells (HUVEC) when encapsulated in three-dimensional peptide hydrogel matrices (PuraMatrix) were able to support cell survival, migration, and capillary network formation and to regenerate vascularized pulp-like tissue after transplantation in mice [126]. Iohara et al. [127] were able to isolate and characterize a highly vasculogenic subfraction of side population (SP) of CD31−/CD146− porcine tooth germ-derived dental MSCs, while in later study the CD31− pulp fraction was used successfully to reconstitute blood flow and capillary density in a mouse hindlimb ischemia model, to induce neurogenesis in a cerebral ischemia model, and finally to reinstitute a vascularized pulp in an ectopic root transplantation model [116]. Similar results were reported for human DPSCs, which showed ability to induce angiogenesis and reduce infarct size in a myocardial infarction rat model [128].

3.2.2. Angiogenic Properties of Dental MSC Secretome. Despite encouraging data on the endothelial transdifferentiation potential of dental MSCs, significant lines of evidence indicate that the rate of MSC engraftment after local or systemic delivery *in vivo* remains problematically low at <10% [129]. This contrasts with several other lines of evidence suggesting that the angiogenic effects of MSCs are primarily derived from secretion of several soluble factors, such as growth factors, cytokines, chemokines, Extracellular Matrix proteins and proteases, or even genetic material [130] as a response to various microenvironmental cues (summarized in Table 1), rather than their endothelial transdifferentiation. There is growing interest in the investigation of MSC "secretome" with the increasing recognition of the paracrine/autocrine role of MSCs to many biological functions, including cell proliferation, differentiation, signaling, apoptosis, angiogenesis, and neurogenesis. Furthermore, the use of cell-free approaches offers several advantages with respect to concerns related to immunogenicity, tumorigenicity, and transmission of infections, which, although currently considered very low for autologous therapies with adult MSCs, are still under investigation in "proof-of-concept" clinical studies being underway in various fields of Medicine and Dentistry.

Dental MSCs (DPSCs and SCAP) have been shown by recent studies to secrete, under various stress conditions, several pro- and antiangiogenetic factors able to stimulate endothelial motility and function [58, 121]. In particular, it has

been shown that DPSCs secrete several proangiogenic factors (VEGF, Monocyte Chemotactic Protein 1- (MCP-1), IL-8, Insulin-Like Growth Factor Binding Protein 3 (IGFBP-3), and Urokinase Plasminogen Activator (uPA)) and antiangiogenic factors (Tissue Inhibitor Of Metalloproteinase 1 (TIMP-1), Plasminogen Activator Inhibitor-1 (PAI-1), endostatin, and Thrombospondin-1 (TSP-1)), under serum deprivation conditions [117], while in a later study by the same group differential angiogenic secretome expression was observed among various dental MSC types, including DPSCs, SCAP, and DFSCs [121]. Interestingly, DPSCs and SCAP elicited a predominant proangiogenic effect *in vitro* and *in vivo* compared to DFSCs, which renders them an attractive cell source for angiogenesis applications. Subsequently, it has been shown that, under serum, glucose, and oxygen deprivation (SGOD) conditions, SCAP release higher numbers and amounts of proangiogenic factors (Angiogenin, IGFBP-3, and VEGF) and lower amounts of antiangiogenic factors (Serpin-E1, TIMP-1, and TSP-1) in comparison with SOD or SD alone, providing insights into the optimal preconditioning strategies for SC-based treatment of damaged/ischemic tissues [58]. Most recently, SCAP secretome has been extensively profiled [131]; it was found that a total of 2,046 proteins are released, including chemokines, angiogenic, immunomodulatory, antiapoptotic, and neuroprotective factors, and ECM proteins. SCAP secreted significantly larger amounts of chemokines and neurotrophins than BMMSCs, whereas BMMSCs secreted more ECM proteins and proangiogenic factors.

It is significant to note that secretion of various soluble factors by MSCs may occur either via exocytosis or via release of extracellular vesicles (EVs). These include either exosomes (30–100 nm in size, originating from intracellular microvesicles) or microvesicles (100–1000 nm in size, originating from the plasma membranes) [132]. A recent study showed that DPSC-derived exosomes suppress carrageenan-induced acute inflammation in mice [133]. This was among other reasons attributed to the fact that SHED exosomes contain annexin A1 that acts as mediator of the antimigratory effects of glucocorticoids, thereby suppressing edema formation.

3.3. Neurogenic Properties of Dental MSCs

3.3.1. Neurogenic Transdifferentiation Potential of Dental MSCs. Numerous studies so far have highlighted the inherent neurogenic differentiation potential of dental MSCs (summarized in Table 2), attributed to their neural crest embryonic origin [134]. *DPSCs* [51, 135–141], *SHED* [36, 57, 142, 143], and *SCAP* [4, 47, 125, 144–146] have shown enhanced potential for differentiation into a variety of neural lineages, including functionally active dopaminergic cells and glial cells, leading proposals for dental MSCs to be used for regenerative therapy of several neurodegenerative diseases [37]. Notably, dental MSCs, while still in an undifferentiated state, constitutively express markers of neural stem/progenitor, as well as mature neural cells, including SOX-2, tenascin C, ENO-2, MAP2ab, c-FOS, Nestin, Neurofilament (NEF-H and NEF-L), Glial Fibrillary Acidic Protein (GFAP), bIII-tubulin, Microtubule-Associated Protein 2 (MAP-2), and many others [143]. However, the data regarding the neural differentiation potential of dental MSCs seem to vary for different cell types and their subpopulations in the vast body of studies published to date [37], preventing safe comparative conclusions regarding the superiority of any one cell type in regenerating neural tissues.

An overview of existing literature actually reveals the wide range of diversity encountered in the neural differentiation protocols used so far by different research groups. This complexity is connected to (1) the culture microenvironment, (2) the application of either single- or in most recent studies multiple-stage differentiation protocols often alternating cell suspension (in the form of spheroids/neurospheres) with adherent cell culture systems, and (3) the biological endpoints explored by each study.

Regarding the cell culture conditions, a variety of substrates, predominantly poly-l-lysine [36, 57, 140], poly-l-ornithine with/without lamin [147], gelatin [4, 47], and more rarely other substrates, have been used, while in most studies direct culture in culture-treated polystyrene [61, 125, 135, 137, 138, 141, 144] forms the commonest practice. However, the absence of comparative studies makes conclusions about the superiority of one substrate over the other impossible. Regarding the neuroinductive culture media, most studies use either the Neurobasal A or conventional primarily Dulbecco's Modified Eagle's Medium (DMEM)/F12 media in their neural differentiation protocols. These are used in conjunction with various neural supplements (most commonly the B27 [36, 125, 142, 144, 148, 149], but also the N2 consisting of a mixture of insulin, transferrin, progesterone, selenium, and putrescine [137] and the insulin-transferrin-selenium (ITS) supplement [54] or their combinations [143]) in a serum-free approach. Alternatively, in other studies, the media are supplemented with conventional fetal calf (FCS) or Fetal Bovine Serum (FBS) at least for the first-stage preincubation phase [135]. In addition to these supplements, various growth factors, mainly Epidermal Growth Factor (EGF) and basic Fibroblast Growth Factor (FGF-2) and, to a less extent, Nerve Growth Factor (NGF), Neurotrophin 3 (NT-3), Brain-Derived Neurotrophic Factor (BDNF), Sonic Hedgehog (SHH), Glial Cell Line-Derived Neurotrophic Factor (GDNF), and so forth, have been used to induce neural maturation. These are additionally supported by neuroinductive small molecules, such as beta-mercaptoethanol, 5-azacytidine, retinoic acid, dibutyryl cyclic adenosine monophosphate (dbcAMP), 3-Isobutyl-1-Methylxanthine (IBMX), Dimethyl Sulfoxide (DMSO), Butylated Hydroxyanisole (BHA), forskolin, and hydrocortisone [37]. All these factors and supplements have been variously used in a number of studies, overall making it impossible to define an ideal culture microenvironment or neural induction approach.

Neural differentiation in the majority of these studies is evaluated by the expression of neural markers (such as NCAM, GFAP, GAP-43, GABA, NeuN, bIII-tubulin, synapsin, NSE, and NFL [37]), while very few have carried out functional assessments. Methods most applied to confirm functional neural transformation include the patch-clamp

analysis of the voltage-dependent Na^+ or K^+ channels [139–141, 147] and the fluorescent detection of intracellular Ca^{2+} flux upon stimulation with neurotransmitters [135].

Finally, a small number of studies have performed *in vivo* transplantation of dental MSCs to assess cell engraftment and neural marker expression [140] but also for neural disease treatment in various experimental animal models. Predifferentiated SHED-derived neurospheres were applied into the striatum of parkinsonian rats and significant improvement in behavioral impairment as compared to the implantation of control undifferentiated SHED was reported [36]. Similar results were achieved after inducing neural maturation of SHED into dopaminergic neuron-like cells and transplantation in parkinsonian rats [150]. Moreover, transplantation of neural-induced SHED in a rat Spinal Cord Injury (SCI) model led to complete recovery of hindlimb motor function [148]. All of the above studies support that neural preinduction of undifferentiated MSCs before *in vivo* transplantation increases the expression of neural surface receptors and therefore the grafting efficiency into the nervous system, potentially improving clinical outcomes. In a very interesting recent study, the entire apical papilla was transplanted in a SCI (hemisection) model, in comparison to transplantation of human SCAP inside fibrin hydrogels [146]. Significantly, the delivery of SCAP in their original niche (entire apical papilla) improved gait and reduced glial reactivity, as compared to the classical TE approach of cell expansion and delivery in 3D scaffolds. This highlights the importance of the 3D organization of stem cells and the surrounding microenvironment. Finally, another important set of *in vivo* studies were carried out by Sasaki et al. [151, 152]. They used silicone tube conduits filled with a collagen gel containing rat DPSCs and managed to bridge an experimental gap in the rat facial nerve. In a subsequent study, [152] the same group replaced the nonabsorbable silicon material with a degradable PLGA tube that was readily resorbed simultaneously promoting nerve regeneration.

3.3.2. Neurogenic Properties of Dental MSC Secretome. There is a growing body of evidence questioning the ability of dental MSCs to differentiate into functional neurons after transplantation *in vivo* and supports the idea that their neurogenic action is primarily exerted as in the case of angiogenesis through multiple neurotrophic factors found in their secretion products and acting in a paracrine manner (Table 1). Sakai et al. [53] showed that transplantation of DPSCs into rat SCI lesions lead to functional recovery despite only glial rather than neuronal differentiation being observed under these extreme conditions, suggesting a paracrine-mediated action. Mead et al. [153] contended that DPSCs have limited potential to differentiate into neurons and fail to integrate into the retina, after transplantation. The same group found that DPSCs have a more favorable neurotrophic secretome, rich in NGF, BDNF, and NT-3, in comparison with BMMSCs [154], which is efficient in promoting survival and neuritogenesis/axogenesis of bIII-tubulin positive retinal cells after transplantation into the vitreous body of the eye; this effect was neutralized after the addition of specific Fc-receptor inhibitors, overall suggesting a paracrine effect. Various other studies have reported on the existence of multiple neurotrophic factors, including NGF, BDNF, NT-3, CNTF, GDNF, VEGF, and FGF-2 [53, 154–156] in DPSC secretome. Finally, DPSCs mobilized by G-CSF were shown to secrete a panel of neurotrophic and angiogenic factors (BDNF, GDNF, IGF, NGF, and VEGF) capable of regenerating myelinated fibers in a rat sciatic nerve defect model [157].

A series of studies on the neuroregenerative/neuroprotective properties of SHED secretome have been also published by the group of Mita et al. using various experimental neural disease models. They have found that SHED-derived, serum-free Conditioned Medium (SHED-CM) improved cognitive function in an Alzheimer's disease mouse model [158] and enhanced recovery of focal cerebral ischemia in rats after intranasal administration [159]. Additionally, SHED-CM after intracerebral administration in mice with perinatal hypoxia-ischemia-induced brain injury generated an anti-inflammatory environment, reduced tissue loss, and significantly improved the neurological outcome by converting a M1 proinflammatory to an M2 anti-inflammatory environment. The latter was primarily attributed to the combined secretion of MCP-1 and the Secreted Ectodomain of Sialic Acid-Binding Ig-Like Lectin-9 (ED-Siglec-9) among 28 proteins detected in SHED-CM [160]. These results have been also validated by other groups that used SHED-CM for peripheral nerve regeneration across nerve gaps on rat sciatic nerve gap models [161]. A recent study also investigated the neuroprotective role of SHED-derived exosomes, highlighting another mechanism of their paracrine-mediated action [162].

In contrast to DPSCs and SHED, little data exist so far on the neurogenic activity of SCAP secretome. A recent study [145] demonstrated that SCAP release BDNF responsible for triggering directed axonal targeting both *in vitro* and *in vivo*, as shown by microfluidic and Matrigel implant experiments. Yu et al. also detected several neurotrophic factors in SCAP secretome, including Midkine (MDK), Pleiotrophin (PTN), Mesencephalic Astrocyte-Derived Neurotrophic Factor (MANF), Neuroblast Differentiation-Associated Protein (AHNAK), and Neurophilin 2 (NRP2).

Thus, we seem to be able to conclude that the neuroregenerative/neuroprotective properties of dental MSCs are primarily exerted through a paracrine mechanism rather than on their potential for *in vivo* differentiation into mature neural phenotypes. Current research trends are focusing on the preconditioning strategies to enhance neurogenic properties of dental MSC secretome, as an effective surrogate therapeutic module for stem cell transplantation therapies in the treatment of neurodegenerative diseases.

4. Establishment of Clinical-Grade Dental MSCs and Challenges to Be Overcome before Clinical Application

Despite the very promising results of the plethora of TE approaches published to date on the application of dental MSCs for the regeneration of various tissues, very few clinical trials mainly in the form of new methodological paradigms

or "proof-of-concept" (phase I/II, safety/efficacy) studies have been conducted or are currently being conducted. This is in complete contrast to the rapidly growing number of clinical trials using other MSC sources (mainly BM-MSCs) in treatment of various bone/articular, cardiovascular, neurological, immune, and blood pathologies (data found on https://clinicaltrials.gov/). The unique biological value of MSCs lies in the combination of differentiation potential into tissue-forming cells and the paracrine-mediated revascularization/reinnervation of the regenerated tissues, under an immunosuppressive/immunoregulatory "deck" limiting probability for adverse reactions [163].

However, one of the basic factors still hindering extensive clinical application of MSC-based therapies is among others the difficulty encountered in the *ex vivo* expansion of clinical-grade, xeno-free MSCs under Good Manufacturing Practice (GMP) conditions, as described in the EU Regulation 2003/94/EC [164] (*GMP is that part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by the marketing authorization*) and in compliance to the EU regulations (1394/2007) [165] established for the clinical use of Advanced Therapy Medicinal Products (ATMPs). These have been defined as "*biological medicinal products containing or consisting of living cells or sub-cellular fractions with biological functions*." AMTPs do not belong to the same category of drugs or transplants because (1) they contain viable allogeneic or autologous cells undergoing *ex vivo* substantial manipulations (as defined in the EU Regulation 1394/2007, Annex 1) and (2) they may be applied in "non-homologous use," that is, at sites not physiologically present or to perform biological functions they do not usually participate in. ATMPs are considered Cell-Based Medicinal Products (CBMPs) when containing living cells or tissues. CBMPs are "*medicinal products presented as having properties for, or used in or administered to, human beings with a view to treating, preventing or diagnosing a disease in which the pharmacological, immunological or metabolic actions are carried out by cells or tissues*" [166].

The recent literature on the subject has questioned whether epigenetic (e.g., homing receptor/ligand expression, cytokine/growth factor production, lineage commitment/differentiation, and programmed senescence) [20, 21] and genetic alterations (e.g., transformation, fusion, and gene transfer) occurring during expansion culture [167] may affect the therapeutic potential of stem cells in a positive or negative way. For example, the changes shown might be beneficial for site-specific application depending on the target tissues but adverse for systemic administration or vice versa. Since development of adequate numbers of high quality SCs at early passages is a prerequisite for any safe cell therapy treatment, considerable effort has been put into evaluating the consequences of the cultivation process on stem cell behavior, in particular, in developing reliable standardization protocols in the form of Standard Operating Procedures (SOPs) to be routinely applied to characterize (1) phenotypic and genetic stability of cultured dental MSCs, (2) efficacy in regenerating target tissues, (3) the permitted population doubling before senescence becomes a problem, (4) the absence of microbial, viral, fungal, mycoplasma, endotoxin, or other contamination in cultured cells, and (5) lack of tumorigenicity, toxicity, and immunogenicity, something highlighted in recent reports discussing current challenges towards clinical application of dental MSCs [168, 169]. It becomes clear from these reports that the lack of reliable characterization methods and reference standards for the evaluation of each of the above mentioned important parameters presents a major hurdle for the development of cGMP-grade cells and respective CBMPs.

Among other parameters, significant efforts have been made to replace animal sera used in conventional media due to their highly variable and often unknown composition, the immunological risks associated with serum proteins, and the potential of transmission of prion diseases [170]. Considering the significant impact of serum components in MSC maintenance and multilineage differentiation [171], efforts to replace it with autologous or allogeneic sera or with proprietary serum-free media of unknown formulations by different companies have yet to be validated for their efficacy, while their use is still restricted by the prohibitive cost. The need for development of chemically defined media which can maintain "stemness" without adversely affecting MSC function, immunoregulatory properties, and phenotype remains a significant problem to be overcome for cGMP production of MSCs [172].

Apart from establishment of clinical-grade dental MSCs, SOPs must be also developed for each of the successive steps leading to clinical application including (1) scaling-up of culture systems to produce the desired cell numbers based on the targeted therapeutic goal (upstream process); this might range from thousands to billions of cells depending on the size of the defect; a major problem to achieve this lies on the significant variability in donors and the derived cell lines, which may significantly complicate the culture scale up for high-throughput production in automated and parallel culturing systems [173]; (2) harvesting (preferably by mechanical means or by a cGMP enzymatic process using recombinant enzymes and avoiding porcine-derived trypsin or similar reagents [174]), volume reduction, and isolation of the desired cell populations (downstream possess); in particular, for cell isolation, molecular-tagging based methods have been employed to purify dental MSCs by using specific molecular markers; among these methods, fluorescence-activated cell sorting (FACS) has been mostly used offering the advantage of multiparametric analysis for several markers [175]; although FACS systems have been recently upgraded to cGMP function [176], they have limited capacity for large-scale MSC processing and significantly high costs; the same reservations can be made for the magnetism and adsorption-based cell separation systems (MACS), which are considered to represent the "gold" standard method for cell purification, but they also have limited scalability and low efficacy to obtain high cell numbers [177]; (3) loading into appropriate carriers and preserving the final ATMP in safe conditions for immediate or later application. The latter requires robust cryopreservation processes with minimal adverse effects on cell survival and "stemness" characteristics [178]. While the conventional slow-freezing and rapid-thawing method

in liquid nitrogen or its vapor phase is the "state-of-the-art" method [179], other methods such as vitrification by the "open pulled straw" method using high cryoprotectant concentrations and ROCK inhibitor treatment together with flash freezing in liquid nitrogen have been proposed to result in higher cell survival rates [180]. However, direct contact with liquid nitrogen is considered a major drawback, as it may increase the risk of cross-contamination among samples. It still remains quite challenging that all of the above-mentioned steps, which are routinely used for research purposes, have to be optimized, upgraded, and standardized to be carried out under cGMP conditions and followed by quality assessments to secure safety and efficacy of the delivered cell-based products, making the whole process quite complicated and extremely time-consuming.

Other scientific, technological, policy, and commercial development challenges and hurdles have also to be addressed before extensive clinical application of dental MSC therapies using commercially available ATPMs to replace the biomaterial-based treatment modalities currently being used in clinical dentistry, in a solid, evidence-based manner. Another challenging point to be considered before application of dental MSC therapies in clinical dentistry is that most currently applied biomaterials and clinical methodologies have despite reported biological and technical complications high overall survival and success rates [181]. In addition, they are related to nonlife threatening diseases; therefore any novel alternative therapies need to be shown to have marked superiority to be established as clinically routine processes.

In contrast to medical literature, very limited published work exists so far on the development of clinical-grade dental MSCs and related ATMPs. In an effort to avoid serum-containing media, Tarle et al. [182] evaluated the capacity of chemically defined serum-free culture systems to effectively expand and maintain the stem cell properties of SHED and PDLSCs. Although these cells proliferated at lower rates in serum-free conditions, their multilineage differentiation potential and differential expression of 84 stem cell-associated genes showed only minor differences compared to the serum-containing medium, thus validating application of such serum-free, cGMP conditions for their safe and effective expansion. The same group proposed use of fibronectin an important serum component for optimizing the initial recovery of DPSCs from pulp biopsies under serum-free conditions [183]. Lizier et al. [184] developed a protocol of scaling-up large numbers of dental MSCs at early passages by mechanical transfer (i.e., without enzymatic treatment) into new culture dishes, thus minimizing risk of loss of their "stemness." Other novel cell culture systems for large-scale expansion such as cell factories and bioreactors have been proposed as extremely effective for other oral MSC types [169]. However, no studies exist so far on the application of these systems to dental MSC expansion, which would be important towards optimizing 3D microenvironments for targeted dental TE.

A recent report [185] described manufacturing strategies of DPSC-based ATMPs to improve safety, efficacy, and consistency of their GMP production. The authors proposed the use of impacted third molars of young healthy donors between 5 and 7 Nolla's developmental stage (i.e., from complete crown upto one third of root completed) as ideal dental MSC sources. Regarding culture conditions, they proposed explant culture instead of enzymatic dissociation, although both methods have been associated with advantages and disadvantages [186, 187], both being capable of recovering approximately 1 million cells from one third molar within 2 weeks. The authors also proposed the precoating of culture plates with a mixture of human placental collagens I and III, use of GMP reagents, such as TrypLe® or Accutase®, and serum-free, clinical-grade culture media. Finally, they considered typical MSC markers such as CD105, CD90, and CD73 proposed by ISCT as being expressed by several MSC populations and therefore being nonspecific and proposed a large and multiparametric immunophenotyping as crucially important.

Based on the above, the process for the development of clinical-grade, xeno-free, GMP-compliant dental MSCs cultures and of the respective dental MSC-based CBPMs for preclinical and clinical evaluation is illustrated in Figure 1.

5. Dental MSCs-Based Clinical Trials

A significant number of studies have already been published using MSCs for the regeneration of orofacial bones, including sinus augmentation and regeneration of large- (cleft palate, alveolar ridge augmentation, maxillary replacement, mandibular fracture, replacement, and osteoradionecrosis cases) or small-size bone defects. These studies, mainly comprised of case reports/series together with few randomized controlled clinical trials (RCTs), have been systematically reviewed by Padial-Molina et al. [188] and Jakobsen et al. [189]. In the majority of these studies, BMMSCs and to a lesser extent other MSC types such as periosteum-derived MSCs or adipose tissue-derived MSCs have been used. These cells were cultured in growth media containing bovine serum, autologous serum, or other growth media and the cells either were preinduced or were not preinduced towards osteogenic differentiation before cell transplantation.

In contrast, very few clinical studies using dental MSCs have been published so far. Two successive studies by the group of Papaccio et al. [190, 191] reported on the use of autologous DPSCs, combined with a collagen sponge, to repair human mandible bone defects after extraction of third molars. The authors reported optimal vertical bone repair three months after surgery and complete restoration of periodontal tissue back to the second molars. They also evaluated the bone quality three years after transplantation and found that an entirely compact rather than spongy bone was the final outcome, without any serious clinical implications. Notably, these studies were performed in the absence of the above-described universally accepted protocols of GMP-compatible production of DPSCs. Nakashima et al. [192] published a series of studies in the potential of mobilized DPSCs to regenerate pulp in dog pulpectomized teeth and based on this they have initiated a clinical trial with pending announcement. This will provide significant insight into the potential for bringing dental MSC-based pulp regeneration into clinical reality. Finally, besides already published studies,

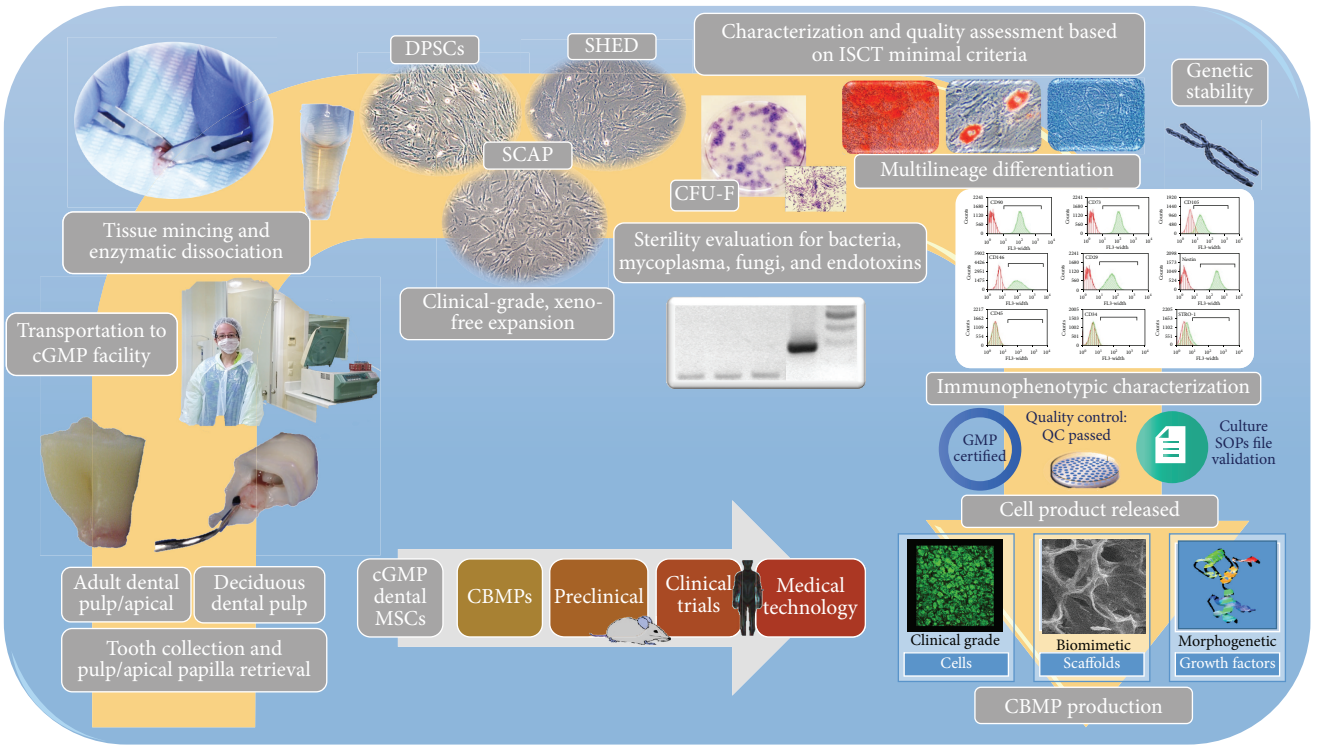


Figure 1: Process for the development of clinical-grade, xeno-free, GMP-compliant dental MSC cultures and of the respective dental MSC-based CBPMs for preclinical and clinical evaluation.

an electronic search in the https://clinicaltrials.gov/ database under the key words "mesenchymal stem cells" resulted in 595 clinical trials (excluding 11 which have been withdrawn), applying MSCs in various medical conditions, while only 4 clinical trials have been initiated using dental stem cells, as analytically described in Table 3.

6. Conclusions

Despite the constraints and limitations of current research approaches, it is safe to conclude that dental MSCs, including DPSCs, SHED, and SCAP, have been extensively studied in the past years by the dental research community using highly sophisticated *in vitro* and *in vivo* systems; this has led to a substantial understanding of their unique biological properties. As a result, bioengineering of various constituents of dental tissues such as dentin, pulp, or alveolar bone using dental MSC-based TE approaches has now been achieved. In addition, "proof-of-concept" studies for whole-tooth regeneration [193–195] are among the most fascinating recent advances, however, despite the intriguing possibilities that are opened up, there is still considerable need for further work to attain "clinical reality." Nevertheless, the major challenge still remains: how can and will the results of this extremely time-consuming, laborious, and costly research be translated into clinical therapeutic modules available to the patient; who is the final recipient of this groundbreaking technology. To consolidate the clinical utility of dental MSCs and/or their secretome in Regenerative Dentistry, there is pressing need for the initiation of well-designed RCTs aiming at the regenerative treatment of various oral tissues. This will allow a full understanding of the potential risks involved in the use of these technologies and spur efforts to surmount any problems and create a viable therapy option, a potential milestone in the application of science to clinical settings.

Abbreviations

Ac-LPL:	Acetylated-Low Density LipoProtein Lipase
AHNAK:	Neuroblast Differentiation-Associated Protein
ANG:	Angiogenin
ANGPT-1:	Angiopoietin 1
ASCs:	Adult Stem Cells
ATMPs:	Advanced Therapy Medicinal Products
BDNF:	Brain-Derived Neurotrophic Factor
BHA:	Butylated Hydroxyanisole
BMMSCs:	Bone Marrow Mesenchymal Stem Cells
BMP-2:	Bone Morphogenetic Protein 2
CAM:	Chicken Chorioallantoic Membrane
CAMs:	Cell Adhesion Molecules
CBMPs:	Cell-Based Medicinal Products
CFUs:	Colony Forming Units
CM:	Conditioned Medium
CNTF:	Ciliary Neurotrophic Factor

Table 3: Clinical trials using dental MSCs currently being registered at https://clinicaltrials.gov/ database.

	Title	Type of cells	Treatment	Disease	Stage	Number of subjects	(Estimated) completion date	Endpoints	Results	Clinicaltials.gov identifier
(1)	Periodontal Regeneration of Chronic Periodontal Disease Patients Receiving Stem Cells Injection Therapy	Allogeneic human DPSCs	Local injection at the local periodontal defects	Chronic periodontal disease	Recruiting	40	Dec. 2016	Improvement of baseline alveolar bone volume and clinical parameters, including probing depth (PD), Clinical Attachment Level (CAL), Quigley-Hein plaque Index (QHI), Bleeding on Probing (BoP)	Not reported yet	NCT02523651
(2)	Use of Mesenchymal Stem Cells for Alveolar Bone Tissue Engineering for Cleft Lip and Palate Patients	SHED (autologous)	Application of MSCs inside a collagen and hydroxyapatite biomaterial (Geistlich Bio-Oss®) into the defect	Alveolar bone TE for cleft lip and palate patients (secondary alveolar graft after completion of orthodontic treatment)	Unknown	5	Mar. 16	Amount and quality of regenerated bones (CT scans)	In all 5 patients bone formation closing the alveolar cleft was observed after 6 months	NCT01932164
(3)	Revitalization of Immature Permanent Teeth with Necrotic Pulps Using SHED Cells	SHED (autologous)	Application of scaffold-free SHED-derived pellet	Immature permanent teeth with pulp necrosis	Recruiting	80	Jul. 2017	Pulp status evaluated by dental pulp vitality tester; pulp revascularization examined by laser Doppler flowmeter; and the index of clinical examination Also, the degree of apical closure; the rate of increase in root length; and the change of root canal wall thickness.	Not reported yet	NCT01814436
(4)	Effect on Allogenic Mesenchymal Stem Cells on Osseointegration of Dental Implants	Allogeneic human DPSCs	The implant is dipped in the stem cell solution for 3 minutes so that the cells adhere to the titanium implant surface before placement at the osteotomy site	Improvement of implant osseointegration	Enrolling by invitation	10	Feb. 2017	Evaluation of primary and secondary stability is measured using Resonance Frequency Analysis (RFA).	Not reported yet	NCT02731586

CTDM: Cryopreserved Treated Dentine Matrices
CXCL-16: Chemokine (C-X-C motif) Ligand 16
dbcAMP: Dibutyryl Cyclic Adenosine Monophosphate
DCX: Doublin or Lissencephalin-X (encoded by *DCX* gene), most known as Doublecortin
DFSCs: Dental Follicle Stem Cells
DMEM: Dulbecco's Modified Eagle's Medium
DMP-1: Dentin Matrix Protein 1
DMSO: Dimethyl Sulfoxide
DPPIV: Dipeptidyl Peptidase-4
DPSCs: Dental Pulp Stem Cells
DSP: Dentin Sialoprotein
ECM: Extracellular Matrix
EDN-1: Endothelin 1
ED-Siglec-9: Secreted Ectodomain of Sialic Acid-Binding Ig-Like Lectin-9
EGF: Epidermal Growth Factor
EG-VEGF (PK1): Endocrine Gland-Derived Vascular Endothelial Growth Factor or Prokineticin-1
EVs: Extracellular Vesicles
FBS: Fetal Bovine Serum
FCS: Fetal Calf Serum
FGF: Fibroblast Growth Factor
FGFR-1: Fibroblast Growth Factor Receptor 1
GABA: Gamma-Aminobutyric Acid
GAP-43: Growth Associated Protein 43
G-CSF: Granulocyte-Colony Stimulating Factor
GDF-15: Growth Differentiation Factor 15
GDNF: Glial Cell Line-Derived Neurotrophic Factor
GFAP: Glial Fibrillary Acidic Protein
GMP: Good Manufacturing Practice
GMSCs: Gingival Mesenchymal Stem Cells
HA/TCP: Hydroxyapatite/Tricalcium Phosphate
HGF: Hepatocyte Growth Factor
HMGA-2: High-Mobility Group AT-hook 2
HUVEC: Human Umbilical Vein Endothelial Cells
IBMX: 3-Isobutyl-1-Methylxanthine
IGF-1: Insulin-like Growth Factor 1
IGFBP: Insulin-like Growth Factor Binding Protein
IGFR-1: Insulin Growth Factor Receptor 1
IL: Interleukin
ISCT: International Society of Cellular Therapy
MANF: Mesencephalic Astrocyte-derived Neurotrophic Factor
MAP-2: Microtubule-Associated Protein 2
MCP-1: Monocyte Chemotactic Protein 1
MDK: Midkine
MEPE: Matrix Extracellular Phosphoglycoprotein
MMP: Matrix Metalloproteinase
MSCs: Mesenchymal Stem Cells or Mesenchymal Stromal Cells
NCAM-1: Neural Cell Adhesion Molecule 1
NFIC: Nuclear Factor I-C
NFL: Neurofilament
NGF: Nerve Growth Factor
NRCAM: Neuronal Cell Adhesion Molecule
NRG-1-B-1: Neuregulin Beta 1
NRP-2: Neurophilin 2
NSE: Neuron Specific Enolase
NT-3: Neurotrophin 3
OMSCs: Oral Mucosa Stem Cells
PAI-1 (serpin E1): Plasminogen Activator Inhibitor-1
PDGF: Platelet-Derived Growth Factor
PDLSCs: Periodontal Ligament Stem Cells
PEDF (serpin F1): Pigment Epithelium-Derived Factor
PGA: Polyglycolic Acid
PIGF: PhosphatidylInositol-Glycan Biosynthesis Class F
PLA: Polylactic Acid
PRP: Platelet-Rich Plasma
PSCs: Periosteum-Derived Stem Cells
PTN: Pleiotrophin
PTX-3: Pentraxin 3
SCAP: Stem Cells from Apical Papilla
SCF: Stem Cell Factor
SCI: Spinal Cord Injury
SCs: Stem cells
SDF-1a: Stromal Cell-Derived Factor-1a
SGSCs: Salivary Gland-Derived Stem Cells
SHED: Stem Cells from Human Exfoliated Deciduous teeth
SHH: Sonic Hedgehog
SOPs: Standard Operating Procedures
TACE: Tumor Necrosis Factor-A Converting Enzyme
TDMs: Treated Dentin Matrices
TE: Tissue Engineering
TGFb: Transforming Growth Factor Beta
TGFbRII: Transforming Growth Factor Beta Receptor II
TIMP: Tissue Inhibitor of Metalloproteinase
TSP-1: Thrombospondin-1
uPA: Urokinase Plasminogen Activator
VE-cadherin: Vascular Endothelial cadherin
VEGF: Vascular Endothelial Growth Factor
VEGFR-1: Vascular Endothelial Growth Factor Receptor I
vWF: von Willebrand Factor.

Competing Interests

The authors declare no competing interests.

References

[1] S. Gronthos, M. Mankani, J. Brahim, P. G. Robey, and S. Shi, "Postnatal human dental pulp stem cells (DPSCs) in vitro and

in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13625–13630, 2000.

[2] M. Miura, S. Gronthos, M. Zhao et al., "SHED: stem cells from human exfoliated deciduous teeth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 5807–5812, 2003.

[3] G. T.-J. Huang, W. Sonoyama, Y. Liu, H. Liu, S. Wang, and S. Shi, "The hidden treasure in apical papilla: the potential role in pulp/dentin regeneration and bioroot engineering," *Journal of Endodontics*, vol. 34, no. 6, pp. 645–651, 2008.

[4] W. Sonoyama, Y. Liu, T. Yamaza et al., "Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study," *Journal of Endodontics*, vol. 34, no. 2, pp. 166–171, 2008.

[5] C. Morsczeck, W. Götz, J. Schierholz et al., "Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth," *Matrix Biology*, vol. 24, no. 2, pp. 155–165, 2005.

[6] B.-M. Seo, M. Miura, S. Gronthos et al., "Investigation of multipotent postnatal stem cells from human periodontal ligament," *The Lancet*, vol. 364, no. 9429, pp. 149–155, 2004.

[7] Q. Zhang, S. Shi, Y. Liu et al., "Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis," *The Journal of Immunology*, vol. 183, no. 12, pp. 7787–7798, 2009.

[8] K. Marynka-Kalmani, S. Treves, M. Yafee et al., "The lamina propria of adult human oral mucosa harbors a novel stem cell population," *STEM CELLS*, vol. 28, no. 5, pp. 984–995, 2010.

[9] S. O. Akintoye, T. Lam, S. Shi, J. Brahim, M. T. Collins, and P. G. Robey, "Skeletal site-specific characterization of orofacial and iliac crest human bone marrow stromal cells in same individuals," *Bone*, vol. 38, no. 6, pp. 758–768, 2006.

[10] E. J. Arnsdorf, L. M. Jones, D. R. Carter, and C. R. Jacobs, "The periosteum as a cellular source for functional tissue engineering," *Tissue Engineering—Part A*, vol. 15, no. 9, pp. 2637–2642, 2009.

[11] A. Sato, K. Okumura, S. Matsumoto et al., "Isolation, tissue localization, and cellular characterization of progenitors derived from adult human salivary glands," *Cloning and Stem Cells*, vol. 9, no. 2, pp. 191–205, 2007.

[12] E. M. Horwitz, K. Le Blanc, M. Dominici et al., "Clarification of the nomenclature for MSC: The International Society for Cellular Therapy position statement," *Cytotherapy*, vol. 7, no. 5, pp. 393–395, 2005.

[13] D. J. Alongi, T. Yamaza, Y. Song et al., "Stem/progenitor cells from inflamed human dental pulp retain tissue regeneration potential," *Regenerative Medicine*, vol. 5, no. 4, pp. 617–631, 2010.

[14] A. Malekfar, K. S. Valli, M. M. Kanafi, and R. R. Bhonde, "Isolation and characterization of human dental pulp stem cells from cryopreserved pulp tissues obtained from teeth with irreversible pulpitis," *Journal of Endodontics*, vol. 42, no. 1, pp. 76–81, 2016.

[15] M. Marrelli, F. Paduano, and M. Tatullo, "Cells isolated from human periapical cysts express mesenchymal stem cell-like properties," *International Journal of Biological Sciences*, vol. 9, no. 10, pp. 1070–1078, 2013.

[16] S. Shi and S. Gronthos, "Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp," *Journal of Bone and Mineral Research*, vol. 18, no. 4, pp. 696–704, 2003.

[17] J. Feng, A. Mantesso, C. De Bari, A. Nishiyama, and P. T. Sharp, "Dual origin of mesenchymal stem cells contributing to organ growth and repair," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 16, pp. 6503–6508, 2011.

[18] M. Dominici, K. Le Blanc, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.

[19] G. T.-J. Huang, S. Gronthos, and S. Shi, "Mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in regenerative medicine," *Journal of Dental Research*, vol. 88, no. 9, pp. 792–806, 2009.

[20] H. F. Duncan, A. J. Smith, G. J. Fleming, and P. R. Cooper, "Epigenetic modulation of dental pulp stem cells: implications for regenerative endodontics," *International Endodontic Journal*, vol. 49, no. 5, pp. 431–446, 2016.

[21] G. Gopinathan, A. Kolokythas, X. Luan, and T. G. H. Diekwisch, "Epigenetic marks define the lineage and differentiation potential of two distinct neural crest-derived intermediate odontogenic progenitor populations," *Stem Cells and Development*, vol. 22, no. 12, pp. 1763–1778, 2013.

[22] I. About, "Dentin regeneration in vitro: the pivotal role of supportive cells," *Advances in Dental Research*, vol. 23, no. 3, pp. 320–324, 2011.

[23] C. Jeanneau, P. Rufas, C. Rombouts, T. Giraud, J. Dejou, and I. About, "Can pulp fibroblasts kill cariogenic bacteria? Role of complement activation," *Journal of Dental Research*, vol. 94, no. 12, pp. 1765–1772, 2015.

[24] F. Chmilewsky, C. Jeanneau, P. Laurent, M. Kirschfink, and I. About, "Pulp progenitor cell recruitment is selectively guided by a C5a gradient," *Journal of Dental Research*, vol. 92, no. 6, pp. 532–539, 2013.

[25] F. Chmilewsky, C. Jeanneau, P. Laurent, and I. About, "Pulp fibroblasts synthesize functional complement proteins involved in initiating dentin-pulp regeneration," *The American Journal of Pathology*, vol. 184, no. 7, pp. 1991–2000, 2014.

[26] X. Wang, X.-J. Sha, G.-H. Li et al., "Comparative characterization of stem cells from human exfoliated deciduous teeth and dental pulp stem cells," *Archives of Oral Biology*, vol. 57, no. 9, pp. 1231–1240, 2012.

[27] M. F. Abdullah, S. F. Abdullah, N. S. Omar et al., "Proliferation rate of stem cells derived from human dental pulp and identification of differentially expressed genes," *Cell Biology International*, vol. 38, no. 5, pp. 582–590, 2014.

[28] H. Egusa, W. Sonoyama, M. Nishimura, I. Atsuta, and K. Akiyama, "Stem cells in dentistry—part I: stem cell sources," *Journal of Prosthodontic Research*, vol. 56, no. 3, pp. 151–165, 2012.

[29] S. Kim, S.-J. Shin, Y. Song, and E. Kim, "In vivo experiments with dental pulp stem cells for pulp-dentin complex regeneration," *Mediators of Inflammation*, vol. 2015, Article ID 409347, 6 pages, 2015.

[30] K. M. Fawzy El-Sayed, K. Jakusz, A. Jochens, C. Dörfer, and F. Schwendicke, "Stem cell transplantation for pulpal regeneration: a systematic review," *Tissue Engineering Part B: Reviews*, vol. 21, no. 5, pp. 451–460, 2015.

[31] G. Morad, L. Kheiri, and A. Khojasteh, "Dental pulp stem cells for in vivo bone regeneration: a systematic review of literature," *Archives of Oral Biology*, vol. 58, no. 12, pp. 1818–1827, 2013.

[32] A. H.-H. Yen and P. T. Sharpe, "Stem cells and tooth tissue engineering," *Cell and Tissue Research*, vol. 331, no. 1, pp. 359–372, 2008.

[33] A. Bronckaers, P. Hilkens, W. Martens et al., "Mesenchymal stem/stromal cells as a pharmacological and therapeutic approach to accelerate angiogenesis," *Pharmacology and Therapeutics*, vol. 143, no. 2, pp. 181–196, 2014.

[34] M. A. Saghiri, A. Asatourian, C. M. Sorenson, and N. Sheibani, "Role of angiogenesis in endodontics: contributions of stem cells and proangiogenic and antiangiogenic factors to dental pulp regeneration," *Journal of Endodontics*, vol. 41, no. 6, pp. 797–803, 2015.

[35] I. About, "Pulp vascularization and its regulation by the microenvironment," in *The Dental Pulp: Biology, Pathology, and Regenerative Therapies*, M. Goldberg, Ed., pp. 61–74, Springer, Berlin, Germany, 2014.

[36] J. Wang, X. Wang, Z. Sun et al., "Stem cells from human-exfoliated deciduous teeth can differentiate into dopaminergic neuron-like cells," *Stem Cells and Development*, vol. 19, no. 9, pp. 1375–1383, 2010.

[37] B. C. Heng, L. W. Lim, W. Wu, and C. Zhang, "An overview of protocols for the neural induction of dental and oral stem cells *in vitro*," *Tissue Engineering Part B: Reviews*, vol. 22, no. 3, pp. 220–250, 2016.

[38] C. Tran and M. S. Damaser, "Stem cells as drug delivery methods: application of stem cell secretome for regeneration," *Advanced Drug Delivery Reviews*, vol. 82-83, pp. 1–11, 2015.

[39] M. C. M. Conde, L. A. Chisini, F. F. Demarco, J. E. Nör, L. Casagrande, and S. B. C. Tarquinio, "Stem cell-based pulp tissue engineering: Variables enrolled in translation from the bench to the bedside, a systematic review of literature," *International Endodontic Journal*, vol. 49, no. 6, pp. 543–550, 2015.

[40] S. R. Simon, A. Berdal, P. R. Cooper, P. J. Lumley, P. L. Tomson, and A. J. Smith, "Dentin-pulp complex regeneration: from lab to clinic," *Advances in Dental Research*, vol. 23, no. 3, pp. 340–345, 2011.

[41] L. Xiao and M. Nasu, "From regenerative dentistry to regenerative medicine: progress, challenges, and potential applications of oral stem cells," *Stem Cells and Cloning: Advances and Applications*, vol. 7, pp. 89–99, 2014.

[42] J. Liu, F. Yu, Y. Sun et al., "Concise reviews: characteristics and potential applications of human dental tissue-derived mesenchymal stem cells," *Stem Cells*, vol. 33, no. 3, pp. 627–638, 2015.

[43] A. Di Benedetto, G. Brunetti, F. Posa et al., "Osteogenic differentiation of mesenchymal stem cells from dental bud: role of integrins and cadherins," *Stem Cell Research*, vol. 15, no. 3, pp. 618–628, 2015.

[44] E. Prateeptongkum, C. Klingelhöffer, and C. Morsczeck, "The influence of the donor on dental apical papilla stem cell properties," *Tissue and Cell*, vol. 47, no. 4, pp. 382–388, 2015.

[45] P. J. Simmons and B. Torok-Storb, "Identification of stromal cell precursors in human bone marrow by a novel monoclonal antibody, STRO-1," *Blood*, vol. 78, no. 1, pp. 55–62, 1991.

[46] S. Mathieu, C. Jeanneau, N. Sheibat-Othman, N. Kalaji, H. Fessi, and I. About, "Usefulness of controlled release of growth factors in investigating the early events of dentin-pulp regeneration," *Journal of Endodontics*, vol. 39, no. 2, pp. 228–235, 2013.

[47] A. Bakopoulou, G. Leyhausen, J. Volk, P. Koidis, and W. Geurtsen, "Comparative characterization of STRO-1(neg)/CD146(pos) and STRO-1(pos)/CD146(pos) apical papilla stem cells enriched with flow cytometry," *Archives of Oral Biology*, vol. 58, no. 10, pp. 1556–1568, 2013.

[48] A. I. Caplan, "All MSCs are pericytes?" *Cell Stem Cell*, vol. 3, no. 3, pp. 229–230, 2008.

[49] C. V. Machado, S. T. Passos, T. M. C. Campos et al., "The dental pulp stem cell niche based on aldehyde dehydrogenase 1 expression," *International Endodontic Journal*, vol. 49, no. 8, pp. 755–763, 2015.

[50] M. Jamal, S. M. Chogle, S. M. Karam, and G. T. Huang, "NOTCH3 is expressed in human apical papilla and in subpopulations of stem cells isolated from the tissue," *Genes & Diseases*, vol. 2, no. 3, pp. 261–267, 2015.

[51] J. Karbanová, T. Soukup, J. Suchánek, R. Pytlík, D. Corbeil, and J. Mokrý, "Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium," *Cells Tissues Organs*, vol. 193, no. 6, pp. 344–365, 2011.

[52] S. Abe, K. Hamada, M. Miura, and S. Yamaguchi, "Neural crest stem cell property of apical pulp cells derived from human developing tooth," *Cell Biology International*, vol. 36, no. 10, pp. 927–936, 2012.

[53] K. Sakai, A. Yamamoto, K. Matsubara et al., "Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms," *Journal of Clinical Investigation*, vol. 122, no. 1, pp. 80–90, 2012.

[54] W. Zhang, X. F. Walboomers, S. Shi, M. Fan, and J. A. Jansen, "Multilineage differentiation potential of stem cells derived from human dental pulp after cryopreservation," *Tissue Engineering*, vol. 12, no. 10, pp. 2813–2823, 2006.

[55] Y. Yamada, S. Nakamura, K. Ito et al., "A feasibility of useful cell-based therapy by bone regeneration with deciduous tooth stem cells, dental pulp stem cells, or bone-marrow-derived mesenchymal stem cells for clinical study using tissue engineering technology," *Tissue Engineering—Part A*, vol. 16, no. 6, pp. 1891–1900, 2010.

[56] I. Kerkis, A. Kerkis, D. Dozortsev et al., "Isolation and characterization of a population of immature dental pulp stem cells expressing OCT-4 and other embryonic stem cell markers," *Cells Tissues Organs*, vol. 184, no. 3-4, pp. 105–116, 2006.

[57] N. Nourbakhsh, M. Soleimani, Z. Taghipour et al., "Induced in vitro differentiation of neural-like cells from human exfoliated deciduous teeth-derived stem cells," *International Journal of Developmental Biology*, vol. 55, no. 2, pp. 189–195, 2011.

[58] A. Bakopoulou, A. Kritis, D. Andreadis et al., "Angiogenic potential and secretome of human apical papilla mesenchymal stem cells in various stress microenvironments," *Stem Cells and Development*, vol. 24, no. 21, pp. 2496–2512, 2015.

[59] M. E. Yalvac, M. Ramazanoglu, A. A. Rizvanov et al., "Isolation and characterization of stem cells derived from human third molar tooth germs of young adults: implications in neo-vascularization, osteo-, adipo-and neurogenesis," *Pharmacogenomics Journal*, vol. 10, no. 2, pp. 105–113, 2010.

[60] G. Laino, A. Graziano, R. d'Aquino et al., "An approachable human adult stem cell source for hard-tissue engineering," *Journal of Cellular Physiology*, vol. 206, no. 3, pp. 693–701, 2006.

[61] A. Pisciotta, G. Carnevale, S. Meloni et al., "Human dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations integrative control of development," *BMC Developmental Biology*, vol. 15, article 14, 2015.

[62] R. d'Aquino, A. Graziano, M. Sampaolesi et al., "Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation," *Cell Death and Differentiation*, vol. 14, no. 6, pp. 1162–1171, 2007.

[63] C.-S. Lin, H. Ning, G. Lin, and T. F. Lue, "Is CD34 truly a negative marker for mesenchymal stromal cells?" *Cytotherapy*, vol. 14, no. 10, pp. 1159–1163, 2012.

[64] R. Alvarez, H. L. Lee, C. Hong, and C. Y. Wang, "Single CD271 marker isolates mesenchymal stem cells from human dental pulp," *International Journal of Oral Science*, vol. 7, no. 4, pp. 205–212, 2015.

[65] E. Flores-Torales, A. Orozco-Barocio, O. R. Gonzalez-Ramella, A. Carrasco-Yalan, K. Gazarian, and S. Cuneo-Pareto, "The CD271 expression could be alone for establisher phenotypic marker in Bone Marrow derived mesenchymal stem cells," *Folia Histochemica et Cytobiologica*, vol. 48, no. 4, pp. 682–686, 2010.

[66] M. Ramamoorthi, M. Bakkar, J. Jordan, and S. D. Tran, "Osteogenic potential of dental mesenchymal stem cells in preclinical studies: a systematic review using modified ARRIVE and CONSORT guidelines," *Stem Cells International*, vol. 2015, Article ID 378368, 28 pages, 2015.

[67] S. Shi, P. M. Bartold, M. Miura, B. M. Seo, P. G. Robey, and S. Gronthos, "The efficacy of mesenchymal stem cells to regenerate and repair dental structures," *Orthodontics and Craniofacial Research*, vol. 8, no. 3, pp. 191–199, 2005.

[68] G. T.-J. Huang, W. Sonoyama, J. Chen, and S. H. Park, "In vitro characterization of human dental pulp cells: various isolation methods and culturing environments," *Cell and Tissue Research*, vol. 324, no. 2, pp. 225–236, 2006.

[69] S. Batouli, M. Miura, J. Brahim et al., "Comparison of stem-cell-mediated osteogenesis and dentinogenesis," *Journal of Dental Research*, vol. 82, no. 12, pp. 976–981, 2003.

[70] K. Iohara, M. Nakashima, M. Ito, M. Ishikawa, A. Nakasima, and A. Akamine, "Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2," *Journal of Dental Research*, vol. 83, no. 8, pp. 590–595, 2004.

[71] K. Iohara, K. Imabayashi, R. Ishizaka et al., "Complete pulp regeneration after pulpectomy by transplantation of $CD105^{+}$ stem cells with stromal cell-derived factor-1," *Tissue Engineering—Part A*, vol. 17, no. 15-16, pp. 1911–1920, 2011.

[72] K. Iohara, M. Murakami, N. Takeuchi et al., "A novel combinatorial therapy with pulp stem cells and granulocyte colony stimulating factor for total pulp regeneration," *Stem Cells Translational Medicine*, vol. 2, no. 7, pp. 521–533, 2013.

[73] K. Iohara, M. Murakami, K. Nakata, and M. Nakashima, "Age-dependent decline in dental pulp regeneration after pulpectomy in dogs," *Experimental Gerontology*, vol. 52, pp. 39–45, 2014.

[74] H. Horibe, M. Murakami, K. Iohara et al., "Isolation of a stable subpopulation of Mobilized Dental Pulp Stem Cells (MDPSCs) with high proliferation, migration, and regeneration potential is independent of age," *PLoS ONE*, vol. 9, no. 5, Article ID e98553, 2014.

[75] R. S. Prescott, R. Alsanea, M. I. Fayad et al., "In vivo generation of dental pulp-like tissue by using dental pulp stem cells, a collagen scaffold, and dentin matrix protein 1 after subcutaneous transplantation in mice," *Journal of Endodontics*, vol. 34, no. 4, pp. 421–426, 2008.

[76] G. T. Huang, T. Yamaza, L. D. Shea et al., "Stem/progenitor cell–mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an in vivo model," *Tissue Engineering A*, vol. 16, no. 2, pp. 605–615, 2010.

[77] X. Petridis, E. Diamanti, G. C. Trigas, D. Kalyvas, and E. Kitraki, "Bone regeneration in critical-size calvarial defects using human dental pulp cells in an extracellular matrix-based scaffold," *Journal of Cranio-Maxillofacial Surgery*, vol. 43, no. 4, pp. 483–490, 2015.

[78] A. De Mendonça Costa, D. F. Bueno, M. T. Martins et al., "Reconstruction of large cranial defects in nonimmunosuppressed experimental design with human dental pulp stem cells," *Journal of Craniofacial Surgery*, vol. 19, no. 1, pp. 204–210, 2008.

[79] T. Maraldi, M. Riccio, A. Pisciotta et al., "Human amniotic fluid-derived and dental pulp-derived stem cells seeded into collagen scaffold repair critical-size bone defects promoting vascularization," *Stem Cell Research and Therapy*, vol. 4, no. 3, article 53, 2013.

[80] H.-C. Liu, L.-L. E, D.-S. Wang et al., "Reconstruction of alveolar bone defects using bone morphogenetic protein 2 mediated rabbit dental pulp stem cells seeded on nano-hydroxyapatite/collagen/ poly(l-lactide)," *Tissue Engineering—Part A*, vol. 17, no. 19-20, pp. 2417–2433, 2011.

[81] K. Ito, Y. Yamada, S. Nakamura, and M. Ueda, "Osteogenic potential of effective bone engineering using dental pulp stem cells, bone marrow stem cells, and periosteal cells for osseointegration of dental implants," *The International Journal of Oral & Maxillofacial Implants*, vol. 26, no. 5, pp. 947–954, 2011.

[82] Y. Zheng, Y. Liu, C. M. Zhang et al., "Stem cells from deciduous tooth repair mandibular defect in swine," *Journal of Dental Research*, vol. 88, no. 3, pp. 249–254, 2009.

[83] S. Sharma, D. Srivastava, S. Grover, and V. Sharma, "Biomaterials in tooth tissue engineering: a review," *Journal of Clinical and Diagnostic Research*, vol. 8, no. 1, pp. 309–315, 2014.

[84] K. M. Galler, J. D. Hartgerink, A. C. Cavender, G. Schmalz, and R. N. D'Souza, "A customized self-assembling peptide hydrogel for dental pulp tissue engineering," *Tissue Engineering Part A*, vol. 18, no. 1-2, pp. 176–184, 2012.

[85] V. Rosa, Z. Zhang, R. H. M. Grande, and J. E. Nör, "Dental pulp tissue engineering in full-length human root canals," *Journal of Dental Research*, vol. 92, no. 11, pp. 970–975, 2013.

[86] R. Li, W. Guo, B. Yang et al., "Human treated dentin matrix as a natural scaffold for complete human dentin tissue regeneration," *Biomaterials*, vol. 32, no. 20, pp. 4525–4538, 2011.

[87] L. Jiao, L. Xie, B. Yang et al., "Cryopreserved dentin matrix as a scaffold material for dentin-pulp tissue regeneration," *Biomaterials*, vol. 35, no. 18, pp. 4929–4939, 2014.

[88] K. M. Galler, M. Widbiller, W. Buchalla et al., "EDTA conditioning of dentine promotes adhesion, migration and differentiation of dental pulp stem cells," *International Endodontic Journal*, vol. 49, no. 6, pp. 581–590, 2016.

[89] M. M. Cordeiro, Z. Dong, T. Kaneko et al., "Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth," *Journal of Endodontics*, vol. 34, no. 8, pp. 962–969, 2008.

[90] L. Casagrande, F. F. Demarco, Z. Zhang, F. B. Araujo, S. Shi, and J. E. Nör, "Dentin-derived BMP-2 and odontoblast differentiation," *Journal of Dental Research*, vol. 89, no. 6, pp. 603–608, 2010.

[91] J. Y. Kim, X. Xin, E. K. Moioli et al., "Regeneration of dental-pulp-like tissue by chemotaxis-induced cell homing," *Tissue Engineering—Part A*, vol. 16, no. 10, pp. 3023–3031, 2010.

[92] J.-W. Yang, Y.-F. Zhang, C.-Y. Wan et al., "Autophagy in SDF-1α-mediated DPSC migration and pulp regeneration," *Biomaterials*, vol. 44, pp. 11–23, 2015.

[93] M. Seo, W. Sonoyama, T. Yamaza et al., "SHED repair critical-size calvarial defects in mice," *Oral Diseases*, vol. 14, no. 5, pp. 428–434, 2008.

[94] A. Behnia, A. Haghighat, A. Talebi, N. Nourbakhsh, and F. Heidari, "Transplantation of stem cells from human exfoliated

deciduous teeth for bone regeneration in the dog mandibular defect," *World Journal of Stem Cells*, vol. 26, no. 4, pp. 505–510, 2014.

[95] A. Alkaisi, A. R. Ismail, S. S. Mutum, Z. A. Rifin Ahmad, S. Masudi, and N. H. Abd Razak, "Transplantation of human dental pulp stem cells: enhance bone consolidation in mandibular distraction osteogenesis," *Journal of Oral and Maxillofacial Surgery*, vol. 71, no. 10, pp. 1758.e1–1758.e13, 2013.

[96] N. Kaukua, M. Chen, P. Guarnieri et al., "Molecular differences between stromal cell populations from deciduous and permanent human teeth," *Stem Cell Research and Therapy*, vol. 6, no. 1, article 59, 2015.

[97] A. Bakopoulou, G. Leyhausen, J. Volk et al., "Comparative analysis of in vitro osteo/odontogenic differentiation potential of human dental pulp stem cells (DPSCs) and stem cells from the apical papilla (SCAP)," *Archives of Oral Biology*, vol. 56, no. 7, pp. 709–721, 2011.

[98] A. A. Volponi, E. Gentleman, R. Fatscher, Y. W. Y. Pang, M. M. Gentleman, and P. T. Sharpe, "Composition of mineral produced by dental mesenchymal stem cells," *Journal of Dental Research*, vol. 94, no. 11, pp. 1568–1574, 2015.

[99] S. Wang, J. Mu, Z. Fan et al., "Insulin-like growth factor 1 can promote the osteogenic differentiation and osteogenesis of stem cells from apical papilla," *Stem Cell Research*, vol. 8, no. 3, pp. 346–356, 2012.

[100] W. Sonoyama, Y. Liu, D. Fang et al., "Mesenchymal stem cell-mediated functional tooth regeneration in Swine," *PLoS ONE*, vol. 1, no. 1, article e79, 2006.

[101] T. Yagyuu, E. Ikeda, H. Ohgushi et al., "Hard tissue-forming potential of stem/progenitor cells in human dental follicle and dental papilla," *Archives of Oral Biology*, vol. 55, no. 1, pp. 68–76, 2010.

[102] W. Wang, M. Dang, Z. Zhang et al., "Dentin regeneration by stem cells of apical papilla on injectable nanofibrous microspheres and stimulated by controlled BMP-2 release," *Acta Biomaterialia*, vol. 36, pp. 63–72, 2016.

[103] J. Wang, H. Zhang, W. Zhang et al., "Bone morphogenetic protein-9 effectively induces osteo/odontoblastic differentiation of the reversibly immortalized stem cells of dental apical papilla," *Stem Cells and Development*, vol. 23, no. 12, pp. 1405–1416, 2014.

[104] W. Zhang, X. Zhang, J. Ling, X. Wei, and Y. Jian, "Osteo-/odontogenic differentiation of BMP2 and VEGF gene-co-transfected human stem cells from apical papilla," *Molecular Medicine Reports*, vol. 13, no. 5, pp. 3747–3754, 2016.

[105] J. Zhang, Z. Wang, Y. Jiang et al., "Nuclear Factor I-C promotes proliferation and differentiation of apical papilla-derived human stem cells in vitro," *Experimental Cell Research*, vol. 332, no. 2, pp. 259–266, 2015.

[106] W. He, J. Zhang, Z. Niu et al., "Regulatory interplay between NFIC and TGF-β1 in apical papilla-derived stem cells," *Journal of Dental Research*, vol. 93, no. 5, pp. 496–501, 2014.

[107] B. Jin and P. Choung, "Recombinant human plasminogen activator inhibitor-1 accelerates odontoblastic differentiation of human stem cells from apical papilla," *Tissue Engineering Part A*, vol. 22, no. 9-10, pp. 721–732, 2016.

[108] H.-H. Chang, M.-C. Chang, I.-H. Wu et al., "Role of ALK5/Smad2/3 and MEK1/ERK signaling in transforming growth factor beta 1-modulated growth, collagen turnover, and differentiation of stem cells from apical papilla of human tooth," *Journal of Endodontics*, vol. 41, no. 8, pp. 1272–1280, 2015.

[109] H. Zhang, J. Wang, F. Deng et al., "Canonical Wnt signaling acts synergistically on BMP9-induced osteo/odontoblastic differentiation of stem cells of dental apical papilla (SCAPs)," *Biomaterials*, vol. 39, pp. 145–154, 2015.

[110] F. Sun, M. Wan, X. Xu et al., "Crosstalk between miR-34a and notch signaling promotes differentiation in apical papilla stem cells (SCAPs)," *Journal of Dental Research*, vol. 93, no. 6, pp. 589–595, 2014.

[111] J. Li, M. Yan, Z. Wang et al., "Effects of canonical NF-κB signaling pathway on the proliferation and odonto/osteogenic differentiation of human stem cells from apical papilla," *BioMed Research International*, vol. 2014, Article ID 319651, 12 pages, 2014.

[112] C. Mu, T. Lv, Z. Wang et al., "Mechanical stress stimulates the osteo/odontoblastic differentiation of human stem cells from apical papilla via ERK 1/2 and JNK MAPK pathways," *BioMed Research International*, vol. 2014, Article ID 494378, 10 pages, 2014.

[113] J. Yu, Z. Deng, J. Shi et al., "Differentiation of dental pulp stem cells into regular-shaped dentin-pulp complex induced by tooth germ cell conditioned medium," *Tissue Engineering*, vol. 12, no. 11, pp. 3097–3105, 2006.

[114] K. Kodonas, C. Gogos, S. Papadimitriou, K. Kouzi-Koliakou, and D. Tziafas, "Experimental formation of dentin-like structure in the root canal implant model using cryopreserved swine dental pulp progenitor cells," *Journal of Endodontics*, vol. 38, no. 7, pp. 913–919, 2012.

[115] C. Marchionni, L. Bonsi, F. Alviano et al., "Angiogenic potential of human dental pulp stromal (stem) cells," *International Journal of Immunopathology and Pharmacology*, vol. 22, no. 3, pp. 699–706, 2009.

[116] R. Ishizaka, Y. Hayashi, K. Iohara et al., "Stimulation of angiogenesis, neurogenesis and regeneration by side population cells from dental pulp," *Biomaterials*, vol. 34, no. 8, pp. 1888–1897, 2013.

[117] A. Bronckaers, P. Hilkens, Y. Fanton et al., "Angiogenic properties of human dental pulp stem cells," *PLoS ONE*, vol. 8, no. 8, article e71104, 2013.

[118] K. Janebodin, Y. Zeng, W. Buranaphatthana, N. Ieronimakis, and M. Reyes, "VEGFR2-dependent angiogenic capacity of pericyte-like dental pulp stem cells," *Journal of Dental Research*, vol. 92, no. 6, pp. 524–531, 2013.

[119] V. T. Sakai, Z. Zhang, Z. Dong et al., "SHED differentiate into functional odontoblasts and endothelium," *Journal of Dental Research*, vol. 89, no. 8, pp. 791–796, 2010.

[120] L. W. Bento, Z. Zhang, A. Imai et al., "Endothelial differentiation of SHED requires MEK1/ERK signaling," *Journal of Dental Research*, vol. 92, no. 1, pp. 51–57, 2013.

[121] P. Hilkens, Y. Fanton, W. Martens et al., "Pro-angiogenic impact of dental stem cells in vitro and in vivo," *Stem Cell Research*, vol. 12, no. 3, pp. 778–790, 2014.

[122] C. Gorin, G. Y. Rochefort, R. Bascetin et al., "Priming dental pulp stem cells with fibroblast growth factor-2 increases angiogenesis of implanted tissue-engineered constructs through hepatocyte growth factor and vascular endothelial growth factor secretion," *Stem Cells Translational Medicine*, vol. 5, no. 3, pp. 392–404, 2016.

[123] W. L. Dissanayaka, X. Zhan, C. Zhang, K. M. Hargreaves, L. Jin, and E. H. Y. Tong, "Coculture of dental pulp stem cells with endothelial cells enhances osteo-/odontogenic and angiogenic potential in vitro," *Journal of Endodontics*, vol. 38, no. 4, pp. 454–463, 2012.

[124] C. Yuan, P. Wang, L. Zhu et al., "Coculture of stem cells from apical papilla and human umbilical vein endothelial cell under hypoxia increases the formation of three-dimensional vessel-like structures in vitro," *Tissue Engineering Part A*, vol. 21, no. 5-6, pp. 1163–1172, 2015.

[125] J. Vanacker, A. Viswanath, P. De Berdt et al., "Hypoxia modulates the differentiation potential of stem cells of the apical papilla," *Journal of Endodontics*, vol. 40, no. 9, pp. 1410–1418, 2014.

[126] W. L. Dissanayaka, K. M. Hargreaves, L. Jin, L. P. Samaranayake, and C. Zhang, "The interplay of dental pulp stem cells and endothelial cells in an injectable peptide hydrogel on angiogenesis and pulp regeneration in vivo," *Tissue Engineering—Part A*, vol. 21, no. 3-4, pp. 550–563, 2015.

[127] K. Iohara, L. Zheng, H. Wake et al., "A novel stem cell source for vasculogenesis in ischemia: subfraction of side population cells from dental pulp," *Stem Cells*, vol. 26, no. 9, pp. 2408–2418, 2008.

[128] C. Gandia, A. N. A. Armiñan, J. M. García-Verdugo et al., "Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction," *Stem Cells*, vol. 26, no. 3, pp. 638–645, 2008.

[129] E. Eggenhofer, V. Benseler, A. Kroemer et al., "Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion," *Frontiers in Immunology*, vol. 3, article 297, 2012.

[130] S. H. Ranganath, O. Levy, M. S. Inamdar, and J. M. Karp, "Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease," *Cell Stem Cell*, vol. 10, no. 3, pp. 244–258, 2012.

[131] S. Yu, Y. Zhao, Y. Ma, and L. Ge, "Profiling the secretome of human stem cells from dental apical papilla," *Stem Cells and Development*, vol. 25, no. 6, pp. 499–508, 2016.

[132] G. Raposo and W. Stoorvogel, "Extracellular vesicles: exosomes, microvesicles, and friends," *Journal of Cell Biology*, vol. 200, no. 4, pp. 373–383, 2013.

[133] U. Pivoraitė, A. Jarmalavičiūtė, V. Tunaitis et al., "Exosomes from human dental pulp stem cells suppress carrageenan-induced acute inflammation in mice," *Inflammation*, vol. 38, no. 5, pp. 1933–1941, 2015.

[134] M. La Noce, L. Mele, V. Tirino et al., "Neural crest stem cell population in craniomaxillofacial development and tissue repair," *European Cells and Materials*, vol. 28, pp. 348–357, 2014.

[135] T. Osathanon, C. Sawangmake, N. Nowwarote, and P. Pavasant, "Neurogenic differentiation of human dental pulp stem cells using different induction protocols," *Oral Diseases*, vol. 20, no. 4, pp. 352–358, 2014.

[136] L. Xiao and T. Tsutsui, "Characterization of human dental pulp cells-derived spheroids in serum-free medium: stem cells in the core," *Journal of Cellular Biochemistry*, vol. 114, no. 11, pp. 2624–2636, 2013.

[137] C.-C. Chang, K.-C. Chang, S.-J. Tsai, H.-H. Chang, and C.-P. Lin, "Neurogenic differentiation of dental pulp stem cells to neuron-like cells in dopaminergic and motor neuronal inductive media," *Journal of the Formosan Medical Association*, vol. 113, no. 12, pp. 956–965, 2014.

[138] M. Kanafi, D. Majumdar, R. Bhonde, P. Gupta, and I. Datta, "Midbrain cues dictate differentiation of human dental pulp stem cells towards functional dopaminergic neurons," *Journal of Cellular Physiology*, vol. 229, no. 10, pp. 1369–1377, 2014.

[139] N. Urraca, R. Memon, I. El-Iyachi et al., "Characterization of neurons from immortalized dental pulp stem cells for the study of neurogenetic disorders," *Stem Cell Research*, vol. 15, no. 3, pp. 722–730, 2015.

[140] M. Király, K. Kádár, D. B. Horváthy et al., "Integration of neuronally predifferentiated human dental pulp stem cells into rat brain in vivo," *Neurochemistry International*, vol. 59, no. 3, pp. 371–381, 2011.

[141] A. Arthur, G. Rychkov, S. Shi, S. A. Koblar, and S. Gronthose, "Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues," *Stem Cells*, vol. 26, no. 7, pp. 1787–1795, 2008.

[142] Y. Isobe, N. Koyama, K. Nakao et al., "Comparison of human mesenchymal stem cells derived from bone marrow, synovial fluid, adult dental pulp, and exfoliated deciduous tooth pulp," *International Journal of Oral and Maxillofacial Surgery*, vol. 45, no. 1, pp. 124–131, 2016.

[143] X. Feng, J. Xing, G. Feng et al., "Age-dependent impaired neurogenic differentiation capacity of dental stem cell is associated with wnt/β-catenin signaling," *Cellular and Molecular Neurobiology*, vol. 33, no. 8, pp. 1023–1031, 2013.

[144] C. Yang, L. Sun, X. Li et al., "The potential of dental stem cells differentiating into neurogenic cell lineage after cultivation in different modes in vitro," *Cellular Reprogramming*, vol. 16, no. 5, pp. 379–391, 2014.

[145] J. F. A. De Almeida, P. Chen, M. A. Henry, and A. Diogenes, "Stem cells of the apical papilla regulate trigeminal neurite outgrowth and targeting through a BDNF-dependent mechanism," *Tissue Engineering—Part A*, vol. 20, no. 23-24, pp. 3089–3100, 2014.

[146] P. De Berdt, J. Vanacker, B. Ucakar et al., "Dental apical papilla as therapy for spinal cord injury," *Journal of Dental Research*, vol. 94, no. 11, pp. 1575–1581, 2015.

[147] P. Gervois, T. Struys, P. Hilkens et al., "Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons," *Stem Cells and Development*, vol. 24, no. 3, pp. 296–311, 2015.

[148] Z. Taghipour, K. Karbalaie, A. Kiani et al., "Transplantation of undifferentiated and induced human exfoliated deciduous teeth-derived stem cells promote functional recovery of rat spinal cord contusion injury model," *Stem Cells and Development*, vol. 21, no. 10, pp. 1794–1802, 2012.

[149] G. J. Brewer, J. R. Torricelli, E. K. Evege, and P. J. Price, "Optimized survival of hippocampal neurons in B27-supplemented Neurobasal™, a new serum-free medium combination," *Journal of Neuroscience Research*, vol. 35, no. 5, pp. 567–576, 1993.

[150] H. Fujii, K. Matsubara, K. Sakai et al., "Dopaminergic differentiation of stem cells from human deciduous teeth and their therapeutic benefits for Parkinsonian rats," *Brain Research*, vol. 1613, pp. 59–72, 2015.

[151] R. Sasaki, S. Aoki, M. Yamato et al., "Tubulation with dental pulp cells promotes facial nerve regeneration in rats," *Tissue Engineering Part A*, vol. 14, no. 7, pp. 1141–1147, 2008.

[152] R. Sasaki, S. Aoki, M. Yamato et al., "PLGA artificial nerve conduits with dental pulp cells promote facial nerve regeneration," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 5, no. 10, pp. 823–830, 2011.

[153] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury," *Investigative Ophthalmology and Visual Science*, vol. 54, no. 12, pp. 7544–7556, 2013.

[154] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells," *PLoS ONE*, vol. 9, no. 10, article e109305, 2014.

[155] A. H.-C. Huang, B. R. Snyder, P.-H. Cheng, and A. W. S. Chan, "Putative dental pulp-derived stem/stromal cells promote proliferation and differentiation of endogenous neural cells in the hippocampus of mice," *STEM CELLS*, vol. 26, no. 10, pp. 2654–2663, 2008.

[156] Z. Gale, P. R. Cooper, and B. A. A. Scheven, "Effects of glial cell line-derived neurotrophic factor on dental pulp cells," *Journal of Dental Research*, vol. 90, no. 10, pp. 1240–1245, 2011.

[157] T. Yamamoto, Y. Osako, M. Ito et al., "Trophic effects of dental pulp stem cells on Schwann cells in peripheral nerve regeneration," *Cell Transplantation*, vol. 25, no. 1, pp. 183–193, 2016.

[158] T. Mita, Y. Furukawa-Hibi, H. Takeuchi et al., "Conditioned medium from the stem cells of human dental pulp improves cognitive function in a mouse model of Alzheimer's disease," *Behavioural Brain Research*, vol. 293, pp. 189–197, 2015.

[159] T. Inoue, M. Sugiyama, H. Hattori, H. Wakita, T. Wakabayashi, and M. Ueda, "Stem cells from human exfoliated deciduous tooth-derived conditioned medium enhance recovery of focal cerebral ischemia in rats," *Tissue Engineering Part A*, vol. 19, no. 1-2, pp. 24–29, 2013.

[160] K. Matsubara, Y. Matsushita, K. Sakai et al., "Secreted ectodomain of sialic acid-binding Ig-like lectin-9 and monocyte chemoattractant protein-1 promote recovery after rat spinal cord injury by altering macrophage polarity," *Journal of Neuroscience*, vol. 35, no. 6, pp. 2452–2464, 2015.

[161] Y. Sugimura-Wakayama, W. Katagiri, M. Osugi et al., "Peripheral nerve regeneration by secretomes of stem cells from human exfoliated deciduous teeth," *Stem Cells and Development*, vol. 24, no. 22, pp. 2687–2699, 2015.

[162] A. Jarmalavičiūtė, V. Tunaitis, U. Pivoraitė, A. Venalis, and A. Pivoriūnas, "Exosomes from dental pulp stem cells rescue human dopaminergic neurons from 6-hydroxy-dopamine-induced apoptosis," *Cytotherapy*, vol. 17, no. 7, pp. 932–939, 2015.

[163] M. P. de Miguel, S. Fuentes-Julián, A. Blázquez-Martínez et al., "Immunosuppressive properties of mesenchymal stem cells: advances and applications," *Current Molecular Medicine*, vol. 12, no. 5, pp. 574–591, 2012.

[164] Regulation (EC) No 2003/94/EC of the European Parliament and of the Council of 8 October 2003 laying down the principles and guidelines of good manufacturing practice in respect of medicinal products for human use and investigational medicinal products for human use.

[165] Regulation (EC) No 1394/2007 of the European parliament and of the council of 13 November 2007 on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004.

[166] S. Pacini, "Deterministic and stochastic approaches in the clinical application of mesenchymal stromal cells (MSCs)," *Frontiers in Cell and Developmental Biology*, vol. 2, article 50, 2014.

[167] E. H. Javazon, K. J. Beggs, and A. W. Flake, "Mesenchymal stem cells: paradoxes of passaging," *Experimental Hematology*, vol. 32, no. 5, pp. 414–425, 2004.

[168] P. Hilkens, N. Meschi, P. Lambrechts, A. Bronckaers, and I. Lambrichts, "Dental stem cells in pulp regeneration: near future or long road ahead?" *Stem Cells and Development*, vol. 24, no. 14, pp. 1610–1622, 2015.

[169] P. G. Robey, S. A. Kuznetsov, J. Ren, H. G. Klein, M. Sabatino, and D. F. Stroncek, "Generation of clinical grade human bone marrow stromal cells for use in bone regeneration," *Bone*, vol. 70, pp. 87–92, 2015.

[170] A. Shahdadfar, K. Frønsdal, T. Haug, F. P. Reinholt, and J. E. Brinchmann, "In vitro expansion of human mesenchymal stem cells: choice of serum is a determinant of cell proliferation, differentiation, gene expression, and transcriptome stability," *Stem Cells*, vol. 23, no. 9, pp. 1357–1366, 2005.

[171] P. A. Sotiropoulou, S. A. Perez, M. Salagianni, C. N. Baxevanis, and M. Papamichail, "Characterization of the optimal culture conditions for clinical scale production of human mesenchymal stem cells," *Stem Cells*, vol. 24, no. 2, pp. 462–471, 2006.

[172] F. Mannello and G. A. Tonti, "Concise review: No breakthroughs for human mesenchymal and embryonic stem cell culture: Conditioned medium, feeder layer, or feeder-free; medium with fetal calf serum, human serum, or enriched plasma; serum-free, serum replacement nonconditioned medium, or ad hoc formula? All that glitters is not gold!," *Stem Cells*, vol. 25, no. 7, pp. 1603–1609, 2007.

[173] R. Brandenberger, S. Burger, A. Campbell, T. Fong, E. Lapinskas, and J. A. Rowley, "Cell therapy bioprocessing," *BioProcess International*, vol. 3, pp. 30–36, 2011.

[174] C. Capelli, O. Pedrini, R. Valgardsdottir, F. Da Roit, J. Golay, and M. Introna, "Clinical grade expansion of MSCs," *Immunology Letters*, vol. 168, no. 2, pp. 222–227, 2015.

[175] N. Kawashima, "Characterisation of dental pulp stem cells: a new horizon for tissue regeneration?" *Archives of Oral Biology*, vol. 57, no. 11, pp. 1439–1458, 2012.

[176] C. A. McIntyre, B. T. Flyg, and T. C. Fong, "Fluorescence-activated cell sorting for CGMP processing of therapeutic cells," *BioProcess International*, vol. 8, no. 6, pp. 44–53, 2010.

[177] S.-Y. Lee, G.-W. Huang, J.-N. Shiung et al., "Magnetic cryopreservation for dental pulp stem cells," *Cells Tissues Organs*, vol. 196, no. 1, pp. 23–33, 2012.

[178] T. K. Chatzistamatiou, A. C. Papassavas, E. Michalopoulos et al., "Optimizing isolation culture and freezing methods to preserve Wharton's jelly's mesenchymal stem cell (MSC) properties: an MSC banking protocol validation for the Hellenic Cord Blood Bank," *Transfusion*, vol. 54, no. 12, pp. 3108–3120, 2014.

[179] S. Abbasalizadeh and H. Baharvand, "Technological progress and challenges towards cGMP manufacturing of human pluripotent stem cells based therapeutic products for allogeneic and autologous cell therapies," *Biotechnology Advances*, vol. 31, no. 8, pp. 1600–1623, 2013.

[180] R. Martín-Ibáñez, A. M. Strömberg, O. Hovatta, and J. M. Canals, "UNIT 1C.8 cryopreservation of dissociated human embryonic stem cells in the presence of ROCK inhibitor," *Current Protocols in Stem Cell Biology*, 2009.

[181] C. A. Lemos, V. E. de Souza Batista, D. A. Almeida, J. F. Santiago Júnior, F. R. Verri, and E. P. Pellizzer, "Evaluation of cement-retained versus screw-retained implant-supported restorations for marginal bone loss," *The Journal of Prosthetic Dentistry*, vol. 115, no. 4, pp. 419–427, 2016.

[182] S. A. Tarle, S. Shi, and D. Kaigler, "Development of a serum-free system to expand dental-derived stem cells: PDLSCs and SHEDs," *Journal of Cellular Physiology*, vol. 226, no. 1, pp. 66–73, 2011.

[183] E. J. Eubanks, S. A. Tarle, and D. Kaigler, "Tooth storage, dental pulp stem cell isolation, and clinical scale expansion without

animal serum," *Journal of Endodontics*, vol. 40, no. 5, pp. 652–657, 2014.

[184] N. F. Lizier, A. Kerkis, C. M. Gomes et al., "Scaling-up of dental pulp stem cells isolated from multiple niches," *PLoS ONE*, vol. 7, no. 6, article e39885, 2012.

[185] M. Ducret, H. Fabre, O. Degoul et al., "Manufacturing of dental pulp cell-based products from human third molars: current strategies and future investigations," *Frontiers in Physiology*, vol. 6, article 213, 2015.

[186] A. Bakopoulou, G. Leyhausen, J. Volk et al., "Assessment of the impact of two different isolation methods on the osteo/odontogenic differentiation potential of human dental stem cells derived from deciduous teeth," *Calcified Tissue International*, vol. 88, no. 2, pp. 130–141, 2011.

[187] P. Hilkens, P. Gervois, Y. Fanton et al., "Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells," *Cell and Tissue Research*, vol. 353, no. 1, pp. 65–78, 2013.

[188] M. Padial-Molina, F. O'Valle, A. Lanis et al., "Clinical application of mesenchymal stem cells and novel supportive therapies for oral bone regeneration," *BioMed Research International*, vol. 2015, Article ID 341327, 16 pages, 2015.

[189] C. Jakobsen, J. A. Sørensen, M. Kassem, and T. H. Thygesen, "Mesenchymal stem cells in oral reconstructive surgery: a systematic review of the literature," *Journal of Oral Rehabilitation*, vol. 40, no. 9, pp. 693–706, 2013.

[190] R. d'Aquino, A. De Rosa, V. Lanza et al., "Human mandible bone defect repair by the grafting of dental pulp stem/progenitor cells and collagen sponge biocomplexes," *European Cells and Materials*, vol. 18, pp. 75–83, 2009.

[191] A. Giuliani, A. Manescu, M. Langer et al., "Three years after transplants in human mandibles, histological and in-line holotomography revealed that stem cells regenerated a compact rather than a spongy bone: biological and clinical implications," *Stem Cells Translational Medicine*, vol. 2, no. 4, pp. 316–324, 2013.

[192] M. Nakashima and K. Iohara, "Mobilized dental pulp stem cells for pulp regeneration: initiation of clinical trial," *Journal of Endodontics*, vol. 40, no. 4, pp. S26–S32, 2014.

[193] S. E. Duailibi, M. T. Duailibi, W. Zhang, R. Asrican, J. P. Vacanti, and P. C. Yelick, "Bioengineered dental tissues grown in the rat jaw," *Journal of Dental Research*, vol. 87, no. 8, pp. 745–750, 2008.

[194] E. Ikeda, R. Morita, K. Nakao et al., "Fully functional bioengineered tooth replacement as an organ replacement therapy," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 106, no. 32, pp. 13475–13480, 2009.

[195] S. A. C. Modino and P. T. Sharpe, "Tissue engineering of teeth using adult stem cells," *Archives of Oral Biology*, vol. 50, no. 2, pp. 255–258, 2005.

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 6921097, 12 pages
http://dx.doi.org/10.1155/2016/6921097

Research Article

Characterization of Neurogenic Potential of Dental Pulp Stem Cells Cultured in Xeno/Serum-Free Condition: *In Vitro* and *In Vivo* Assessment

Jieun Jung,[1,2] Jong-Wan Kim,[1,2] Ho-Jin Moon,[3] Jin Young Hong,[2] and Jung Keun Hyun[1,2,4]

[1] *Institute of Tissue Regeneration Engineering, Dankook University, Cheonan, Republic of Korea*
[2] *Department of Nanobiomedical Science and BK21 PLUS NBM Global Research Center for Regenerative Medicine, Dankook University, Cheonan, Republic of Korea*
[3] *Department of Restorative Dentistry & Endodontics, College of Dentistry, Dankook University, Cheonan, Republic of Korea*
[4] *Department of Rehabilitation Medicine, College of Medicine, Dankook University, Cheonan, Republic of Korea*

Correspondence should be addressed to Jung Keun Hyun; rhhyun@dankook.ac.kr

Received 28 March 2016; Revised 27 June 2016; Accepted 10 August 2016

Academic Editor: George Huang

Neural stem cells (NSCs) have a high potency for differentiation to neurons and glial cells for replacement of damaged cells and paracrine effects for the regeneration and remyelination of host axons. Dental pulp is known to have a potential to differentiate into neural-like cells; therefore, dental pulp may be used as an autologous cell source for neural repair. In this study, we selectively expanded stem cells from human dental pulp in an initial culture using NSC media under xeno- and serum-free conditions. At the initial step of primary culture, human dental pulp was divided into two groups according to the culture media: 10% fetal bovine serum medium group (FBS group) and NSC culture medium group (NSC group). In the NSC group relative to the FBS group, the expression of NSC markers and the concentrations of leukemia inhibitory factor, nerve growth factor, and stem cell factor were higher, although their expression levels were lower than those of human fetal NSCs. The transplanted cells of the NSC group survived well within the normal brain and injured spinal cord of rats and expressed nestin and Sox2. Under the xeno- and serum-free conditions, autologous human dental pulp-derived stem cells might prove useful for clinical cell-based therapies to repair damaged neural tissues.

1. Introduction

Stem cell-based therapies using neural stem cells (NSCs) are considered to be one of the most promising strategies for treatment of the lesions of the central and peripheral nervous systems [1, 2]. Endogenous NSCs are known to exist within the adult brain and even in the spinal cord; unfortunately, their capacity for neural regeneration following stroke or spinal cord injury in adults is very limited, as they are difficult to stimulate within *in vivo* microenvironments [2, 3]. Exogenous NSCs from embryos or fetus which have been shown to be effective for neural regeneration [4, 5] still have immunological, ethical, and political problems [6]. Recently developed induced pluripotent stem cells (iPSCs) and direct reprogrammed NSCs [7–9] also have potential risks of viral integration, tumor formation, and genomic instability which remain hurdles to clinical translation [10]. Among other concerns about the clinical application of stem cells, there are animal components such as fetal bovine serum (FBS) which can incur the risk of transmitting pathogens and immune responses to recipients [11, 12].

Various cell types including endothelial cells, fibroblasts, odontoblasts, mesenchymal stem cells (MSCs), and neural cells are contained in dental pulp easily obtainable from human adults and infants. Stem cells within dental pulp (i.e., dental pulp stem cells: DPSCs) have a high potential for proliferation and differentiation into neural-like cells and as such might be a good source for neural regeneration [13]. Previous studies in fact have demonstrated successful differentiation of human dental pulp-derived stem cells into neural-like cells in both *in vitro* and *in vivo* conditions [14–17], and other studies, moreover, have found that neural-like

cells can effectively promote functional improvements in rodent nerve injury models [18, 19].

The aim of this study was to establish a method for isolation and expansion of stem cells from human dental pulp under xeno- and serum-free conditions as well as investigate whether these cells express key neural genes after transplantation into neural tissues of rats.

2. Materials and Methods

2.1. DPSC Isolation and Culture. This study was approved by the Institutional Review Board of Dankook University Dental Hospital in Korea (approval number H-1304/005/003). Normal third molars were extracted and collected from three healthy patients (aged 22-23 years). One oral surgeon gently separated the dental pulp from the periodontal ligament and gingival tissue without contamination, subsequently cutting it into small pieces for incubation in mixed collagenase type I/dispase solution for 1 hour. Large aggregates and debris were removed by passing the cells through a 70-μM strainer. The obtained single cells were divided equally into two groups: a control group and an experimental group. In the control group (FBS group), cells were suspended and cultured with FBS media: Dulbecco's modified Eagle's medium/Ham's F12 medium (DMEM/F12) supplemented with 10% FBS and 1% penicillin/streptomycin (P/S, all from Invitrogen). In the experimental group (NSC group), cells were cultured in NSC media: DMEM/F12 supplemented with 1% P/S, 20 ng/mL basic fibroblast growth factor (bFGF, R&D Systems), 20 ng/mL epidermal growth factor (EGF, R&D Systems), N2 (Invitrogen), and B27 (Invitrogen), under the xeno- and serum-free conditions. All of the cells of both groups, FBS and NSC, were used at passage 5 for later analysis. Two control cell lines were used to compare the cell metabolic activity and quantitative real-time polymerase chain reaction (qRT-PCR) analysis: bone-marrow-derived MSCs (BM-MSCs, from ATCC) which were cultured in DMEM/F12 medium supplemented with 10% FBS, 1% P/S, and human fetal brain-derived NSCs (hNSCs) (from Professor Cho) which were cultured in DMEM/F12 medium supplemented with 1% P/S, bFGF (20 ng/mL), leukemia inhibitory factor (10 ng/mL, Sigma), and N2.

2.2. Neural Differentiation of DPSCs. For neural differentiation, plastic film-surfaced coverslips were placed on a 24-well plate and coated with laminin and fibronectin at 4°C overnight after coating with type I and IV collagen (all 10 μg/mL) for 1 h at room temperature (RT). The cells at passage 5 in FBS and NSC group were seeded at 2×10^4 cells/well and cultured in DMEM/F12 medium supplemented with 1% P/S, N2, B27, retinoic acid (100 nM), sonic hedgehog (100 ng/mL), brain-derived neurotrophic factor (10 ng/mL), glial-cell-derived neurotrophic factor (10 ng/mL), and insulin-like growth factor-1 (10 ng/mL) for 2 weeks and subsequently supplemented with N^6,2′-O-dibutyryladenosine 3′,5′-cyclic monophosphate (dbcAMP, 1 mM) and forskolin (10 μM) for 1 week.

2.3. MTT Assay. To determine the cellular metabolic activity, 3(-4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay was performed. Cells in FBS or NSC groups or BM-MSCs were seeded at 2×10^3 concentrations in a 96-well plate and cultured for 1 to 5 days in appropriate media. Then, the cells were dissolved with 100 μL DMSO after 2-hour incubation with MTT (0.5 mg/mL) and measured by spectrophotometry (OD 570 nm, Bio-Rad Laboratories). The values were expressed as fold changes. The analysis was performed in three independent experiments using three separate wells at each time point.

2.4. Total RNA Isolation. The cells were seeded at 2×10^6 concentrations on a 100 mm culture plate and then lysed in TRIzol (Invitrogen) three days later. Total RNA was extracted with chloroform, precipitated with isopropanol, washed in 75% ethanol, and dissolved in RNase- and DNase-free distilled water (Invitrogen). The RNA concentration was quantified by UV spectrophotometry (NanoDrop Technologies) at 260 nm. The total RNA was stored at −80°C after extraction.

2.5. qRT-PCR Analysis. Complementary DNA (cDNA) was synthesized with 80 ng of RNA by 20 μL reverse transcription reaction using the High-Capacity RNA-to-cDNA Kit (Applied Biosystems). The qRT-PCR of FBS and NSC groups ($n = 3$ each) was performed in StepOnePlus (Applied Biosystems) using the SYBR® Green PCR master mix (Applied Biosystems) containing 0.5 mM of each primer and 2 μL of template in 20 μL of final volume. The sequences of the forward and reverse primers are listed in Table 1. The triplicate reaction for the amplification conditions proceeded as follows: 5 minutes at 95°C, with 25–32 cycles at 94°C for 30 seconds, at 55–60°C for 1 minute, and at 72°C for 1 minute. The expression level of each gene was normalized to 18s ribosomal RNA.

2.6. Fluorescence-Activated Cell Sorting (FACS) Analyses. The FACS analysis was performed on cells seeded at 2×10^6 concentrations in a 100 mm dish after 3 days of culture. FBS and NSC medium cells were stained for 1 hour with the prescribed antibodies (listed in Table 2) and appropriate isotype controls as per the manufacturer's instructions. After being washed twice in phosphate-buffered saline (PBS), the cells were analyzed using FACSCalibur and CellQuest software (BD Biosciences).

2.7. Multiplex Supernatant Cytokine Assay. For the purposes of a cytokine assay, 2×10^4 cells in FBS or NSC groups ($n = 3$) were seeded in a 24-well plate with 0.2 mL of DMEM-F12 supplemented with 1% penicillin/streptomycin (P/S) media, and the individual supernatants were harvested at 24 hours of culture. Cell-supernatant samples of 50 μL were combined with coated beads using the MILLIPLEX™ MAP kit (Millipore) and analyzed. DMEM-F12 supplemented with 1% P/S was used both as a control and as a diluent for the standard samples. After incubation and washing instances, beads from the wells were resuspended in a 125 μL cuvette of the Luminex apparatus. An acquisition gate was set between

Table 1: Sequence of primers used for quantitative real-time PCR in this study.

Gene	Sequence
Dcx	F: 5′-AGC CAA GAG CCC TGG TCC TAT-3′
	R: 5′-TGG AGG TTC CGT TTG CTG AGT-3′
Fibronectin	F: 5′-CAG TGG GAG ACC TCG AGA AG-3′
	R: 5′- CAA AGA CTA CAA GGC TCC CT-3′
Map2	F: 5′- AAC CCT TTG AGA ACA CGA CA-3′
	R: 5′-TCT TTC CGT TCA TCT GCC A-3′
Mash1	F: 5′-CCA GTT GTA CTT CAG CAC C-3′
	R: 5′-TGC CAC TTT GAG TTT GGA C-3′
NCAD	F: 5′-ACA GTG GCC ACC TAC AAA GG-3′
	R: 5′- GTA ATA GTT GGG GTA GAG CC-3′
NCAM	F: 5′-CAG CCA GCA GAT TAC AAT GC-3′
	R: 5′-TGG CTG GGA ACA ATA TCC AC-3′
Nestin	F: 5′-CTG GAG CAG GAG AAA CAG G-3′
	R: 5′-TGG GAG CAA AGA TCC AAG AC-3′
NeuroD1	F: 5′- CCA CGG ATC AAT CTT CTC AG-3′
	R: 5′-CAT GAT GTG AAT GGC TAT CG-3′
Olig2	F: 5′-GGT AAG TGC GCA ATG CTA AGC TGT-3′
	R: 5′-TAC AAA GCC CAG TTT GCA ACG CAG-3′
Pax6	F: 5′-ATG TGT GAG TAA AAT TCT GGG CA-3′
	R: 3′-GCT TAC AAC TTC TGG AGT CGC TA-3′
Sox1	F: 5′-AAT TTT ATT TTC GGC GTT GC-3′
	R: 5′-TGG GCT CTG TCT CTT AAA TTT GT-3′
Sox2	F: 5′-CCC AGC AGA CTT CAC ATG T-3′
	R: 5′-CCT CCC ATT TCC CTC GTT TT-3′
Vimentin	F: 5′-GAG AAC TTT GCC GTT GAA GC-3′
	R: 5′- CTA ACG GTG GAT GTC CTT CG -3′
18S rRNA	F: 5′-CGG CTA CAT CCA AGG AA-3′
	R: 5′-GCT GGA ATT ACC GCG GCT-3′

Dcx: doublecortin; Map2: microtubule associated protein 2; NeuroD1: neuronal differentiation 1; NCAD: neural cadherin; NCAM: neural cell adhesion molecule; Olig2: oligodendrocyte transcription factor 2; Pax6: paired box protein 6; Sox: sex-determining region Y-box.

Table 2: Antibodies used in this study.

Antibody	Dilution	Application	Company
CD95	1 : 100	FACS	BD Biosciences
CD105	1 : 100	FACS	BD Biosciences
CD54	1 : 100	FACS	BD Biosciences
CD56	1 : 100	FACS, IF	BD Biosciences
Nestin	1 : 200	IF	Millipore
Pax6	1 : 500	IF	Covance
Sox2	1 : 200	IF	Millipore
βIII-Tubulin	1 : 1,000	IF	Covance

FACS: fluorescence-activated cell sorting; IF: immunofluorescence staining; Pax6: paired box protein 6; Sox2: sex-determining region Y-box 2.

7500 and 13,500 for doublet discrimination; the sample volume was 75 μL, and 100 events/region were acquired. To obtain the concentration values, raw data (mean fluorescence intensity) from all of the bead combinations tested were analyzed using Master Plex QT3.0 quantification software (MiraiBio Inc.).

2.8. In Vivo Transplantation. To investigate the cell survivability under the *in vivo* condition, cells were transplanted into the neonatal brain (2 days, $n = 3$) and injured spinal cord (12 weeks, $n = 3$) of Sprague-Dawley (SD) rats. All of the animal care and surgical procedures were approved by the Institutional Animal Care and Use Committee of Dankook University in Korea (approval number DKU-12-019) and conformed to the ARRIVE guidelines. The contusion injury was applied to the T9 spinal cord level using the Infinite Horizon impactor (IH-400, Precision Systems and Instrumentation) as previously described [20]. Cells of both groups, NSC or FBS medium cells (5×10^5 cells in 5 μL of PBS), were transplanted into the cortex region at first, and then NSC medium cells were transplanted into the epicenter of the injured spinal cord at 9 days after contusion injury at a rate of 1 μL/min using a Hamilton syringe (Hamilton Company). Cyclosporine A (Cipol Inj™, Chong Kun Dang Pharmaceutical Corp.) was administered subcutaneously at 10 mg kg^{-1} day^{-1} until the animals were sacrificed.

2.9. Immunofluorescence Staining. Cells were fixed in 4% paraformaldehyde (PFA) for 30 minutes at RT and washed with PBS 3 times. The rats were perfused and the brains removed 1 week after cell transplantation. The removed brain and spinal cord tissues were postfixed, immersed, and then embedded, whereupon coronal sectioning of the brain tissue and sagittal sectioning of the spinal cord tissue were performed serially. The prepared cell and tissue samples were incubated with primary antibody as listed in Table 2. Following incubation, the slides were washed with PBS and incubated with fluorescent-dye-conjugated secondary antibodies and stained with 4′,6-diamidino-2′-phenylindole dihydrochloride (DAPI) staining. Images were taken using a confocal microscope (Carl Zeiss Inc.).

2.10. Statistical Analysis. Statistical analyses were performed using PASW Statistics 18 (SPSS Inc., Chicago, IL, USA). The Shapiro-Wilk test was conducted to reveal the normal distribution of all quantitative data from MTT assay, qRT-PCR, and cytokine assays from *in vitro* studies, and Levene's test was conducted for homogeneity of variance of fold changes from MTT assay. According to the result, two-way repeated-measures ANOVA (cell types and time point) was performed to compare the fold changes from MTT assay among FBS and NSC groups and BM-MSC control, and individual comparisons were made at each time point by one-way ANOVA with Welch statistic and Games-Howell post hoc test. An independent t-test was performed to compare data from qRT-PCR and cytokine assays between FBS and NSC groups. P value less than 0.05 was considered significant.

3. Results

3.1. Morphology and Cellular Metabolic Activity of DPSCs Cultured under Different Media Conditions. The cells from human dental pulp were firstly passaged at 1 week for

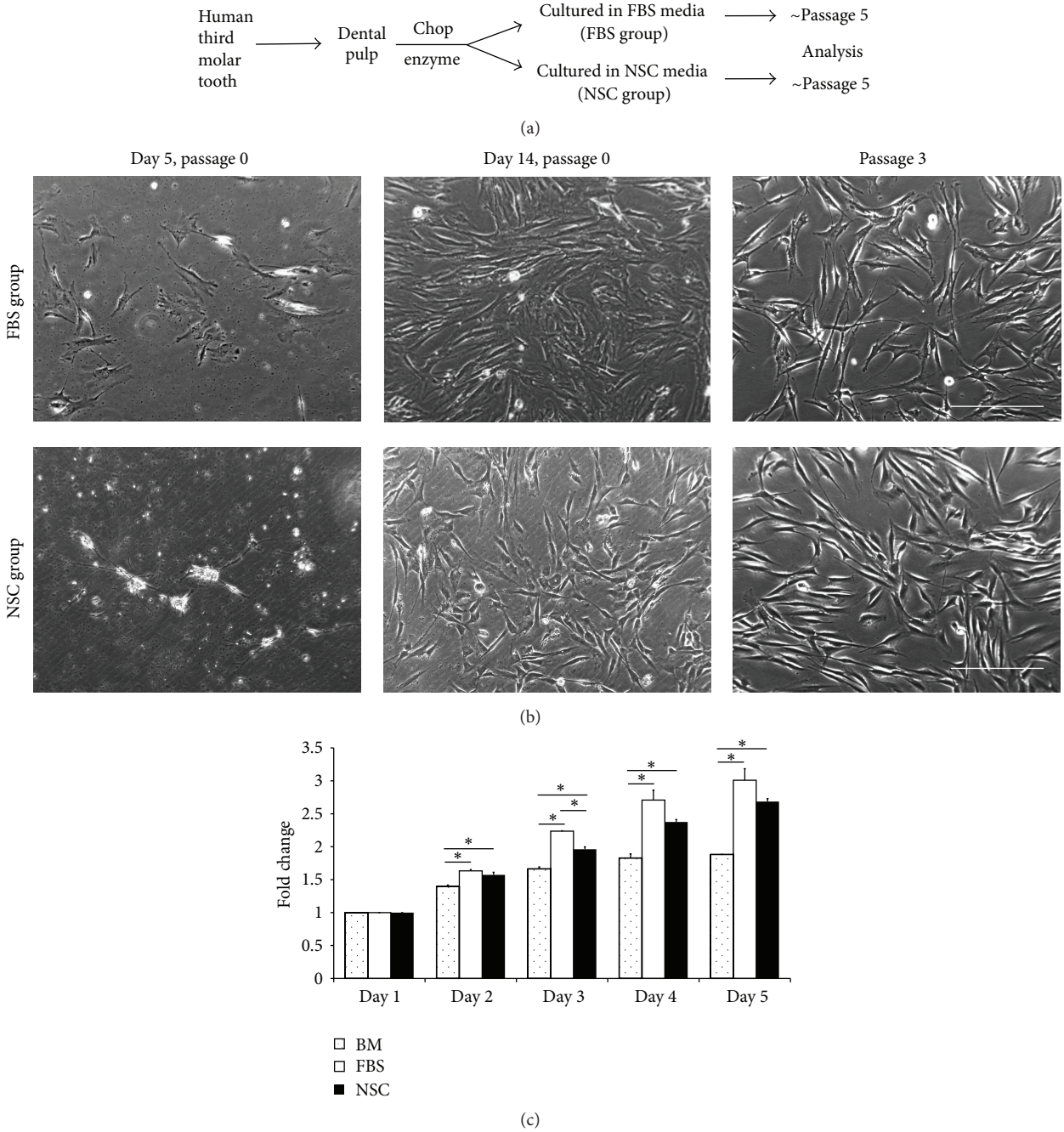


Figure 1: Strategy for derivation of neural stem-like cells from dental pulp of extracted human third molar. (a) Experimental scheme for classification of fetal bovine serum (FBS) and neural stem cell (NSC) groups. (b) Comparison of primary cell expansion and morphology between FBS and NSC medium cells. Scale bar = 100 μm. (c) MTT assay to measure cellular metabolic activities of FBS medium cells, NSC medium cells at passage 5, and human bone-marrow-derived mesenchymal stem cells (BM-MSCs). The error bars represent the standard deviation of mean from three independent experiments from one donor (FBS and NSC groups) or one cell line (BM). Two-way repeated-measures ANOVA (Greenhouse-Geisser) revealed significant effects of day, cell group, and day × cell group interaction ($P < 0.05$). The asterisk (∗) indicates significant difference in comparison with BM-MSC at each time point according to one-way ANOVA and Games-Howell post hoc test ($P < 0.05$).

FBS group and 2 weeks for NSC group after isolation (Figure 1(a)). Although the first passaging of the cells of NSC group required more time than for FBS medium cells (Figure 1(b)), we could obtain many strongly proliferative cells under NSC media condition (Figures 1(b) and 1(c)). At passage 3, FBS medium cells exhibited a rough surface and flattened fibroblast-like morphology, whereas NSC medium cells manifested a smooth surface and more convex shape (Figure 1(b)); non-pulp-tissue-derived cells, for example, from the periodontal ligament and gingiva, however, failed to

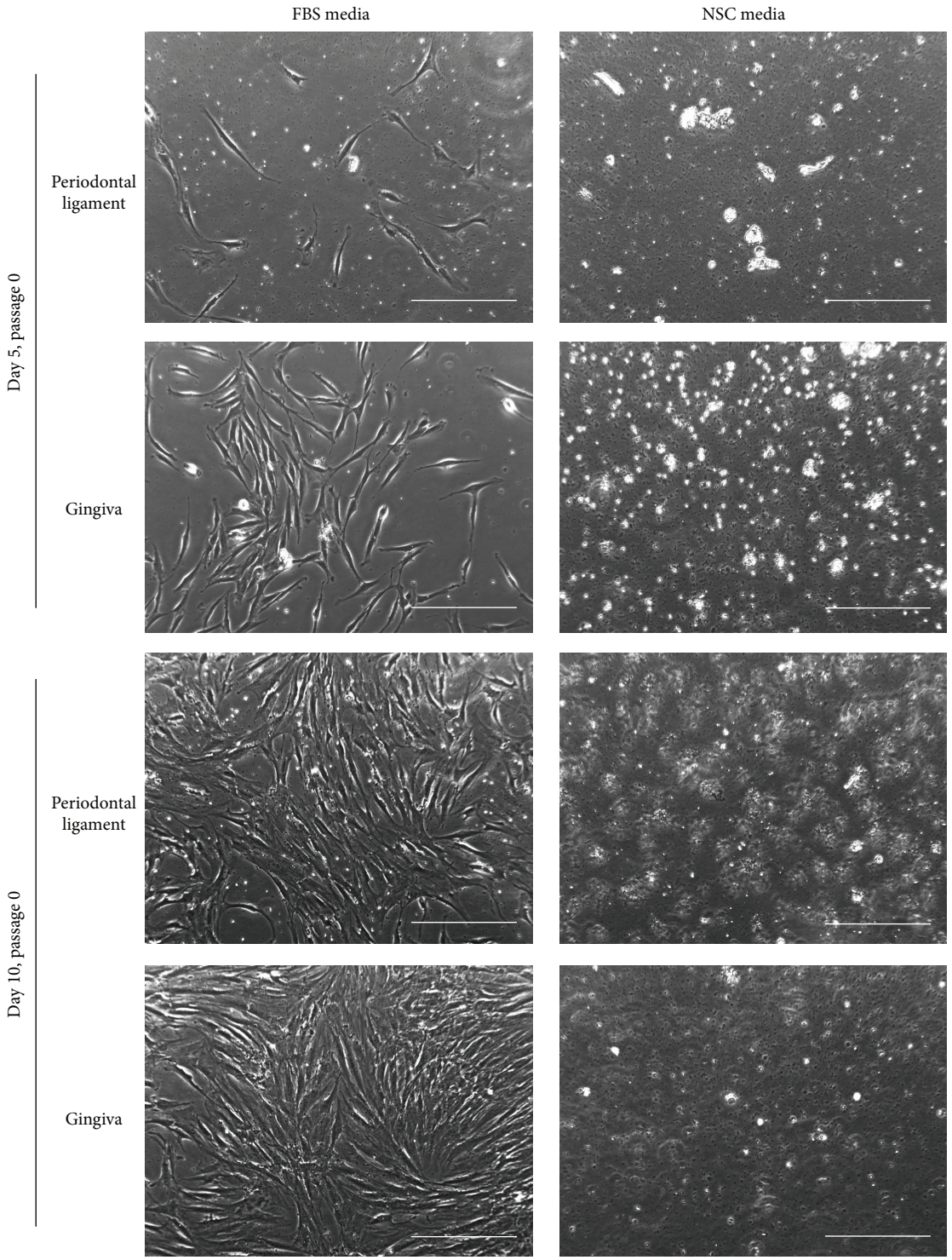


Figure 2: Primary cell adherent culture and morphology of periodontal ligament and gingival cells in FBS media or NSC media from day 5 to day 10. Scale bar = 100 μm.

grow in the NSC media (Figure 2). The MTT assay revealed that NSC medium cells' metabolic activity was similar to that of FBS medium cells during the initial 5-day culture period except for day 3 and was significantly higher than that of the human BM-MSCs (Figure 1(c)). These data indicate, significantly, that highly proliferative cells can be obtained from human dental pulp in a serum-free NSC medium from the initial culture stage.

3.2. Comparison of Marker Expressions between FBS and NSC Medium Cells. The qRT-PCR revealed that the NSC markers, including doublecortin (Dcx), microtubule associated

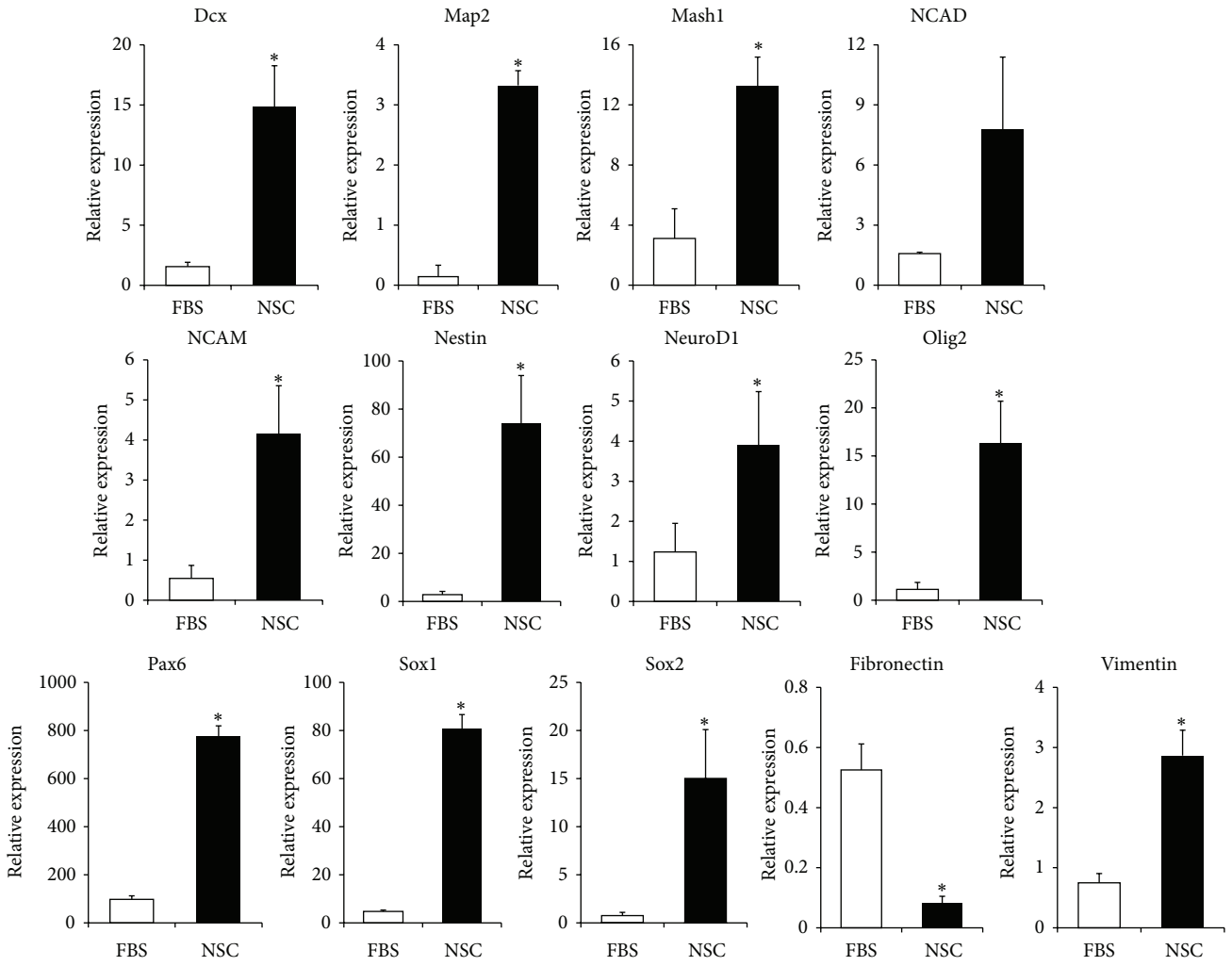


Figure 3: Comparison of marker expressions between FBS group (FBS, white bar) and NSC group (NSC, black bar) at passage 5. qRT-PCR was applied for measurement of NSC or neuronal markers (Dcx, Map2, Mash1, NCAD, NCAM, nestin, NeuroD1, Olig2, Pax6, Sox1, and Sox2), MSC marker (fibronectin), and both NSC and MSC markers (vimentin) in the FBS and NSC groups. The results are displayed relative to the expressions in FBS medium cells via calculation using the ΔΔCT and 2-(ΔΔCT) methods and normalized to 18s ribosomal RNA expression, and the error bars represent the standard deviation of mean from three donors in each group ($n = 3$). The asterisk (∗) indicates significant difference in comparison with the FBS group according to the independent t-test ($P < 0.05$).

protein 2 (Map2), Mash1, neural cell adhesion molecule (NCAM, also known as CD56), nestin, neuronal differentiation 1 (NeuroD1), paired box protein 6 (Pax6), sex-determining region Y-box 1 (Sox1), Sox2, NSC and glial-cell marker including oligodendrocyte transcription factor-2 (Olig2), and vimentin, which is a marker of both NSC and MSC, were all expressed significantly higher in NSC group than in FBS group, whereas fibronectin, which is known as an MSC marker, was expressed significantly lower in NSC group than in FBS group (Figure 3). And individual expression patterns of FBS and NSC groups were shown in Supplemental Figure 1, in Supplementary Material available online at http://dx.doi.org/10.1155/2016/6921097, with the expression patterns of BM-MSC and fetal brain-derived NSCs (Supplemental Figure 1), and the expression level of most of the NSC markers in NSC group seemed to be lower than in human fetal brain-derived NSCs.

Next, NSC- and MSC-specific surface markers were analyzed by FACS. In NSC group, the CD15-, CD54-, CD56-, CD95-, and CD133-positive cell proportions were much higher than those in FBS group. By contrast, the expression level of the MSC marker CD105 was superior in FBS group in comparison to the NSC group. The hematopoietic stem cell markers CD34 and CD45, meanwhile, were expressed negatively in both FBS and NSC medium cells (Figure 4(a)). As demonstrated in Figure 4(b), the immunocytochemical results for Pax6 were consistent with the qRT-PCR data for FBS and NSC groups, though nestin was similarly stained in both groups. These data indicate that the cells cultured in xeno- and serum-free NSC media from the initial step of primary culture showed higher NSC features compared with the cells cultured in the FBS-containing media.

3.3. Factors Secreted from FBS and NSC Medium Cells and Neural Differentiation. The present multiplex supernatant

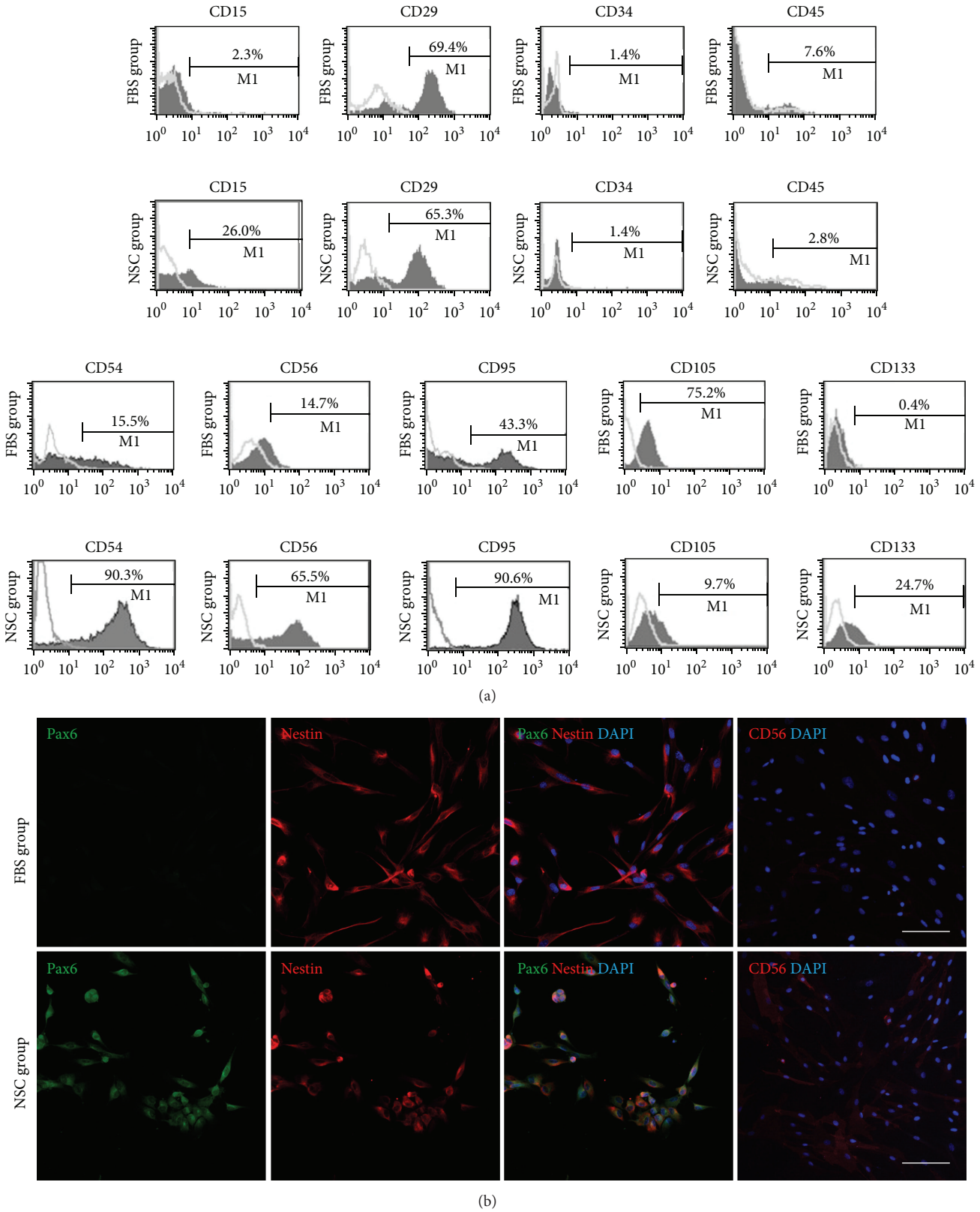


Figure 4: FACS analysis and immunocytofluorescence of FBS and NSC groups. (a) Expression of surface markers in FBS and NSC groups at passage 5 as analyzed by FACS. The NSC markers (CD15, CD29, CD54, CD56, and CD95), MSC marker (CD105), and hematopoietic markers (CD34, CD45, and CD133) in the FBS and NSC medium cells. (b) Immunocytofluorescence findings of FBS and NSC medium cells at passage 5. The cells were stained with Pax6, nestin, and CD56. Scale bar = 100 μm.

human cytokine assay was performed to identify the factors secreted from FBS and NSC medium cells. In the cell supernatant of NSC group, the cytokines involved in self-renewal and differentiation of NSC, such as leukemia inhibitory factor (LIF), nerve growth factor (NGF), and stem cell factor (SCF) [21], were significantly more evident ($P < 0.05$) than in FBS group; meanwhile, the cytokines mainly involved in inflammation, such as interleukin-6 (IL-6), IL-9, granulocyte-macrophage colony-stimulating factor (GM-CSF), growth-regulated oncogene α (GROα), and tumor necrosis factor α (TNF-α) [22], were significantly less abundant ($P < 0.05$) (Figure 5(a)).

Further, the neural differentiation potential of FBS and NSC medium cells was investigated, to which end βIII-tubulin-positive cells were examined 3 weeks after neural differentiation (Figure 5(b)). Before neural differentiation, no clear βIII-tubulin-positive cells were found in either FBS or NSC groups (Supplemental Figure 2). Three weeks after the differentiation process, we found that βIII-tubulin-positive cells were visible in both FBS and NSC groups; however, the number of positive cells was higher in NSC group than in FBS group (Figure 5(b)). The data obtained indicated that NSC medium cells secreted multiple growth factors and stem cell-niche-related cytokines at higher levels than did FBS medium cells, thereby demonstrating, via manifested bipolar and multipolar morphologies, their neural differentiation potential.

3.4. In Vivo Transplantation of FBS and NSC Medium Cells into Rat CNS. One week after transplantation of NSC medium cells, the transplanted cells had survived well within the brain and injured spinal cord and also had successfully expressed NSC markers (Figure 6). The engrafted NSC medium cells expressed Sox2, which were costained with nestin (Figure 6(a)) or human nucleus (Figure 6(b)); however, their staining intensity was lower than Sox2-positive host cells (Figure 6). We also transplanted FBS medium cells within the normal neonatal brain of rats, and transplanted cells survived with a small amount within the transplanted site 1 week after transplantation (Supplemental Figure 3). These data reflect NSC medium cells' favorable indications for survival and integration within the rat CNS.

4. Discussion

Dental pulp, which is cell-rich soft tissue generated in the cranial neural crest during tooth development, contains heterogeneous populations such as odontoblast, fibroblast, pericyte, and neural cells as well as stem cells persisting in the adult dental pulp niche [23]. For culturing of DPSCs, most studies have utilized animal-serum-containing media to obtain sufficient cells during primary culturing, even for research on neural regeneration [14, 17]. However, serum is more suitable for MSC than for NSC growth; furthermore, animal serum incurs safety risks due to the possibility of the transmission of pathogens such as prions, viruses, and zoonoses. Also, animal-serum proteins can induce immunogenic reactions, which might reject transplanted cells even if autologous [12]. In light of these factors, establishment of a xeno-free culture system is required for any cell therapeutic strategies. In the present study then, in order to exclude animal components and to inhibit the expansion of MSC or fibroblasts within dental pulp, we strictly avoided the use of synthetic-, human-, or other animal-origin serums for the expansion of stem cells. Notwithstanding the many successful studies that have already investigated DPSC properties, the present investigation, to our knowledge, is the first to undertake the selective expansion of neural stem-like cells from human dental pulp without any exposure of animal serum from the initial step of primary culture, an approach that is highly advantageous from the clinical application safety standpoint. Although a previous study revealed the expansion abilities of DPSCs in a serum-free medium, the cells were cultured with 10%-FBS-supplemented medium on the first day before culturing them in the serum-free medium [23].

Herein, we hypothesize that stem cells can be expanded among the heterogeneous cells of dental pulp in serum-free NSC media from the initial step of primary culture. In the present study accordingly, two different culture conditions, FBS media and NSC media, were applied to dental pulp that had been divided from three independent donors. The results showed that the cells were greatly expanded in the NSC media until at least the 10th passage, though further study will be needed to determine the exact role in neural repair of NSC media. Significantly nonetheless, we found that no cells had grown from other dental tissues such as periodontal ligament- or gingival-derived tissues in the serum-free NSC media, whereas, in the animal-serum-containing media, the cells, as already demonstrated in relevant earlier studies, were well expanded (Figure 2) [24, 25]. These differences between dental pulp and other dental tissues might be caused by the diversity of cell types; certainly, the results tell us that dental pulp, as opposed to other oral-origin tissues, is favorable for obtainment of responsive cells under the neural basal condition. In further results, whereas the NSC medium cells only slowly expanded in the initial step of primary culture, they acquired a high growth capacity after adaptation to *in vitro* culture system. At the 5th passage, we investigated, by FACS analysis, whether cell surface markers were expressed differently in FBS and NSC medium cells. Surface markers act as essential compounds and receptors for various cellular metabolic processes such as proliferation and differentiation. Our data revealed, in fact, that a large number of DPSCs expressed the MSC marker when they were cultured in 10%-FBS-supplemented medium; however, the NSC markers were dominantly expressed when they were expanded in the NSC medium, even though the CD29-positive populations in the respective groups were similar. These marker patterns were shown in the qRT-PCR and immunofluorescence results concurrently with the FACS data. Overall, these data support the hypothesis that neuroectodermal origin human DPSCs presenting the typical profiles of NSC markers for gene and protein expression can be selectively expanded in xeno- and serum-free NSC media.

For regulation of the environmental niche for stem cells' survival and differentiation, paracrine effects are important. Previous studies analyzed the cytokine release profiles of

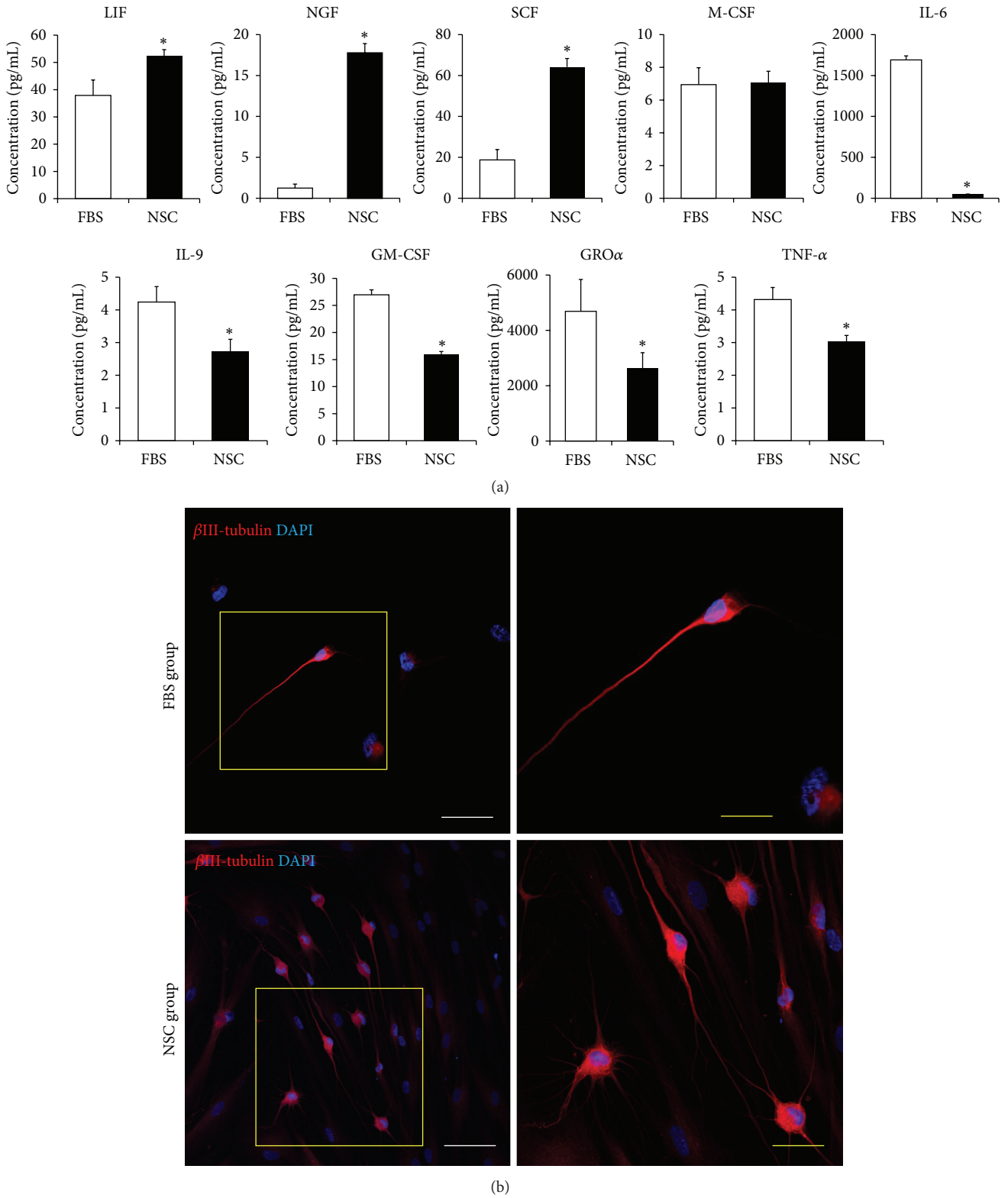


Figure 5: Growth factors, cytokine-secretion capacity, and neural differentiation potency. (a) Multiplex supernatant cytokine assay in FBS group (FBS, white bar) and NSC group (NSC, black bar) at passage 5. The error bars represent the standard deviation of mean from three donors in each group ($n = 3$). The asterisk ($*$) indicates significant difference in comparison with the FBS group according to the independent t-test ($P < 0.05$). (b) Immunocytofluorescence findings of FBS and NSC medium cells after 3-week procedure for differentiation to neural-like cells. βIII-Tubulin positive cells were stained in red, and nuclear counterstaining was performed with DAPI (blue). White scale bars = 100 μm; yellow scale bar = 50 μm.

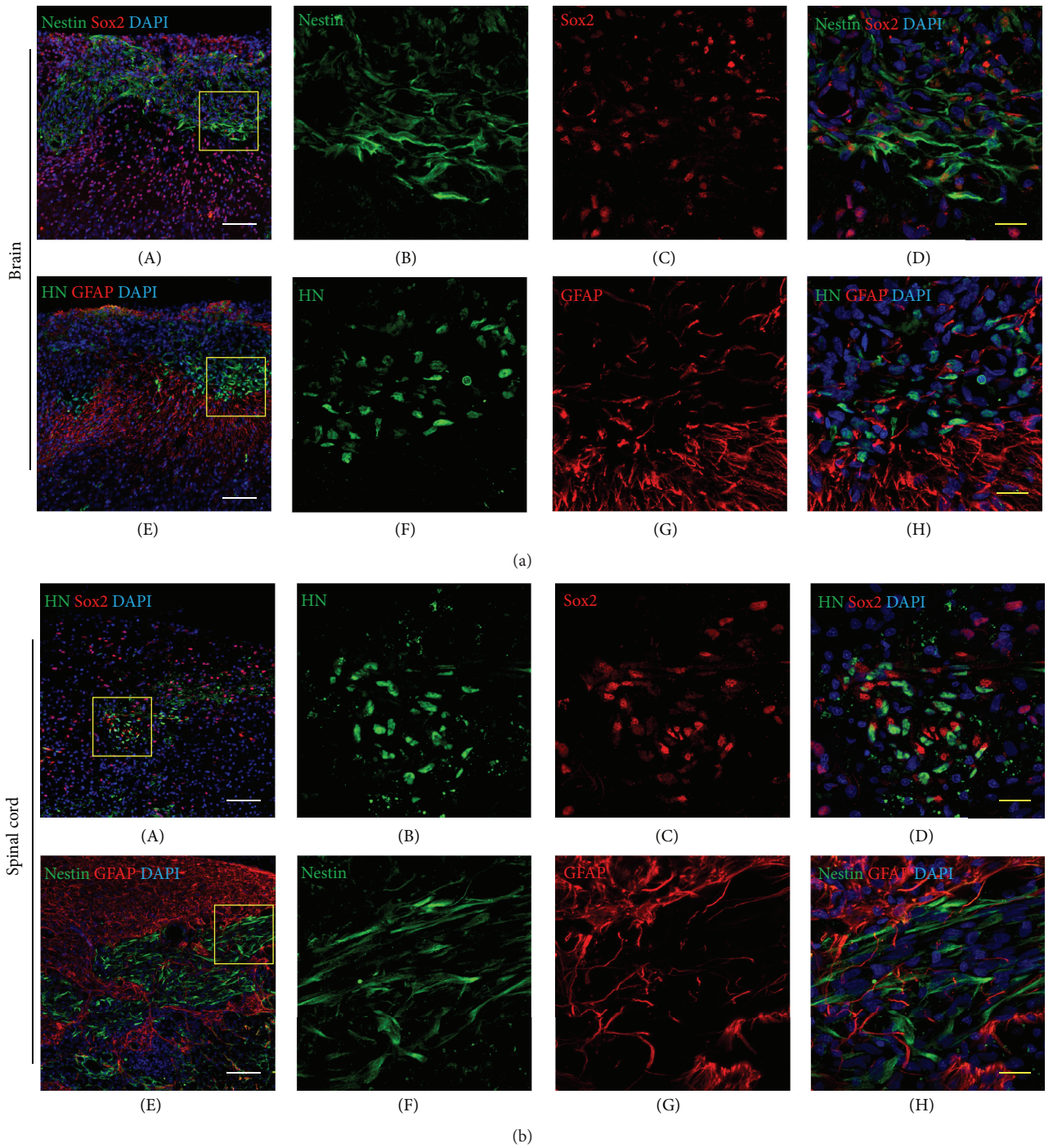


FIGURE 6: Immunohistofluorescence findings of the neonatal brain (a) and injured spinal cord tissues (b) of rats at 1 week after transplantation of NSC medium cells. The tissues were stained with human nuclei (HN, green, (a)(F) and (b)(B); GFAP, red, (a)(G) and (b)(G); nestin, green, (a)(B) and (b)(F); and Sox2, red, (a)(C) and (b)(C)), and nuclear counterstaining was performed with DAPI (blue). (a)(B–D), (a)(F–H), (b)(B–D), and (b)(F–H) show magnified images of yellow-line boxes in (a)(A), (a)(E), (b)(A), and (b)(E) images, respectively. White scale bars = 100 μm; yellow scale bar = 20 μm.

DPSCs during osteo/odontogenic differentiation [26] or secretomes in the conditioned medium from stem cells from human exfoliated deciduous teeth [27]; however, there has been no study on NSC-related secretomes on DPSCs. In the current study, because secretion of cytokines related to self-renewal and tissue-repair and immunomodulatory factors are important for stem cell transplantation, we evaluated cytokines known to be secreted by NSCs and to affect neural activity [28–30]. In the results, cytokines known to be secreted by NSCs were highly contained in the supernatant

of NSC medium cells, suggesting the possibility of neuroregenerative activities with paracrine effects. We additionally confirmed the neural differentiation potential (Figure 5(b)) and *in vivo* experiments, as an a priori study, revealed the survival ability of dental pulp-derived NSC medium cells within the rat CNS, specifically the normal brain and injured spinal cord (Figure 6). However, the expression level of NSC markers in NSC medium cells, as shown in qRT-PCR and immunohistochemistry, seemed to be lower than endogenous neural stem cells, and further *in vivo* researches will be needed to clarify the potential usefulness of human DPSC-derived NSC medium cells to promote functional restoration of CNS lesions.

Taken together, our investigations and results would seem to confirm the hypothesis that human dental pulp tissue contains stem cell populations applicable to neuroregenerative medicine. Optimization of cell-isolation and culture methods for such application, therefore, is called for. Nonetheless, small number of samples, which were obtained from only three subjects, might raise power issues, and future studies are required for elucidation of the potencies for differentiation to specific types of neural cells, for the therapeutic function on neural injury over the long term, and also for derivation of a more time-efficient method of initial expansion for safer application of NSC medium cells.

5. Conclusion

Here, we identified a novel method for safe and reliable cell expansion of stem cells from dental pulp in an initial culture using NSC media using, for the first time, xeno- and serum-free culture systems. This study may help pave the way for the clinical translation using cell-based therapies to repair and regenerate damaged neural tissues.

Competing Interests

The authors have no competing interests to declare.

Acknowledgments

This research is supported by the National Research Foundation of Korea (NRF), Contracts 2014R1A1A3052554, 2009-0093829, NRF-2015R1A2A1A15053883, and NRF-2015R1D1A1A02061196, and the Korea Health Technology R&D Project (HI14C0522) by the Ministry of Health & Welfare, Republic of Korea. The authors thank Professor Seung-Woo Cho (Yonsei University, Korea) for the donation of the human fetal brain-derived neural stem cells and Professor Gi Jin Kim (Cha University, Korea) for the support of this project.

References

[1] T. H. Mouhieddine, F. H. Kobeissy, M. Itani, A. Nokkari, and K. K. W. Wang, "Stem cells in neuroinjury and neurodegenerative disorders: challenges and future neurotherapeutic prospects," *Neural Regeneration Research*, vol. 9, no. 9, pp. 901–906, 2014.

[2] C.-A. Grégoire, B. L. Goldenstein, E. M. Floriddia, F. Barnabé-Heider, and K. J. L. Fernandes, "Endogenous neural stem cell responses to stroke and spinal cord injury," *Glia*, vol. 63, no. 8, pp. 1469–1482, 2015.

[3] Y. A.-Z. Wang, J. M. Plane, P. Jiang, C. J. Zhou, and W. Deng, "Concise review: quiescent and active states of endogenous adult neural stem cells: identification and characterization," *Stem Cells*, vol. 29, no. 6, pp. 907–912, 2011.

[4] W. Tetzlaff, E. B. Okon, S. Karimi-Abdolrezaee et al., "A systematic review of cellular transplantation therapies for spinal cord injury," *Journal of Neurotrauma*, vol. 28, no. 8, pp. 1611–1682, 2011.

[5] T. K. Kelly, S. L. Karsten, D. H. Geschwind, and H. I. Kornblum, "Cell lineage and regional identity of cultured spinal cord neural stem cells and comparison to brain-derived neural stem cells," *PLoS ONE*, vol. 4, no. 1, article e4213, 2009.

[6] K. A. Galvin and D. G. Jones, "Adult human neural stem cells for cell-replacement therapies in the central nervous system," *Medical Journal of Australia*, vol. 177, no. 6, pp. 316–318, 2002.

[7] T. Matsui, W. Akamatsu, M. Nakamura, and H. Okano, "Regeneration of the damaged central nervous system through reprogramming technology: basic concepts and potential application for cell replacement therapy," *Experimental Neurology*, vol. 260, pp. 12–18, 2014.

[8] M. Ruggieri, G. Riboldi, S. Brajkovic et al., "Induced neural stem cells: methods of reprogramming and potential therapeutic applications," *Progress in Neurobiology*, vol. 114, pp. 15–24, 2014.

[9] D. W. Han, N. Tapia, A. Hermann et al., "Direct reprogramming of fibroblasts into neural stem cells by defined factors," *Cell Stem Cell*, vol. 10, no. 4, pp. 465–472, 2012.

[10] A. S. Lee, C. Tang, M. S. Rao, I. L. Weissman, and J. C. Wu, "Tumorigenicity as a clinical hurdle for pluripotent stem cell therapies," *Nature Medicine*, vol. 19, no. 8, pp. 998–1004, 2013.

[11] G. A. Tonti and F. Mannello, "From bone marrow to therapeutic applications: different behaviour and genetic/epigenetic stability during mesenchymal stem cell expansion in autologous and foetal bovine sera?" *International Journal of Developmental Biology*, vol. 52, no. 8, pp. 1023–1032, 2008.

[12] M. H. Mohd Yunus, K. Chan Siang, N. I. Hashim et al., "The effects of human serum to the morphology, proliferation and gene expression level of the respiratory epithelium in vitro," *Tissue and Cell*, vol. 46, no. 4, pp. 233–240, 2014.

[13] G. Ibarretxe, O. Crende, M. Aurrekoetxea, V. García-Murga, J. Etxaniz, and F. Unda, "Neural crest stem cells from dental tissues: a new hope for dental and neural regeneration," *Stem Cells International*, vol. 2012, Article ID 103503, 12 pages, 2012.

[14] P. Gervois, T. Struys, P. Hilkens et al., "Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons," *Stem Cells and Development*, vol. 24, no. 3, pp. 296–311, 2015.

[15] W. K. Leong, T. L. Henshall, A. Arthur et al., "Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms," *Stem Cells Translational Medicine*, vol. 1, no. 3, pp. 177–187, 2012.

[16] A. Arthur, G. Rychkov, S. Shi, S. A. Koblar, and S. Gronthose, "Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues," *Stem Cells*, vol. 26, no. 7, pp. 1787–1795, 2008.

[17] M. Kanafi, D. Majumdar, R. Bhonde, P. Gupta, and I. Datta, "Midbrain cues dictate differentiation of human dental pulp

stem cells towards functional dopaminergic neurons," *Journal of Cellular Physiology*, vol. 229, no. 10, pp. 1369–1377, 2014.

[18] K. Sakai, A. Yamamoto, K. Matsubara et al., "Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms," *Journal of Clinical Investigation*, vol. 122, no. 1, pp. 80–90, 2012.

[19] M. Yamagata, A. Yamamoto, E. Kako et al., "Human dental pulp-derived stem cells protect against hypoxic-ischemic brain injury in neonatal mice," *Stroke*, vol. 44, no. 2, pp. 551–554, 2013.

[20] J. Y. Hong, S. H. Lee, S. C. Lee et al., "Therapeutic potential of induced neural stem cells for spinal cord injury," *The Journal of Biological Chemistry*, vol. 289, no. 47, pp. 32512–32525, 2014.

[21] J. S. Goldberg and K. K. Hirschi, "Diverse roles of the vasculature within the neural stem cell niche," *Regenerative Medicine*, vol. 4, no. 6, pp. 879–897, 2009.

[22] H. Hakimi, N. Zainodini, H. Khorramdelazad, M. K. Arababadi, and G. Hassanshahi, "Seminal levels of pro-inflammatory (CXCL1, CXCL9, CXCL10) and homeostatic (CXCL12) chemokines in men with asymptomatic *Chlamydia trachomatis* infection," *Jundishapur Journal of Microbiology*, vol. 7, no. 12, Article ID e11152, 2014.

[23] V. Bonnamain, R. Thinard, S. Sergent-Tanguy et al., "Human dental pulp stem cells cultured in serum-free supplemented medium," *Frontiers in Physiology*, vol. 4, article 357, 2013.

[24] V. R. Fortino, R.-S. Chen, D. Pelaez, and H. S. Cheung, "Neurogenesis of neural crest-derived periodontal ligament stem cells by EGF and bFGF," *Journal of Cellular Physiology*, vol. 229, no. 4, pp. 479–488, 2014.

[25] X. Xu, C. Chen, K. Akiyama et al., "Gingivae contain neural-crest- and mesoderm-derived mesenchymal stem cells," *Journal of Dental Research*, vol. 92, no. 9, pp. 825–832, 2013.

[26] S. Asgary, H. Nazarian, A. Khojasteh, and N. Shokouhinejad, "Gene expression and cytokine release during odontogenic differentiation of human dental pulp stem cells induced by 2 endodontic biomaterials," *Journal of Endodontics*, vol. 40, no. 3, pp. 387–392, 2014.

[27] K. Matsubara, Y. Matsushita, K. Sakai et al., "Secreted ectodomain of sialic acid-binding Ig-like lectin-9 and monocyte chemoattractant protein-1 promote recovery after rat spinal cord injury by altering macrophage polarity," *Journal of Neuroscience*, vol. 35, no. 6, pp. 2452–2464, 2015.

[28] H.-C. Chen, H.-I. Ma, H.-K. Sytwu et al., "Neural stem cells secrete factors that promote auditory cell proliferation via a leukemia inhibitory factor signaling pathway," *Journal of Neuroscience Research*, vol. 88, no. 15, pp. 3308–3318, 2010.

[29] K. J. Kovacs, A. Földes, and E. S. Vizi, "C-kit ligand (Stem Cell Factor) affects neuronal activity, stimulates pituitary-adrenal axis and prolactin secretion in rats," *Journal of Neuroimmunology*, vol. 65, no. 2, pp. 133–141, 1996.

[30] P. Lu, L. L. Jones, E. Y. Snyder, and M. H. Tuszynski, "Neural stem cells constitutively secrete neurotrophic factors and promote extensive host axonal growth after spinal cord injury," *Experimental Neurology*, vol. 181, no. 2, pp. 115–129, 2003.

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 9762871, 17 pages
http://dx.doi.org/10.1155/2016/9762871

Review Article

The Neurovascular Properties of Dental Stem Cells and Their Importance in Dental Tissue Engineering

Jessica Ratajczak, Annelies Bronckaers, Yörg Dillen, Pascal Gervois, Tim Vangansewinkel, Ronald B. Driesen, Esther Wolfs, Ivo Lambrichts, and Petra Hilkens

Laboratory of Morphology, Biomedical Research Institute (BIOMED), Hasselt University, Diepenbeek, Belgium

Correspondence should be addressed to Petra Hilkens; petra.hilkens@uhasselt.be

Received 26 May 2016; Accepted 1 August 2016

Academic Editor: Imad About

Within the field of tissue engineering, natural tissues are reconstructed by combining growth factors, stem cells, and different biomaterials to serve as a scaffold for novel tissue growth. As adequate vascularization and innervation are essential components for the viability of regenerated tissues, there is a high need for easily accessible stem cells that are capable of supporting these functions. Within the human tooth and its surrounding tissues, different stem cell populations can be distinguished, such as dental pulp stem cells, stem cells from human deciduous teeth, stem cells from the apical papilla, dental follicle stem cells, and periodontal ligament stem cells. Given their straightforward and relatively easy isolation from extracted third molars, dental stem cells (DSCs) have become an attractive source of mesenchymal-like stem cells. Over the past decade, there have been numerous studies supporting the angiogenic, neuroprotective, and neurotrophic effects of the DSC secretome. Together with their ability to differentiate into endothelial cells and neural cell types, this makes DSCs suitable candidates for dental tissue engineering and nerve injury repair.

1. Introduction

The main goal of tissue engineering is to reconstruct natural tissues by combining progenitor/stem cells with growth factors and different biomaterials to serve as a scaffold for novel tissue growth [1]. Selecting a suitable stem cell source is probably the most essential component of a successful tissue engineering approach. The field of tissue engineering is in need of high quality adult stem cells from an easily accessible source. Within the human body a wide variety of stem cell niches have been identified, not only in bone marrow, adipose tissue, and umbilical cord but also in teeth [2–6]. During tooth development, an outer layer of enamel and an inner layer of primary dentin are formed by reciprocal, spatiotemporal interactions between neural crest-derived mesenchyme and embryonic oral epithelium [7, 8]. Primary dentin is produced by odontoblasts, cells that are thought to arise from precursor cells residing in a strongly innervated and vascularized soft connective tissue within the tooth, that is, the dental pulp. In 2000, Gronthos et al. were the first to describe a heterogeneous, clonogenic, and highly proliferative cell population within the dental pulp, namely, dental pulp stem cells (DPSCs) [4]. A similar stem cell population could also be isolated from the dental pulp of human deciduous teeth [9]. In addition to DPSCs and stem cells from human exfoliated deciduous teeth (SHEDs), a number of other distinct stem cell populations have been reported to reside within the human tooth and its surrounding tissues. For example, stem cells from the apical papilla (SCAPs) can be found in the loosely attached soft connective tissue at the apex of developing permanent teeth, that is, the apical papilla [10]. Dental follicle stem cells (FSCs), on the other hand, are isolated from the dental follicle. This is a loose connective tissue which surrounds developing teeth and later on in development gives rise to the periodontal ligament and other tissues of the periodontium [11]. The periodontal ligament, a specialized connective tissue, not only attaches the tooth to the alveolar bone but also has a sensory function. Within this ligament, another stem cell population can be found, namely, periodontal ligament stem cells (PDLSCs) [12]. According to

the minimal criteria defined by the International Society for Cellular Therapy, DPSCs, SHEDs, SCAPs, FSCs, and PDLSCs (collectively referred to as dental stem cells (DSCs)) are considered to be mesenchymal stem cells (MSCs). In addition to their plastic adherence and characteristic expression of surface markers such as CD73, CD90, and CD105, they also display a negative expression of CD14, CD34, and CD45, and they are capable of osteogenic, chondrogenic, and adipogenic differentiation [4, 13–15]. Next to the formation of dental tissue *in vitro* and *in vivo*, DSCs have also been reported to differentiate into myogenic, neurogenic, and endothelial lineages. Due to this multilineage differentiation potential as well as their immunomodulatory properties and minimally invasive isolation from extracted third molars, these stem cells have raised high hopes for potential clinical applications [16–21]. Nevertheless, one should always take into account potential origin-related differences. In general, SCAPs and FSCs are considered to be more immature, given their origin from developing dental tissues, and thus more potent in comparison to DPSCs. SCAPs have already been reported to have a higher proliferation rate, a more distinct doubling capacity, and enhanced migratory properties in comparison to DPSCs [10]. Furthermore, the glial origin of a subpopulation of DPSCs suggests that the tissue of origin is a determining factor for the regenerative potential of DSCs [22, 23]. In order to offer an elaborate overview of the angiogenic and neurogenic properties of different DSC populations as well as their current clinical applications in the dental and neurovascular field, a literature search was performed on PubMed. The following keywords were used: "dental stem cells"; "dental pulp stem cells"; "stem cells from the apical papilla"; "stem cells from human exfoliated deciduous teeth"; "dental follicle stem cells"; "periodontal ligament stem cells". These keywords were subsequently combined with the search terms, "angiogenesis"; "endothelial differentiation"; "neurogenic differentiation"; "neuroregeneration"; "dental tissue engineering"; "dental pulp regeneration"; "periodontal regeneration"; "peripheral nerve injury", without any set limitations regarding the type or year of publication.

2. Dental Stem Cells and Angiogenesis

Within the healthy human body, the most predominant and most studied form of blood vessel formation is angiogenesis. In general, angiogenesis can be defined as the sprouting of new capillaries from preexisting blood vessels in response to specific stimuli such as inflammation or hypoxia [42, 43]. This well-coordinated biological process is regulated by a broad range of proteins, which maintain a natural balance between stimulatory and inhibitory signaling pathways. As the latter are considered to be dominant, endothelial cells normally remain quiescent within the healthy human body [44]. However, in pathological conditions such as ischemic stroke, myocardial infarction, cancer, and diabetes, this balance is disturbed [45].

Since deprivation of oxygen and nutrients, due to a lack in vascular supply, can lead to tissue necrosis, angiogenesis also plays an important role in tissue engineering. However, the limited success of growth factor-based revascularization urged the need to promote angiogenesis with a more regenerative approach by means of stem cell-based therapies [46, 47]. MSCs are considered to establish therapeutic angiogenesis by either paracrine secretion of angiogenic growth factors or differentiation into endothelial cells [48–50].

2.1. Paracrine Mediation of Angiogenesis by Dental Stem Cells. With regard to the angiogenic properties of DSCs, studies have indicated the secretion of a broad range of regulatory proteins. DPSCs, for example, have been reported to express stimulatory growth factors such as platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), and vascular endothelial growth factor (VEGF), either in basal conditions or in response to noxious stimuli, for example, injury or hypoxia [17, 28, 29, 34, 35]. Other angiogenesis-promoting factors that have been detected in DPSCs are angiogenin (ANG), angiopoietin-1 (ANGPT1), colony-stimulating factor (CSF), dipeptidyl peptidase IV (DPPIV), endothelin-1 (EDN1), interleukin-8 (IL-8), insulin-like growth factor binding protein-3 (IGFBP3), monocyte chemoattractant protein-1 (MCP-1), and urokinase-type plasminogen activator (uPA) [24, 30]. Nevertheless, the secretome of DPSCs also comprises several inhibitory proteins, such as endostatin, pentraxin-3 (PTX-3), pigment epithelium-derived factor (PEGF), plasminogen activator inhibitor-1 (PAI-1), tissue inhibitor of matrix metalloproteinase-1/4 (TIMP-1/4), and thrombospondin-1 (THBS-1) [24, 30]. Comparable findings were also described for SCAPs and FSCs, albeit with variable expression levels between the different stem cell populations [16, 24–26, 31, 51]. With regard to the secretome of SHEDs and PDLSCs, literature indicates the expression of ANGPT2 [27], bFGF [16, 27, 32], endostatin [16], hepatocyte growth factor (HGF) [33], insulin-like growth factor-1 (IGF-1) [27], and VEGF [27, 32, 33, 36]. An overview of the DSC secretome and its associated functions can be found in Table 1 [44, 45, 52]. One should take into account the fact that not all growth factor functions have already been described for DSCs.

Since DSCs express a wide variety of angiogenesis regulating proteins, stimulatory as well as inhibitory, it is important to determine their potential impact on the behavior of endothelial cells and angiogenesis altogether. Each well-coordinated event within the angiogenic process can be mimicked by a series of *in vitro* assays. For instance, colorimetric assays are performed to evaluate the effect of DSC-derived growth factors on endothelial proliferation. A significant increase of both survival and proliferation of human umbilical vein endothelial cells (HUVECs) was observed after incubation with conditioned medium (CM) of a $CD31^-/CD146^-$ subpopulation of DPSCs [53]. Aranha et al. also reported a time-dependent increase in the proliferation of human dermal microvascular endothelial cells (HDMECs) when incubated with CM of hypoxia-preconditioned DPSCs [34]. Hilkens et al., on the other hand, reported no pronounced effect of CM of DPSCs, SCAPs, and FSCs on the proliferation of human microvascular endothelial cells (HMECs) [24]. To date, the potential effect of SHEDs and PDLSCs on endothelial proliferation has not been described. In order to evaluate whether endothelial cells migrate along a gradient

Table 1: The secretome of dental stem cells and its associated functions.

Factor	Function	Population	Reference
	Angiogenesis-stimulating factors		
Angiogenin (ANG)	Endothelial proliferation and migration. Activation of smooth muscle cells. Indirect degradation of basement membrane.	DPSCs, SCAPs, and FSCs	[24]
Angiopoietin-1 (ANGPT1)	Endothelial survival, migration, and matrix adhesion. Endothelial sprouting and vessel stabilization.	DPSCs, SCAPs, and FSCs	[24–26]
Angiopoietin-2 (ANGPT2)	Endothelial proliferation, migration, and sprouting in the presence of VEGF.	PDLSCs	[27]
Basic fibroblast growth factor (bFGF)	Endothelial proliferation, migration, and tube formation. Upregulation of uPA, VEGF receptor, and integrins.	DPSCs, SCAPs, SHEDs, and PDLSCs	[16, 26–32]
Colony stimulating factor (CSF)	Endothelial proliferation, migration, and differentiation. Induction of proteolytic enzyme release.	DPSCs	[17]
CXC chemokines, for example, interleukin-8 (IL-8)	Endothelial survival, proliferation, migration, and tube formation. Induction of MMP production.	DPSCs	[30]
Dipeptidyl peptidase IV (DPPIV)	Vascular remodeling. Endothelial proliferation, migration, and tube formation.	DPSCs, SCAPs, and FSCs	[24]
Endothelin-1 (EDN1)	Endothelial proliferation and migration. Stimulation of VEGF-mediated angiogenesis. Stimulation of endothelial MMP2 production. Proliferation of vascular smooth muscle cells.	DPSCs, SCAPs, and FSCs	[24]
Hepatocyte growth factor (HGF)	Endothelial proliferation, migration, and tube formation. Proliferation of vascular smooth muscle cells. Stimulation of VEGF and PlGF production.	SCAPs, SHEDs	[25, 26, 33]
Insulin-like growth factor-1 (IGF-1)	Endothelial proliferation, migration, and tube formation. Stimulation of VEGF and plasminogen activator production. Downregulation of endothelial apoptosis.	PDLSCs	[27]
Insulin-like growth factor binding protein-3 (IGFBP3)	Endothelial migration and tube formation. Stimulation of IGF-1-mediated angiogenesis. Stimulation of VEGF and MMP2 production.	DPSCs, SCAPs, and FSCs	[24, 26, 30]
Matrix metalloproteinases (MMPs)	Extracellular matrix degradation and release of sequestered growth factors.	DPSCs, SCAPs	[17, 25]
Monocyte chemotactic protein (MCP-1)	Endothelial chemotaxis, tube formation, and differentiation. Stimulation of HIF-1α and VEGF production.	DPSCs, SCAPs	[26, 30]
Platelet-derived growth factor (PDGF)	Endothelial proliferation, migration, and differentiation. Stimulation of VEGF expression. Proliferation of vascular smooth muscle cells and pericytes. Vessel stabilization.	DPSCs	[28]
Urokinase-type plasminogen activator (uPA)	Participation in ECM degradation and release of sequestered growth factors. Endothelial migration and invasion. Activation of VEGF and pro-HGF.	DPSCs, SCAPs, and FSCs	[24, 26, 30]
Vascular endothelial growth factor (VEGF)	Endothelial proliferation, migration, and tube formation. Stimulation of NO synthase and plasminogen activator expression. Downregulation of endothelial apoptosis.	DPSCs, SCAPs, FSCs, SHEDs, and PDLSCs	[17, 24–36]
	Angiogenesis-inhibiting factors		
ANGPT2	Natural antagonist of ANGPT1. Upregulation of endothelial apoptosis.	PDLSCs	[27]
DPPIV	Inhibition of endothelial progenitor homing. Inhibition of CXCR3-induced chemotaxis.	DPSCs, SCAPs, and FSCs	[24]
Endostatin	Endothelial proliferation and migration. Upregulation of endothelial apoptosis. Inhibition of MMPS and bFGF-mediated and VEGF-mediated angiogenesis.	DPSCs, SCAPs, and SHEDs	[16, 30]

Table 1: Continued.

Factor	Function	Population	Reference
IGFBP3	Endothelial migration and tube formation. Inhibition of MMP9 and VEGF production.	DPSCs, SCAPs, and FSCs	[24, 26, 30]
MMPs	Inhibition of FGFR1 and uPAR-mediated signaling. Generation of angiogenic inhibitors by proteolytic cleavage.	DPSCs	[17]
Pentraxin-3 (PTX-3)	Inhibition of bFGF-mediated angiogenesis.	DPSCs, SCAPs, and FSCs	[24, 26]
Pigment epithelium-derived factor (PEGF)	Endothelial proliferation and migration. Upregulation of endothelial apoptosis. Inhibition of MMPs and bFGF-mediated and VEGF-mediated angiogenesis.	DPSCs, SCAPs, and FSCs	[24, 26]
Plasminogen activator inhibitor (PAI-1)	Inhibition of uPA.	DPSCs, SCAPs, and FSCs	[24, 26, 30]
Thrombospondin-1 (THBS1)	Endothelial proliferation, migration, and tube formation. Upregulation of endothelial apoptosis.	DPSCs, SCAPs, and FSCs	[24, 26]
Tissue inhibitor of MMPs-1/4 (TIMP-1/4)	Inhibition of MMPs	DPSCs, SCAPs, and FSCs	[24, 26, 30]
	Neurotrophic factors		
Basic fibroblast growth factor (bFGF)	Neuronal differentiation. Neurite outgrowth.	DPSCs, SCAPs, SHEDs, and PDLSCs	[16, 26–32]
Brain-derived neurotrophic factor (BDNF)	Survival of neurons. Differentiation of neuroblasts. Formation of synapses and neuritogenesis.	DPSCs, SCAP	[37–41]
Ciliary neurotrophic factor (CNTF)	Neuronal survival.	DPSCs	[40]
Glial-cell derived neurotrophic factor (GDNF)	Survival of neurons. Differentiation of neuroblasts. Neuritogenesis.	DPSCs, SCAPs	[37, 38]
Nerve growth factor (NGF)	Survival, maintenance, and proliferation of neurons. Neurite outgrowth.	DPSCs, SCAPs	[37, 38, 40, 41]
Neurotrophin-3 (NT-3)	Survival of neurons. Differentiation of neuroblasts. Neuritogenesis.	DPSCs	[38, 40]
Neurotrophin-4 (NT-4)	Survival of neurons. Differentiation of neuroblasts. Neuritogenesis.	DPSCs	[40]
PDGF-AA	Neuronal survival. Neuritogenesis.	DPSCs	[40]
VGF (VGF nerve growth factor inducible)	Neuronal survival. Neuritogenesis.	DPSCs	[40]

of DSC-derived chemokines, a transwell migration assay is performed. DPSCs as well as SCAPs have been shown to significantly augment endothelial migration in comparison to FSCs [24, 30]. In terms of endothelial tubulogenesis, Yuan et al. indicated an increased formation of capillary-like structures during a direct coculture of SCAPs and HUVECs [54]. Similar outcomes were found for DPSCs, SHEDs, and PDLSCs [29, 32, 55–57]. During these direct cocultures, DSCs are thought to adopt a pericyte-like function as they are often found in close proximity to the endothelial cells [54, 56, 57]. Alternatively, endothelial tube formation can also be mediated by paracrine factors, as was shown by Dissanayaka et al. through an indirect coculture of DPSCs and HUVECs [58]. In line with these findings, Tran-Hung et al. and others reported a significant increase in endothelial tubulogenesis caused by CM of DPSCs [24, 29]. With regard to the impact of PDLSCs and SHEDs on the functional behavior of endothelial cells, more extensive research is required.

The angiogenic properties of DSCs have also been elaborately investigated *in vivo*. Yeasmin et al., for example, indicated significant vascularization after subcutaneous transplantation of PDLSCs and endothelial cells. Since there was no detection of human-derived blood vessels, PDLSCs were considered to secrete paracrine mediators or to act as pericytes [32]. Mouse DPSCs were found to induce angiogenesis in a VEGF-dependent manner in a mouse Matrigel

plug assay [56]. DPSCs and SCAPs also caused a significant increase in angiogenesis in a chorioallantoic membrane assay [24, 30]. In terms of more clinically relevant disease models, Gandia et al. demonstrated a significant improvement of left ventricular function after injection of GFP-labeled DPSCs in a rat model of myocardial infarction. Apart from a reduction in infarct size and thickening of the anterior ventricular wall, an increase in capillary density was also detected. As there were no signs of differentiated DPSCs within the heart tissue, the aforementioned improvement was probably mediated by paracrine factors [59]. These findings were supported by Iohara et al., who reported a high capillary density after transplantation of a $CD31^-/CD146^-$ subpopulation of DPSCs in a mouse model of hindlimb ischemia. The close location of the stem cells near the newly formed blood vessels suggests a paracrine role for DPSCs [53]. The abovementioned subpopulation of DPSCs also promoted functional recovery in rats suffering from focal cerebral ischemia. Besides neurotrophic factors, the authors also demonstrated augmented levels of VEGF, which potentially played a role in the stimulation of vasculogenesis and neurogenesis in the ischemic rat brain [60, 61].

2.2. Endothelial Differentiation Potential of Dental Stem Cells. As stated before, MSCs not only contribute to therapeutic angiogenesis by secreting angiogenic growth factors but also are able to differentiate into endothelial cells under specific environmental cues. With regard to the endothelial differentiation potential of DPSCs, d'Aquino et al. were the first to report on the so-called codifferentiation of these cells into osteoblasts and endotheliocytes. Following the *in vitro* osteogenic differentiation of a sorted $CD117^+/CD34^+/VEGFR2^+$ DPSC population, flow cytometric analysis demonstrated a subpopulation of $VEGFR2^+/Stro\text{-}1^+/CD44^+/CD54^+$ endothelial progenitor cells with a marked expression of von Willebrand factor (vWF) and angiotensin-converting enzyme (ACE) [62]. Similar results were found by Marchionni et al., indicating the expression of vWF and CD54 as well as the increased presence of VEGFR1 and VEGFR2, after incubating DPSCs with VEGF for 7 days. In addition, these cells were able to form capillary-like structures when seeded on Matrigel or cultured in a fibrin clot [63]. Extensive capillary network formation was also observed by Barachini et al., as well as the *in vitro* expression of CD31 and VEGFR2. Functionality of the differentiated cells was successfully established with the uptake of acetylated low density lipoprotein [64]. These findings confirm earlier observations made by Iohara et al., reporting the *in vitro* endothelial differentiation potential of a $CD31^-/CD146^-$ subpopulation of DPSCs [53]. However, the efficacy of endothelial differentiation is dependent on not only the addition of specific growth factors to the cell culture medium, but also the concentration of fetal bovine serum (FBS) and the cell seeding density appear to play an important role. Karbanová et al., for example, demonstrated the upregulation of CD31, CD34, CD106, and vWF after culturing DPSCs at a low density in serum-free differentiation medium. When cells were seeded at a higher density, no upregulation of vWF was observed. The addition of FBS maintained cell proliferation and the endothelial phenotype of DPSCs [65]. In addition to DPSCs, SHEDs have also been shown to differentiate into endothelial cells. In 2008, Cordeiro et al. detected beta-galactosidase-positive capillaries in transplanted tooth slices containing LacZ-transduced SHEDs [66]. The same researchers later on confirmed these results *in vitro* and *in vivo*, indicating capillary sprouting and the VEGF-induced expression of VEGFR2, CD31, and VE-cadherin and a continuous expression of VEGFR1 by SHEDs [67, 68]. In particular VEGFR1 appears to play an important role in the endothelial differentiation potential of SHEDs and DPSCs. This was demonstrated by a reduction of human CD31-positive capillaries, following transplantation of VEGFR1-silenced SHEDs in a tooth slice model *in vivo*. The importance of the VEGFR1/MEK1/ERK signaling cascade was illustrated *in vitro* by the complete suppression of endothelial differentiation following the inhibition of this signaling pathway [67]. Recent work of Zhang et al. also revealed the Wnt/β-catenin pathway to be an important regulator of the endothelial fate of DPSCs and SHEDs [69]. With regard to the endothelial differentiation potential of other DSC populations, limited data are available. Bakopoulou et al. recently demonstrated the acquisition of a preendothelial phenotype by SCAPs, after exposure to an angiogenic induction medium for 28 days in normoxic conditions. These cells not only were able to form capillary-like structures but also displayed a time-dependent upregulation of different marker proteins, such as CD31, vWF, VEGFR2, angiopoietin-1/2, and Tie-1. Moreover, when depriving SCAPs of oxygen and nutrients, their endothelial differentiation potential appeared to be more pronounced [26]. After induction of differentiation for different periods of time, both a significant upregulation of endothelial marker proteins and the formation of tubules were observed in a $CD105^+$-enriched subpopulation of PDLSCs. Molecular analysis illustrated the critical role of neuropilin-2 (NRP-2) in the angiogenic fate of these stem cells [70]. To date, evidence for the *in vivo* endothelial transdifferentiation of DSCs remains scarce. As previously mentioned, Zhang and colleagues observed the presence of human $CD31^+$ blood vessels after transplanting human DPSCs and SHEDs in a rodent tooth slice model [67, 69]. However, DSCs are mainly considered to assume a pericyte-like phenotype, as they are often located adjacent to endothelial cells *in vitro* as well as *in vivo* [32, 54–57, 71, 72].

3. Dental Stem Cells and Neuroregeneration

3.1. Paracrine Mediation of Neuroprotection and Neurite Outgrowth by Dental Stem Cells. DSCs also produce a wide variety of neurotrophic factors and therefore they can be used in tissue engineering as a growth factor delivery system. These neurotrophic factors play a pivotal role in protecting neurons from apoptosis and inducing endogenous neural repair and neurite formation. Brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), glial cell-derived neurotrophic factor (GDNF), nerve growth factor (NGF), and various others are abundantly present in the secretome of DPSCs and SCAPs (Table 1) [25, 38, 40, 41, 73–77]. The neurotrophic factors secreted by SHEDs, PDLSCs,

or FSCs remain to be characterized. Mead et al. demonstrated that DPSCs expressed more NGF, BDNF, and VEGF than bone marrow-derived mesenchymal stem cells (BM-MSCs) and adipose tissue-derived mesenchymal stem cells (AMSCs) [40]. Another research group revealed that SCAPs secreted significantly larger amounts of chemokines and neurotrophins in comparison to BM-MSCs, whereas BM-MSCs secreted more extracellular matrix (ECM) proteins and proangiogenic factors [25]. Interestingly, the capacity of DPSCs to increase neurite outgrowth of neurons of dorsal ganglia was even more pronounced after these cells were differentiated into Schwann cells [38]. In addition, another study showed that after differentiation into neurons, DPSCs expressed more VEGF and NGF but less BDNF [78].

In comparison to other DSC populations, there is abundant evidence on the beneficial effect of DPSCs on neuroprotection and neuritogenesis *in vitro*. DPSCs were found to rescue sensory and dopaminergic neurons from apoptosis [79] and induce the survival and sprouting of neurons of trigeminal [75], retinal [40, 80], and sympathetic ganglia [38, 81]. SHED CM also enhances the viability and neuritogenesis of neurons of dorsal root ganglia [82]. A recent paper showed that exosomes derived from SHEDs grown on laminin-coated three-dimensional alginate microcarriers are able to suppress 6-hydroxy-dopamine-induced apoptosis in dopaminergic neurons [83]. DPSCs significantly enhanced neuritogenesis of axotomized rat retinal ganglia compared to BM-MSCs and AMSCs and possessed superior neuroprotective properties. The addition of specific Fc-receptor inhibitors revealed that VGF nerve growth factor inducible (VGF) was the responsible factor released by DPSCs [40]. Finally, DPSCs were shown to be superior to BM-MSCs in rescuing astrocytes from cell death induced by oxygen-glucose deprivation [84]. DPSCs are also able to guide the differentiation process of neural precursor cells: rat neural stem cells cultured on P(EA-co-HEA)90/10 biomaterials covered with DPSCs differentiated into young Tuj1-immunoreactive neurons [73].

Numerous studies have demonstrated the beneficial effects of DPSCs in injuries and pathologies of nervous system. In the pioneer study of Arthur et al., DPSCs were able to attract trigeminal neurons after transplantation into chicken embryos [76, 81]. As mentioned above, neurogenic predifferentiated DPSCs were also able to integrate in the host brain of a rat [85]. Intravitreal injection of DPSCs promotes neuronal survival and axon regeneration of retinal ganglia cells after optic nerve injury in rats [39]. In a rat model of spinal cord injury, transplantation of both SHEDs and DPSCs improved recovery of hindlimb locomotor functions. In the same experiment, BM-MSCs or skin-derived fibroblasts caused substantially less recovery of locomotor function. The proposed mechanisms were inhibition of apoptosis of neurons, astrocytes, and oligodendrocytes and regeneration of transected axons [86]. In rodent models of ischemic stroke, transplantation of DPSCs and SHEDs also led to an improvement in neurobehavioral function [61, 87–90]. This could be due to a decrease of inflammation, an increase of angiogenesis, or a reduction of apoptosis. Nagpal et al. announced the first clinical trial to apply autologous DPSCs as a therapy for patients with chronic disability after stroke, the so-called TOOTH trial (The Open study Of dental pulp stem cell Therapy in Humans, TOOTH) [91]. Both SHEDs and DPSCs differentiated to dopaminergic neurons have been shown to improve functional behavior in a rat model of Parkinson's disease. The therapeutic success was attributed to the induction of neurite outgrowth and neuronal survival caused by various neurotrophic factors [92]. Very recently it was described that a single injection of SHED CM in mice suffering from experimental autoimmune encephalomyelitis not only significantly improved disease scores and reduced demyelination and axonal injury but also reduced inflammatory cell infiltration and proinflammatory cytokine levels *in vivo* [93].

Studies describing the neuroprotective effects of FSCs, SCAPs, or PDLSCs are scarce. In a coculture system with rat trigeminal ganglia, SCAPs were able to stimulate and guide neurite outgrowth *in vitro*. This effect was completely inhibited by neutralizing antibodies directed against BDNF but no effect was observed when NGF and GDNF were blocked. In addition, axonal growth was shown to be triggered after subcutaneous injection of a Matrigel containing SCAPs into immunodeficient mice [37]. Furthermore, SCAPs were also applied in a rat model of spinal cord injury. However, the functional outcome was better in animals which received implantation of a whole apical papilla compared to animals which received *in vitro* expanded SCAPs [94]. FSCs seeded on aligned electrospun poly(ε-caprolactone)/poly-DL-lactide-co-glycolide fibers were also applied as a cellular therapy in a spinal cord injury model, but no significant functional improvement was observed after transplantation [95].

3.2. Neuronal and Neural Differentiation Potential of Dental Stem Cells. As DSCs are embryonically derived from the neural crest and glial tissues, it is not surprising that these cells display neurogenic properties. Multiple protocols are available in literature to induce differentiation of DSCs into neurons *in vitro*. These procedures involve either the incubation of DSC monolayers with a cocktail of various growth factors and pharmacological compounds or the generation of floating neurospheres of DSCs, which recapitulates the embryonic stages of neuroblast formation (for a detailed review, see [74, 96]). In general, the mostly applied proteins to induce neuronal differentiation of DSC monolayers include epidermal growth factor (EGF) and bFGF in combination with culture supplements such as B27, forskolin, and insulin-transferrin-sodium selenite (ITS). All DSC populations have been shown to differentiate into neuron-like cells. Although successful differentiation was verified by means of an increased expression of neuronal markers such as NeuN, neural cell adhesion molecule, neurofilament, synaptophysin, A2B5, and microtubule-associated protein-2, ultrastructural and/or electrophysiological analysis of the differentiated cells was lacking in most of these studies. Furthermore, these differentiation protocols mostly result in a low yield of neurons, which are primitive, immature, and not able to produce a train of action potentials [78]. However, studies of Király et al. demonstrated that predifferentiated DPSCs integrated into the host brain when transplanted into the cerebrospinal fluid

of 3-day-old rats with a cortical injury. These cells displayed neuronal properties, not only by expressing neurofilament and NeuN but also by exhibiting voltage-dependent sodium and potassium channels [85]. DPSCs that were injected into the brain of rodents after stroke predominantly differentiated into astrocytes instead of neurons [87]. As DSCs represent a very heterogeneous stem cell population, sorting the cells prior to neuronal induction might increase the success rate of differentiation.

Recent evidence indicates that DSCs also differentiate into oligodendrocytes and Schwann cells, which myelinate neurons from the central and peripheral nervous system, respectively. Transfection of the helix-loop-helix transcription factor Olig2 in DPSCs resulted in an increased expression of oligodendrocyte markers such as nestin, NG2, and myelin basic protein [97]. In addition, Martens et al. reported the differentiation of DPSCs into Schwann cells. The differentiated cells displayed an increased expression of laminin, low-affinity nerve growth factor receptor p75, glial fibrillary acidic protein, and CD104. Moreover, these cells were able to myelinate neurons *in vitro*, which was confirmed by ultrastructural analysis [38]. The ability of DPSCs to differentiate into Schwann cells might be explained by the fact that a significant population of the DPSCs are embryonically derived from peripheral nerve-associated glia [22] and might thus represent a dedifferentiation. Whether other DSCs are capable of differentiating into myelinating cells remains to be investigated. Because of their capacity to differentiate into Schwann cells, DPSCs might represent a promising strategy to treat peripheral nerve injury. Nerve autografts are the current gold standard treatment in the clinic. As this involves sacrifice of other nerves and the clinical results are usually unsatisfactory, other options are currently under investigation [98]. Despite their key role in endogenous nerve repair, transplantation of Schwann cells themselves is very restricted as their isolation also requires destruction of another nerve and their expansion rates are dramatically low [98–100]. In a rat model of facial nerve injury, DPSCs were shown to promote remyelination, blood vessel formation, and nerve regeneration when applied in combination with silicon, collagen I, or poly(lactic-co-glycolic acid) (PLGA) tubes [101–103]. SHEDs were also applied to treat rat sciatic nerve injury. Bridging the nerve gap with silicon conduits containing CM of SHEDs resulted in a higher number of myelinated axons and better functional recovery compared to silicon conduits containing control medium [82]. Finally, one study reported the use of PDLSCs in peripheral nerve injury. Injection of PDLSCs into the crush-injured left mental nerve of rats significantly improved sensory function and increased the number or retrograde labeled sensory neurons and myelinated axons [104].

Despite the fact that under controlled circumstances DSCs are able to differentiate into cells resembling to neurons, Schwann cells, and oligodendrocytes, the current paradigm is that their beneficial effects in preclinical models of neurodegenerative diseases and traumas are caused by the cytokines and growth factors in their secretome.

4. Preconditioning of Dental Stem Cells to Enhance Their Angiogenic and Neurogenic Properties

One of the major hurdles in the field of tissue engineering is the survival of transplanted cells *in vivo*. To overcome this obstacle, several strategies have been developed to modulate the stem cells prior to transplantation in order to improve cellular survival and engraftment [105]. Genetic modification offers a potential strategy to increase stem cell survival, for example, by overexpressing antiapoptotic genes such as Bcl-2 [106] or Akt [107, 108]. Another possibility is to modify the expression of a key protein of a certain illness such as dopamine for Parkinson's patients or insulin for diabetics [109]. However, genetic modification is a new and developing field and many questions remain to be resolved before clinical applications using genetically modified stem cells can be deemed possible [109].

Preparing stem cells for transplantation by exposing them to a hypoxic environment may be a useful technique to improve the stem cell secretome, since hypoxia is a potent stimulus for the secretion of a variety of trophic factors (Table 2) [105, 124]. Hypoxic preconditioning has already been shown to ameliorate cell survival, paracrine activity, and angiogenic potency in a model of murine hindlimb ischemia [125–127]. Oxygen levels in the dental pulp are lower compared to other tissues, since oxygen can only be supplied via blood vessels running through the rather narrow apical foramen of the tooth [128]. Culturing DPSCs under hypoxic conditions increases their proliferation rate [110, 111], VEGF expression [34], and migration [112]. Moreover, hypoxia also upregulates VEGF production in SCAPs [26, 31] and cells from the periodontal ligament [118]. These reports all support the beneficial effects of hypoxic preconditioning. However, mimicking hypoxia by simply adding a pharmacological agent would greatly increase the feasibility of this approach (Table 2). Prolyl hydroxylase (PHD) inhibitors represent such a group of hypoxia mimicking agents. PHD inhibitors include not only iron chelators such as hinokitiol, deferoxamine, or L-mimosine but also cobalt chloride and dimethyloxalylglycine [119, 129]. These PHD inhibitors promote VEGF secretion and HIF-1α expression in dental pulp-derived cells [113, 114], SCAPs [123], and PDL cells [119] and even in a tooth slice organ culture model [130]. Furthermore, preconditioned DPSCs [115] and SCAPs [123] also enhance capillary network formation by HUVECs. In addition, the application of hinokitiol-stimulated DPSCs in a mouse Matrigel plug assay resulted in an increased hemoglobin content and PECAM-1 expression [114]. Taken together, these reports suggest a promising future for the use of hypoxic mimicry in preparing stem cells for *in vivo* transplantation. HIF-1α and its downstream targets stimulate not only angiogenesis but also neurogenesis. For this reason, hypoxic preconditioning offers new prospects with regard to neuroregeneration. Despite the promising results using BM-MSCs [131–133] and embryonic stem cells [134], no reports were found using preconditioned DSCs for the treatment of neurological disorders.

Table 2: Dental stem cells and the effects of preconditioning.

Priming	(Angiogenic) effect	Reference
	Dental pulp stem cells	
Hypoxia	Increased proliferation rate Increased HIF-1α and VEGF expression/secretion Increased migration	[110, 111] [34] [112]
PHD inhibitors	Increased HIF-1α and VEGF expression/secretion	[113]
Hinokitiol	Increased HIF-1α and VEGF expression/secretion Increased hemoglobin content in mouse matrigel plug assay	[114]
FGF-2	Enhanced EC capillary network formation	[115]
Lipopolysaccharide (LPS)	Increased VEGF expression	[116, 117]
	Stem cells from human exfoliated deciduous teeth	
Hypoxia	Increased migration	[112]
	Periodontal ligament stem cells	
Hypoxia	Increased VEGF expression	[118]
PHD inhibitors	Increased HIF-1α and VEGF expression/secretion	[119]
IL-1α	Increased VEGF expression	[36]
TNF-α	Increased VEGF expression	[120]
Adiponectin	Increased proliferation rate Increased wound healing	[121]
	Follicle stem cells	
Lipopolysaccharide (LPS)	Increased migration	[122]
	Stem cells from the apical papilla	
Hypoxia	Increased VEGF expression	[26, 31]
PHD inhibitors ($CoCl_2$)	Increased HIF-1α and VEGF expression/secretion Enhanced EC capillary network formation	[123]

To conclude, there are several more cytokines, growth factors, or chemical agents that can be used to boost the angiogenic potential of DSCs (Table 2). For example, bacterial lipopolysaccharide (LPS) has been shown to enhance VEGF secretion of DPSC [116, 117] and the migration of FSCs [122]. Pretreatment of PDLSCs with IL-1α [36] and TNF-α [120] leads to a more pronounced VEGF secretion, whereas adiponectin exposure increases PDLC proliferation rate and wound healing capabilities [121].

5. Dental Stem Cells and Pulp Regeneration

Although dental pulp can be characterized as a specialized tissue with a number of important physiological functions, it is also very vulnerable to caries, infections, and trauma. As any of these insults can easily interfere with normal pulp homeostasis and subsequently affect normal root development, the endodontic treatment of necrotic, immature permanent teeth in particular poses many challenges [135–137]. Over the past decade, substantial advances have been made regarding the potential application of DSCs in the regeneration of viable dental tissues. Not only has successful dental pulp regeneration been reported for DPSCs, but also SCAPs and FSCs have proven to be effective in different *in vivo* models of pulp regeneration.

Even before the definition and characterization of DPSCs, Mooney et al. already demonstrated the establishment of pulp-like tissue *in vitro* when culturing human pulp fibroblasts onto polyglycolic acid (PGA) matrices for 60 days [138]. In line with these findings, Buurma et al. reported fibroblast survival and ECM production within the PGA constructs after subcutaneous transplantation in immunocompromised mice [139]. Around the same time, Gronthos et al. and others described the presence of a stem cell population within the dental pulp, namely, DPSCs, which was able to form a vascularized dentin/pulp-like complex *in vivo* when cotransplanted with hydroxyapatite/tricalcium phosphate particles (HA/TCP) in immunocompromised mice [4, 14, 140]. These findings led to the development of other proof-of-principle models such as the tooth slice/scaffold model, which comprises the application of an emptied human tooth slice containing a supportive scaffold [141]. A number of studies have pointed out the regeneration of vascularized pulp-like tissue after subcutaneous implantation of tooth slices containing either DPSCs or SHEDs supported by a biodegradable scaffold [66, 67, 69, 141, 142]. Another proof-of-principle model, which illustrates the limited vascular supply within the tooth, is the ectopic root transplantation model. In 2010, Huang et al. described the *de novo* formation of a vascularized dentin/pulp complex in a subcutaneously

transplanted emptied human root canal enclosing a PLGA scaffold seeded with DPSCs [143]. Similar observations were made by Rosa et al., showing the formation of vascularized dentin/pulp-like tissue after subcutaneous implantation of SHEDs and a self-assembling peptide hydrogel in emptied human root canals [144]. A specific, granulocyte colony-stimulating factor- (G-CSF-) mobilized subpopulation of DPSCs was also found to regenerate vascularized pulp tissue in an ectopic root transplantation model after a short 21-day transplantation period [145]. More recently, Dissanayaka et al. demonstrated the successful regeneration of vascularized dental pulp tissue after transplantation of DPSCs or DPSCs and HUVECs encapsulated in a commercially available hydrogel. Root fragments containing cocultures displayed a more pronounced vascularization, ECM deposition, and tissue mineralization in comparison to DPSCs alone, indicating the importance of coordinated cell-cell interactions [58]. Preconditioning DPSCs by means of hypoxia, prior to transplantation, evoked a higher number of blood vessels in the regenerated tissue compared to the control conditions [146]. Although the ectopic root transplantation model has been widely applied, both the shape of the root canals and the implemented size of the apical foramen have been prone to variability [58, 143–146], which leads to the question whether smaller apical openings would interfere with normal tissue regeneration in the "coronal" part of the emptied root canal [143]. Another important aspect which definitely needs to be taken into account during differentiation and tissue engineering is the specific microenvironment at the time of regeneration. This would require the *in situ* transplantation of DPSCs in (partially) pulpectomized teeth in (larger) animal models. In 2004, Iohara et al. reported the formation of reparative dentin after treatment of an amputated canine pulp with an autologous DPSC pellet incubated with bone morphogenetic protein-2 [147, 148]. Over the past decade this research group and others have successfully performed *in situ* transplantations (of different subpopulations) of DPSCs to completely regenerate vascularized pulp tissue [149–156]. In 2013, the first solid steps towards the clinical application of DPSCs were taken. After careful karyotyping and excluding potential tumor formation by the stem cells, Iohara et al. reported the regeneration of a vascularized and innervated dentin/pulp complex following transplantation of a clinical-grade subpopulation of DPSCs in pulpectomized canine teeth with an apical opening of 0.6 mm [152, 157].

With regard to the regenerative potential of SCAPs, Sonoyama et al. demonstrated their ability to form a dentin/pulp complex when transplanted with HA/TCP particles in immunocompromised mice. The human origin of the dentin-producing cells suggested the differentiation of SCAPs into odontoblast-like cells [158]. Similar results were also found by Huang et al., indicating the *de novo* regeneration of vascularized pulp-like tissue after the ectopic transplantation of an emptied human root canal containing a PLGA scaffold seeded with SCAPs [143]. Moreover, analysis showed a more continuous and thicker layer of dentin matrix in comparison to similar constructs containing DPSCs [143]. Subcutaneous implantation of SCAP-based cell sheet-derived pellets in immunodeficient mice also led to the development of a vascularized dentin/pulp complex with a continuous layer of dentin matrix [159].

As previously mentioned, FSCs originate from the dental follicle, a loose connective tissue surrounding developing teeth [11]. As the dental follicle during tooth development gives rise to the periodontium, research has mainly focused on the ability of FSCs to regenerate cementum and periodontal ligament [160–167]. Regarding their ability to form dental pulp tissue, Guo et al. indicated the establishment of an odontoblast-like cell layer as well as the formation of (pre)dentin in the omental pouch of adult rats after transplantation of rat FSCs and treated dentin matrix (TDM) [168]. In an attempt to engineer a complete tooth root, transplantation of similar constructs containing rat or human FSCs led to the regeneration of a dentin/pulp complex as well as cementum and periodontal ligament (PDL) [169, 170]. In line with these findings, Jiao et al. described the formation of vascularized dental pulp-like tissue after subcutaneous transplantation of human FSCs encapsulated within cryopreserved TDM [171]. When comparing the regenerative potential of FSCs and SCAPs, no significant differences were found despite their differential expression of protein markers *in vitro*. Both stem cell populations were able to regenerate a vascularized dentin/pulp-like complex following 8 weeks of transplantation in nude mice [51]. To date, no reports are available regarding the use of PDLSCs in dental pulp regeneration. Given their developmental origin, these stem cells have been mainly investigated for their potential application in periodontal regeneration.

6. Clinical Application of DSCs and Its Challenges

Despite the promising outcomes of DSC transplantation in a preclinical setting, the progression of DSCs from bench to bedside still holds some major challenges. Standardized cell isolation procedures, for example, are indispensable to safeguard the clinical safety, reproducibility, and efficacy of DSC therapy [172]. However, the extraction of third molars as well as the isolation of DSCs is currently being performed using diverse isolation procedures on donors of different ages with molars at different stages of development, which not only impairs in-depth comparison of experimental outcomes but also hinders the development of a standardized treatment protocol [15, 23, 173, 174]. Next to consistent isolation procedures, the clinical implementation of DSCs also entails the upscale production of these stem cells in xeno-free culture conditions, in order to provide an adequate amount of cells without any contamination of potential infectious agents [175–177]. Nevertheless, due to the inherent batch-to-batch variety as well as the current lack of reliable study protocols regarding the use of human blood-derived products as a potential alternative, more research is required before any educated decision can be taken by both scientists and regulatory agencies [178–180]. In addition to the challenges associated with the processing and culturing of DSCs, one also needs to take into account the intrinsic behavior of the stem cells, as it can be influenced by a broad range of

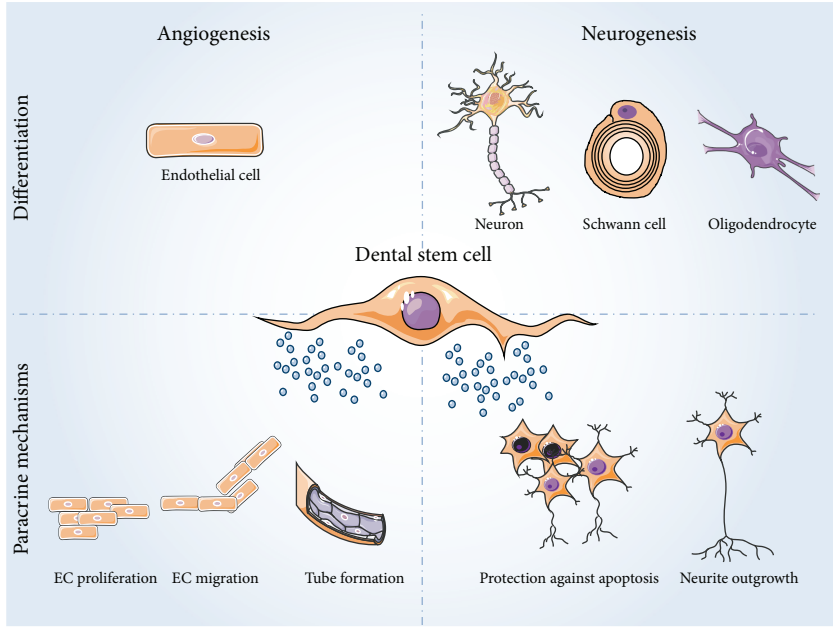


Figure 1: General overview of the angiogenic and neurogenic potential of DSCs. DSCs can differentiate into endothelial cells, neurons, Schwann cells, and oligodendrocytes under specific environmental clues. More relevant for their clinical applications is the fact that DSCs have a secretome rich in proteins which have a beneficial effect on surrounding cells. DSCs secrete a wide variety of angiogenic factors, inducing endothelial cell proliferation, migration, tube formation, and thus blood vessel development. DSCs also express neurotrophic factors which protect neurons from apoptosis and induce neurite outgrowth. This figure was made with images from the Servier Medical Art by Servier.

different donor-related factors, such as (oral) health, age, and orthodontic tooth movement [23].

Although numerous studies have elaborately described the immunomodulatory effects of DSCs *in vitro*, little is known concerning the effects of allogeneic DSC transplantation *in vivo* [19, 181–189]. Tomic et al., for example, reported the formation of granulomatous tissue after xenogeneic transplantation of human DPSCs and FSCs in immunocompetent mice [190]. When transplanting rat DPSCs in mice suffering from colitis, on the other hand, a clear reduction of inflammation was observed [191]. There were also no signs of immune rejection after injection of human SHEDs in a canine model of muscular dystrophy [192]. In line with these findings, conditioned medium of SHEDs was found to alleviate autoimmune encephalomyelitis as well as to improve the cognitive function in a mouse model of Alzheimer's Disease through the induction of anti-inflammatory M2-phenotype microglia [93, 193]. Nevertheless, the outcome of allogeneic DSC transplantation for dental tissue engineering purposes in particular remains largely unknown, as most ectopic transplantation models are performed in immunocompromised mice and most *in situ* models apply autologous DSCs [58, 143–158, 162, 170, 194–197]. More research is thus required with respect to the immunomodulatory behavior of allogeneic DSCs *in vivo* and potential graft-versus-host responses.

When making the switch from bench to bedside it is also important to include sufficient patient-centered outcomes. All too often, dental clinical trials focus on technical, clinician-centered outcomes instead of patient-centered outcomes. Developing a standardized set of core outcomes could help overcome this fixation with clinician-based outcomes and lead to more consistent study designs [198].

Despite these challenges, a few clinical studies using DSC-based therapies are currently recruiting participants (Table 3). In India, a clinical study is currently ongoing in which patients suffering from chronic periodontitis receive a local injection of allogeneic human DPSCs in order to improve periodontal tissue regeneration (ClinicalTrials.gov NCT02523651). Allogeneic DPSCs are also being applied in a clinical trial in China, investigating the effect of DPSCs on osseointegration of dental implants (NCT02731586). Also in China, a second clinical trial focuses on the revitalization of young immature permanent teeth with necrotic pulps using autologous SHEDs (NCT01814436). Finally, Nagpal et al. announced a study protocol for evaluating safety and feasibility of autologous DPSC-based stem cell therapy in patients with chronic disability after stroke; however, this study is not yet recruiting participants [91].

7. Conclusion and Future Perspectives

Taken together, DSCs are considered suitable candidates for cell-based treatment strategies and tissue engineering applications. There is abundant evidence supporting the angiogenic, neuroprotective, and neurotrophic actions of the DSC secretome (Figure 1). These inherent properties can even be augmented by pretreating DSCs prior to their transplantation. In particular hypoxia and hypoxia mimicking agents show great potential to improve stem cell survival and boost the DSC secretome. Up until now, most of the pretreatment studies have been focusing on improving the angiogenic

Table 3: Clinical application of DSCs.

Condition	Cell type	Status	Location	Principal investigator	Identifier
Dental implants	Allogenic DPSCs	Recruiting	India	Mohammed Sufath UR Rehman Venkat Aditya	NCT02731586
Periodontal disease	Allogenic DPSCs	Recruiting	China	Songlin Wang	NCT02523651
Pulp necrosis	Autologous SHEDs	Recruiting	China	Songtao Shi	NCT01814436
Stroke	Autologous DPSCs	/	Australia	Simon Koblar	TBA

effects of DSCs; therefore more research into the possible enhancement of their neurotropic/neuroprotective properties is warranted. In addition to their paracrine effects, DSCs have also been described to have the ability to differentiate into endothelial cells as well as neural cell types (Figure 1). Unfortunately, a wide variety of differentiation protocols have been used, resulting in highly variable outcomes and making it difficult to compare study outcomes but even more so to compare different DSC populations. Furthermore, often different parameters are used to assess successful differentiation.

Based on their origin, DSCs are expected to be ideal candidates for the regeneration of dental tissues such as the dental pulp and the periodontal ligament. Successful dental pulp regeneration has already been reported for DPSCs, SCAPs, and FSCs, whereas PDLSCs hold great potential for the regeneration of periodontal tissues. Furthermore, DPSCs, SHEDs, and PDLSCs have already been reported to improve regeneration after peripheral nerve injury by promoting remyelination, blood vessel formation, and nerve regeneration. These encouraging results contributed to the approval of two clinical studies that are currently recruiting participants and are thereby taking the first steps to introducing DSC-based therapies into the clinic.

Competing Interests

The authors declare that they have no competing interests.

Authors' Contributions

Jessica Ratajczak and Annelies Bronckaers equally contributed to this paper.

References

[1] R. Langer and J. P. Vacanti, "Tissue engineering," *Science*, vol. 260, no. 5110, pp. 920–926, 1993.

[2] A. Erices, P. Conget, and J. J. Minguell, "Mesenchymal progenitor cells in human umbilical cord blood," *British Journal of Haematology*, vol. 109, no. 1, pp. 235–242, 2000.

[3] A. J. Friedenstein, "Osteogenetic activity of transplanted transitional epithelium," *Acta Anatomica*, vol. 45, pp. 31–59, 1961.

[4] S. Gronthos, M. Mankani, J. Brahim, P. G. Robey, and S. Shi, "Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13625–13630, 2000.

[5] K. E. Mitchell, M. L. Weiss, B. M. Mitchell et al., "Matrix cells from Wharton's jelly form neurons and glia," *STEM CELLS*, vol. 21, no. 1, pp. 50–60, 2003.

[6] P. A. Zuk, M. Zhu, H. Mizuno et al., "Multilineage cells from human adipose tissue: implications for cell-based therapies," *Tissue Engineering*, vol. 7, no. 2, pp. 211–228, 2001.

[7] I. Thesleff and P. Nieminen, "Tooth morphogenesis and cell differentiation," *Current Opinion in Cell Biology*, vol. 8, no. 6, pp. 844–850, 1996.

[8] I. Thesleff, A. Vaahtokari, P. Kettunen, and T. Aberg, "Epithelial-mesenchymal signaling during tooth development," *Connective Tissue Research*, vol. 32, no. 1–4, pp. 9–15, 1995.

[9] M. Miura, S. Gronthos, M. Zhao et al., "SHED: stem cells from human exfoliated deciduous teeth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 5807–5812, 2003.

[10] W. Sonoyama, Y. Liu, T. Yamaza et al., "Characterization of the apical papilla and its residing stem cells from human immature permanent teeth: a pilot study," *Journal of Endodontics*, vol. 34, no. 2, pp. 166–171, 2008.

[11] C. Morsczeck, W. Götz, J. Schierholz et al., "Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth," *Matrix Biology*, vol. 24, no. 2, pp. 155–165, 2005.

[12] B.-M. Seo, M. Miura, S. Gronthos et al., "Investigation of multipotent postnatal stem cells from human periodontal ligament," *The Lancet*, vol. 364, no. 9429, pp. 149–155, 2004.

[13] M. Dominici, K. Le Blanc, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.

[14] S. Gronthos, J. Brahim, W. Li et al., "Stem cell properties of human dental pulp stem cells," *Journal of Dental Research*, vol. 81, no. 8, pp. 531–535, 2002.

[15] P. Hilkens, P. Gervois, Y. Fanton et al., "Effect of isolation methodology on stem cell properties and multilineage differentiation potential of human dental pulp stem cells," *Cell and Tissue Research*, vol. 353, no. 1, pp. 65–78, 2013.

[16] G. T.-J. Huang, S. Gronthos, and S. Shi, "Critical reviews in oral biology & medicine: mesenchymal stem cells derived from dental tissues vs. those from other sources: their biology and role in Regenerative Medicine," *Journal of Dental Research*, vol. 88, no. 9, pp. 792–806, 2009.

[17] M. Nakashima, K. Iohara, and M. Sugiyama, "Human dental pulp stem cells with highly angiogenic and neurogenic potential for possible use in pulp regeneration," *Cytokine and Growth Factor Reviews*, vol. 20, no. 5-6, pp. 435–440, 2009.

[18] N. Nuti, C. Corallo, B. M. Chan, M. Ferrari, and B. Gerami-Naini, "Multipotent differentiation of human dental pulp stem cells: a literature review," *Stem Cell Reviews and Reports*, 2016.

[19] L. Pierdomenico, L. Bonsi, M. Calvitti et al., "Multipotent mesenchymal stem cells with immunosuppressive activity can be easily isolated from dental pulp," *Transplantation*, vol. 80, no. 6, pp. 836–842, 2005.

[20] P. T. Sharpe, "Dental mesenchymal stem cells," *Development*, vol. 143, no. 13, pp. 2273–2280, 2016.

[21] G. Ding, J. Niu, and F. Wei, "Current understanding of orofacial tissue derived mesenchymal stem cells: an immunological perspective," *Histology and Histopathology*, vol. 30, no. 3, pp. 255–265, 2015.

[22] N. Kaukua, M. K. Shahidi, C. Konstantinidou et al., "Glial origin of mesenchymal stem cells in a tooth model system," *Nature*, vol. 513, no. 7519, pp. 551–554, 2014.

[23] P. Hilkens, N. Meschi, P. Lambrechts, A. Bronckaers, and I. Lambrichts, "Dental stem cells in pulp regeneration: near future or long road ahead?" *Stem Cells and Development*, vol. 24, no. 14, pp. 1610–1622, 2015.

[24] P. Hilkens, Y. Fanton, W. Martens et al., "Pro-angiogenic impact of dental stem cells in vitro and in vivo," *Stem Cell Research*, vol. 12, no. 3, pp. 778–790, 2014.

[25] S. Yu, Y. Zhao, Y. Ma, and L. Ge, "Profiling the secretome of human stem cells from dental apical papilla," *Stem Cells and Development*, vol. 25, no. 6, pp. 499–508, 2016.

[26] A. Bakopoulou, A. Kritis, D. Andreadis et al., "Angiogenic potential and secretome of human apical papilla mesenchymal stem cells in various stress microenvironments," *Stem Cells and Development*, vol. 24, no. 21, pp. 2496–2512, 2015.

[27] A. Osman, N. Gnanasegaran, V. Govindasamy et al., "Basal expression of growth-factor-associated genes in periodontal ligament stem cells reveals multiple distinctive pathways," *International Endodontic Journal*, vol. 47, no. 7, pp. 639–651, 2014.

[28] L. Tran-Hung, P. Laurent, J. Camps, and I. About, "Quantification of angiogenic growth factors released by human dental cells after injury," *Archives of Oral Biology*, vol. 53, no. 1, pp. 9–13, 2008.

[29] L. Tran-Hung, S. Mathieu, and I. About, "Role of human pulp fibroblasts in angiogenesis," *Journal of Dental Research*, vol. 85, no. 9, pp. 819–823, 2006.

[30] A. Bronckaers, P. Hilkens, Y. Fanton et al., "Angiogenic properties of human dental pulp stem cells," *PLoS ONE*, vol. 8, no. 8, Article ID e71104, 2013.

[31] J. Vanacker, A. Viswanath, P. De Berdt et al., "Hypoxia modulates the differentiation potential of stem cells of the apical papilla," *Journal of Endodontics*, vol. 40, no. 9, pp. 1410–1418, 2014.

[32] S. Yeasmin, J. Ceccarelli, M. Vigen et al., "Stem cells derived from tooth periodontal ligament enhance functional angiogenesis by endothelial cells," *Tissue Engineering—Part A*, vol. 20, no. 7-8, pp. 1188–1196, 2014.

[33] C. Gorin, G. Y. Rochefort, R. Bascetin et al., "Priming dental pulp stem cells with fibroblast growth factor-2 increases angiogenesis of implanted tissue-engineered constructs through hepatocyte growth factor and vascular endothelial growth factor secretion," *Stem Cells Translational Medicine*, vol. 5, no. 3, pp. 392–404, 2016.

[34] A. M. F. Aranha, Z. Zhang, K. G. Neiva, C. A. S. Costa, J. Hebling, and J. E. Nör, "Hypoxia enhances the angiogenic potential of human dental pulp cells," *Journal of Endodontics*, vol. 36, no. 10, pp. 1633–1637, 2010.

[35] K. Matsushita, R. Motani, T. Sakuta et al., "The role of vascular endothelial growth factor in human dental pulp cells: induction of chemotaxis, proliferation, and differentiation and activation of the AP-1-dependent signaling pathway," *Journal of Dental Research*, vol. 79, no. 8, pp. 1596–1603, 2000.

[36] Y. Bando, K. Noguchi, H. Kobayashi, N. Yoshida, I. Ishikawa, and Y. Izumi, "Cyclooxygenase-2-derived prostaglandin E2 is involved in vascular endothelial growth factor production in interleukin-1α-stimulated human periodontal ligament cells," *Journal of Periodontal Research*, vol. 44, no. 3, pp. 395–401, 2009.

[37] J. F. De Almeida, P. Chen, M. A. Henry, and A. Diogenes, "Stem cells of the apical papilla regulate trigeminal neurite outgrowth and targeting through a BDNF-dependent mechanism," *Tissue Engineering—Part A*, vol. 20, no. 23-24, pp. 3089–3100, 2014.

[38] W. Martens, K. Sanen, M. Georgiou et al., "Human dental pulp stem cells can differentiate into Schwann cells and promote and guide neurite outgrowth in an aligned tissue-engineered collagen construct in vitro," *The FASEB Journal*, vol. 28, no. 4, pp. 1634–1643, 2014.

[39] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Intravitreally transplanted dental pulp stem cells promote neuroprotection and axon regeneration of retinal ganglion cells after optic nerve injury," *Investigative Ophthalmology & Visual Science*, vol. 54, no. 12, pp. 7544–7556, 2013.

[40] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Paracrine-mediated neuroprotection and neuritogenesis of axotomised retinal ganglion cells by human dental pulp stem cells: comparison with human bone marrow and adipose-derived mesenchymal stem cells," *PLoS ONE*, vol. 9, no. 10, Article ID e109305, 2014.

[41] C. Nesti, C. Pardini, S. Barachini et al., "Human dental pulp stem cells protect mouse dopaminergic neurons against MPP+ or rotenone," *Brain Research*, vol. 1367, pp. 94–102, 2011.

[42] P. Carmeliet, "Mechanisms of angiogenesis and arteriogenesis," *Nature Medicine*, vol. 6, no. 4, pp. 389–395, 2000.

[43] J. Folkman, "Tumor angiogenesis: therapeutic implications," *The New England Journal of Medicine*, vol. 285, no. 21, pp. 1182–1186, 1971.

[44] J. H. W. Distler, A. Hirth, M. Kurowska-Stolarska, R. E. Gay, S. Gay, and O. Distler, "Angiogenic and angiostatic factors in the molecular control of angiogenesis," *Quarterly Journal of Nuclear Medicine*, vol. 47, no. 3, pp. 149–161, 2003.

[45] S. V. Bhadada, B. R. Goyal, and M. M. Patel, "Angiogenic targets for potential disorders," *Fundamental and Clinical Pharmacology*, vol. 25, no. 1, pp. 29–47, 2011.

[46] N. Ferrara and R. S. Kerbel, "Angiogenesis as a therapeutic target," *Nature*, vol. 438, no. 7070, pp. 967–974, 2005.

[47] D. L. Staudacher and M. Y. Flugelman, "Cell and gene therapies in cardiovascular disease with special focus on the no option patient," *Current Gene Therapy*, vol. 6, no. 6, pp. 609–623, 2006.

[48] A. Giordano, U. Galderisi, and I. R. Marino, "From the laboratory bench to the patient's bedside: an update on clinical trials with mesenchymal stem cells," *Journal of Cellular Physiology*, vol. 211, no. 1, pp. 27–35, 2007.

[49] P. J. Psaltis, A. C. W. Zannettino, S. G. Worthley, and S. Gronthos, "Concise review: mesenchymal stromal cells: potential for cardiovascular repair," *STEM CELLS*, vol. 26, no. 9, pp. 2201–2210, 2008.

[50] D. P. Sieveking and M. K. C. Ng, "Cell therapies for therapeutic angiogenesis: back to the bench," *Vascular Medicine*, vol. 14, no. 2, pp. 153–166, 2009.

[51] L. Guo, J. Li, X. Qiao et al., "Comparison of odontogenic differentiation of human dental follicle cells and human dental papilla cells," *PLoS ONE*, vol. 8, no. 4, Article ID e62332, 2013.

[52] S. Liekens, E. De Clercq, and J. Neyts, "Angiogenesis: regulators and clinical applications," *Biochemical Pharmacology*, vol. 61, no. 3, pp. 253–270, 2001.

[53] K. Iohara, L. Zheng, H. Wake et al., "A novel stem cell source for vasculogenesis in ischemia: subfraction of side population cells from dental pulp," *Stem Cells*, vol. 26, no. 9, pp. 2408–2418, 2008.

[54] C. Yuan, P. Wang, L. Zhu et al., "Coculture of stem cells from apical papilla and human umbilical vein endothelial cell under hypoxia increases the formation of three-dimensional vessel-like structures in vitro," *Tissue Engineering Part A*, vol. 21, no. 5-6, pp. 1163–1172, 2015.

[55] W. L. Dissanayaka, X. Zhan, C. Zhang, K. M. Hargreaves, L. Jin, and E. H. Y. Tong, "Coculture of dental pulp stem cells with endothelial cells enhances osteo-/odontogenic and angiogenic potential in vitro," *Journal of Endodontics*, vol. 38, no. 4, pp. 454–463, 2012.

[56] K. Janebodin, Y. Zeng, W. Buranaphatthana, N. Ieronimakis, and M. Reyes, "VEGFR2-dependent angiogenic capacity of pericyte-like dental pulp stem cells," *Journal of Dental Research*, vol. 92, no. 6, pp. 524–531, 2013.

[57] K. Iwasaki, M. Komaki, N. Yokoyama et al., "Periodontal ligament stem cells possess the characteristics of pericytes," *Journal of Periodontology*, vol. 84, no. 10, pp. 1425–1433, 2013.

[58] W. L. Dissanayaka, K. M. Hargreaves, L. Jin, L. P. Samaranayake, and C. Zhang, "The interplay of dental pulp stem cells and endothelial cells in an injectable peptide hydrogel on angiogenesis and pulp regeneration in vivo," *Tissue Engineering—Part A*, vol. 21, no. 3-4, pp. 550–563, 2015.

[59] C. Gandia, A. N. A. Armiñan, J. M. García-Verdugo et al., "Human dental pulp stem cells improve left ventricular function, induce angiogenesis, and reduce infarct size in rats with acute myocardial infarction," *Stem Cells*, vol. 26, no. 3, pp. 638–645, 2008.

[60] R. Ishizaka, Y. Hayashi, K. Iohara et al., "Stimulation of angiogenesis, neurogenesis and regeneration by side population cells from dental pulp," *Biomaterials*, vol. 34, no. 8, pp. 1888–1897, 2013.

[61] M. Sugiyama, K. Iohara, H. Wakita et al., "Dental pulp-derived $CD31^-/CD146^-$ side population stem/progenitor cells enhance recovery of focal cerebral ischemia in rats," *Tissue Engineering Part A*, vol. 17, no. 9-10, pp. 1303–1311, 2011.

[62] R. d'Aquino, A. Graziano, M. Sampaolesi et al., "Human postnatal dental pulp cells co-differentiate into osteoblasts and endotheliocytes: a pivotal synergy leading to adult bone tissue formation," *Cell Death & Differentiation*, vol. 14, no. 6, pp. 1162–1171, 2007.

[63] C. Marchionni, L. Bonsi, F. Alviano et al., "Angiogenic potential of human dental pulp stromal (stem) cells," *International Journal of Immunopathology and Pharmacology*, vol. 22, no. 3, pp. 699–706, 2009.

[64] S. Barachini, S. Danti, S. Pacini et al., "Plasticity of human dental pulp stromal cells with bioengineering platforms: a versatile tool for regenerative medicine," *Micron*, vol. 67, pp. 155–168, 2014.

[65] J. Karbanová, T. Soukup, J. Suchánek, R. Pytlík, D. Corbeil, and J. Mokrý, "Characterization of dental pulp stem cells from impacted third molars cultured in low serum-containing medium," *Cells Tissues Organs*, vol. 193, no. 6, pp. 344–365, 2011.

[66] M. M. Cordeiro, Z. Dong, T. Kaneko et al., "Dental pulp tissue engineering with stem cells from exfoliated deciduous teeth," *Journal of Endodontics*, vol. 34, no. 8, pp. 962–969, 2008.

[67] L. W. Bento, Z. Zhang, A. Imai et al., "Endothelial differentiation of SHED requires MEK1/ERK signaling," *Journal of Dental Research*, vol. 92, no. 1, pp. 51–57, 2013.

[68] V. T. Sakai, Z. Zhang, Z. Dong et al., "SHED differentiate into functional odontoblasts and endothelium," *Journal of Dental Research*, vol. 89, no. 8, pp. 791–796, 2010.

[69] Z. Zhang, F. Nör, M. Oh, C. Cucco, S. Shi, and J. E. Nör, "Wnt/β-catenin signaling determines the vasculogenic fate of postnatal mesenchymal stem cells," *Stem Cells*, vol. 34, no. 6, pp. 1576–1587, 2016.

[70] B. R. Amorim, K. G. Silvério, M. Z. Casati, E. A. Sallum, K. R. Kantovitz, and F. H. Nociti, "Neuropilin controls endothelial differentiation by mesenchymal stem cells from the periodontal ligament," *Journal of Periodontology*, vol. 87, no. 7, pp. e138–e147, 2016.

[71] M. A. Barros, J. F. Martins, D. A. Maria et al., "Immature dental pulp stem cells showed renotropic and pericyte-like properties in acute renal failure in rats," *Cell Medicine*, vol. 7, no. 3, pp. 95–108, 2015.

[72] S. Shi and S. Gronthos, "Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp," *Journal of Bone and Mineral Research*, vol. 18, no. 4, pp. 696–704, 2003.

[73] J. M. Soria, M. Sancho-Tello, M. A. G. Esparza et al., "Biomaterials coated by dental pulp cells as substrate for neural stem cell differentiation," *Journal of Biomedical Materials Research Part A*, vol. 97, no. 1, pp. 85–92, 2011.

[74] W. Martens, A. Bronckaers, C. Politis, R. Jacobs, and I. Lambrichts, "Dental stem cells and their promising role in neural regeneration: an update," *Clinical Oral Investigations*, vol. 17, no. 9, pp. 1969–1983, 2013.

[75] I. V. Nosrat, J. Widenfalk, L. Olson, and C. A. Nosrat, "Dental pulp cells produce neurotrophic factors, interact with trigeminal neurons in vitro, and rescue motoneurons after spinal cord injury," *Developmental Biology*, vol. 238, no. 1, pp. 120–132, 2001.

[76] A. Arthur, G. Rychkov, S. Shi, S. A. Koblar, and S. Gronthose, "Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues," *Stem Cells*, vol. 26, no. 7, pp. 1787–1795, 2008.

[77] C. Apel, O. V. Forlenza, V. J. R. De Paula et al., "The neuroprotective effect of dental pulp cells in models of Alzheimer's and Parkinson's disease," *Journal of Neural Transmission*, vol. 116, no. 1, pp. 71–78, 2009.

[78] P. Gervois, T. Struys, P. Hilkens et al., "Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons," *Stem Cells and Development*, vol. 24, no. 3, pp. 296–311, 2015.

[79] I. V. Nosrat, C. A. Smith, P. Mullally, L. Olson, and C. A. Nosrat, "Dental pulp cells provide neurotrophic support for dopaminergic neurons and differentiate into neurons in vitro; implications for tissue engineering and repair in the nervous system," *European Journal of Neuroscience*, vol. 19, no. 9, pp. 2388–2398, 2004.

[80] B. Mead, A. Logan, M. Berry, W. Leadbeater, and B. A. Scheven, "Dental pulp stem cells, a paracrine-mediated therapy for the retina," *Neural Regeneration Research*, vol. 9, no. 6, pp. 577–578, 2014.

[81] A. Arthur, S. Shi, A. C. W. Zannettino, N. Fujii, S. Gronthos, and S. A. Koblar, "Implanted adult human dental pulp stem cells induce endogenous axon guidance," *STEM CELLS*, vol. 27, no. 9, pp. 2229–2237, 2009.

[82] Y. Sugimura-Wakayama, W. Katagiri, M. Osugi et al., "Peripheral nerve regeneration by secretomes of stem cells from human

exfoliated deciduous teeth," *Stem Cells and Development*, vol. 24, no. 22, pp. 2687–2699, 2015.

[83] A. Jarmalavičiute, V. Tunaitis, U. Pivoraite, A. Venalis, and A. Pivoriunas, "Exosomes from dental pulp stem cells rescue human dopaminergic neurons from 6-hydroxy-dopamine-induced apoptosis," *Cytotherapy*, vol. 17, no. 7, pp. 932–939, 2015.

[84] M. Song, S.-S. Jue, Y.-A. Cho, and E.-C. Kim, "Comparison of the effects of human dental pulp stem cells and human bone marrow-derived mesenchymal stem cells on ischemic human astrocytes in vitro," *Journal of Neuroscience Research*, vol. 93, no. 6, pp. 973–983, 2015.

[85] M. Király, K. Kádár, D. B. Horváthy et al., "Integration of neuronally predifferentiated human dental pulp stem cells into rat brain in vivo," *Neurochemistry International*, vol. 59, no. 3, pp. 371–381, 2011.

[86] K. Sakai, A. Yamamoto, K. Matsubara et al., "Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms," *The Journal of Clinical Investigation*, vol. 122, no. 1, pp. 80–90, 2012.

[87] W. K. Leong, T. L. Henshall, A. Arthur et al., "Human adult dental pulp stem cells enhance poststroke functional recovery through non-neural replacement mechanisms," *Stem Cells Translational Medicine*, vol. 1, no. 3, pp. 177–187, 2012.

[88] K.-L. Yang, M.-F. Chen, C.-H. Liao, C.-Y. Pang, and P.-Y. Lin, "A simple and efficient method for generating Nurr1-positive neuronal stem cells from human wisdom teeth (tNSC) and the potential of tNSC for stroke therapy," *Cytotherapy*, vol. 11, no. 5, pp. 606–617, 2009.

[89] M. Yamagata, A. Yamamoto, E. Kako et al., "Human dental pulp-derived stem cells protect against hypoxic-ischemic brain injury in neonatal mice," *Stroke*, vol. 44, no. 2, pp. 551–554, 2013.

[90] L.-S. Tseng, S.-H. Chen, M.-T. Lin, and Y.-C. Lin, "Transplantation of human dental pulp-derived stem cells protects against heatstroke in mice," *Cell Transplantation*, vol. 24, no. 5, pp. 921–937, 2015.

[91] A. Nagpal, K. L. Kremer, M. A. Hamilton-Bruce et al., "TOOTH (The Open study Of dental pulp stem cell Therapy in Humans): study protocol for evaluating safety and feasibility of autologous human adult dental pulp stem cell therapy in patients with chronic disability after stroke," *International Journal of Stroke*, vol. 11, no. 5, pp. 575–585, 2016.

[92] H. Fujii, K. Matsubara, K. Sakai et al., "Dopaminergic differentiation of stem cells from human deciduous teeth and their therapeutic benefits for Parkinsonian rats," *Brain Research*, vol. 1613, pp. 59–72, 2015.

[93] C. Shimojima, H. Takeuchi, S. Jin et al., "Conditioned medium from the stem cells of human exfoliated deciduous teeth ameliorates experimental autoimmune encephalomyelitis," *The Journal of Immunology*, vol. 196, no. 10, pp. 4164–4171, 2016.

[94] P. De Berdt, J. Vanacker, B. Ucakar et al., "Dental apical papilla as therapy for spinal cord injury," *Journal of Dental Research*, vol. 94, no. 11, pp. 1575–1581, 2015.

[95] X. Li, C. Yang, L. Li et al., "A therapeutic strategy for spinal cord defect: human dental follicle cells combined with aligned PCL/PLGA electrospun material," *BioMed Research International*, vol. 2015, Article ID 197183, 12 pages, 2015.

[96] B. C. Heng, L. W. Lim, W. Wu, and C. Zhang, "An overview of protocols for the neural induction of dental and oral stem cells in vitro," *Tissue Engineering Part B: Reviews*, vol. 22, no. 3, pp. 220–250, 2016.

[97] N. Askari, M. M. Yaghoobi, M. Shamsara, and S. Esmaeili-Mahani, "Human dental pulp stem cells differentiate into oligodendrocyte progenitors using the expression of Olig2 transcription factor," *Cells Tissues Organs*, vol. 200, no. 2, pp. 93–103, 2014.

[98] R. Deumens, A. Bozkurt, M. F. Meek et al., "Repairing injured peripheral nerves: bridging the gap," *Progress in Neurobiology*, vol. 92, no. 3, pp. 245–276, 2010.

[99] A. D. Gaudet, P. G. Popovich, and M. S. Ramer, "Wallerian degeneration: gaining perspective on inflammatory events after peripheral nerve injury," *Journal of Neuroinflammation*, vol. 8, article 110, 2011.

[100] S. M. Hall, "The effect of inhibiting Schwann cell mitosis on the re-innervation of acellular autografts in the peripheral nervous system of the mouse," *Neuropathology and Applied Neurobiology*, vol. 12, no. 4, pp. 401–414, 1986.

[101] R. Sasaki, S. Aoki, M. Yamato et al., "PLGA artificial nerve conduits with dental pulp cells promote facial nerve regeneration," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 5, no. 10, pp. 823–830, 2011.

[102] R. Sasaki, S. Aoki, M. Yamato et al., "Tubulation with dental pulp cells promotes facial nerve regeneration in rats," *Tissue Engineering Part A*, vol. 14, no. 7, pp. 1141–1147, 2008.

[103] R. Sasaki, H. Matsumine, Y. Watanabe et al., "Electrophysiologic and functional evaluations of regenerated facial nerve defects with a tube containing dental pulp cells in rats," *Plastic and Reconstructive Surgery*, vol. 134, no. 5, pp. 970–978, 2014.

[104] B. H. Li, H.-J. Jung, S.-M. Kim, M.-J. Kim, J. W. Jahng, and J.-H. Lee, "Human periodontal ligament stem cells repair mental nerve injury," *Neural Regeneration Research*, vol. 8, no. 30, pp. 2827–2837, 2013.

[105] C. Muscari, E. Giordano, F. Bonafè, M. Govoni, A. Pasini, and C. Guarnieri, "Priming adult stem cells by hypoxic pretreatments for applications in regenerative medicine," *Journal of Biomedical Science*, vol. 20, article 63, 2013.

[106] W. Li, N. Ma, L.-L. Ong et al., "Bcl-2 engineered MSCs inhibited apoptosis and improved heart function," *Stem Cells*, vol. 25, no. 8, pp. 2118–2127, 2007.

[107] N. Noiseux, M. Gnecchi, M. Lopez-Ilasaca et al., "Mesenchymal stem cells overexpressing Akt dramatically repair infarcted myocardium and improve cardiac function despite infrequent cellular fusion or differentiation," *Molecular Therapy*, vol. 14, no. 6, pp. 840–850, 2006.

[108] M. Gnecchi, H. He, N. Noiseux et al., "Evidence supporting paracrine hypothesis for Akt-modified mesenchymal stem cell-mediated cardiac protection and functional improvement," *The FASEB Journal*, vol. 20, no. 6, pp. 661–669, 2006.

[109] M. I. Phillips and Y. L. Tang, "Genetic modification of stem cells for transplantation," *Advanced Drug Delivery Reviews*, vol. 60, no. 2, pp. 160–172, 2008.

[110] K. Iida, T. Takeda-Kawaguchi, Y. Tezuka, T. Kunisada, T. Shibata, and K.-I. Tezuka, "Hypoxia enhances colony formation and proliferation but inhibits differentiation of human dental pulp cells," *Archives of Oral Biology*, vol. 55, no. 9, pp. 648–654, 2010.

[111] J. B. Sakdee, R. R. White, T. C. Pagonis, and P. V. Hauschka, "Hypoxia-amplified proliferation of human dental pulp cells," *Journal of Endodontics*, vol. 35, no. 6, pp. 818–823, 2009.

[112] M. M. Kanafi, A. Ramesh, P. K. Gupta, and R. R. Bhonde, "Influence of hypoxia, high glucose, and low serum on the growth kinetics of mesenchymal stem cells from deciduous and

permanent teeth," *Cells Tissues Organs*, vol. 198, no. 3, pp. 198–208, 2013.

[113] H.-D. Müller, B. Cvikl, R. Gruber, G. Watzek, and H. Agis, "Prolyl hydroxylase inhibitors increase the production of vascular endothelial growth factor in dental pulp-derived cells," *Journal of Endodontics*, vol. 38, no. 11, pp. 1498–1503, 2012.

[114] M.-K. Kim, H.-J. Park, Y.-D. Kim et al., "Hinokitiol increases the angiogenic potential of dental pulp cells through ERK and p38MAPK activation and hypoxia-inducible factor-1α (HIF-1α) upregulation," *Archives of Oral Biology*, vol. 59, no. 2, pp. 102–110, 2014.

[115] Y.-S. Kim, K.-S. Min, D.-H. Jeong, J.-H. Jang, H.-W. Kim, and E.-C. Kim, "Effects of fibroblast growth factor-2 on the expression and regulation of chemokines in human dental pulp cells," *Journal of Endodontics*, vol. 36, no. 11, pp. 1824–1830, 2010.

[116] K. Matsushita, R. Motani, T. Sakuta et al., "Lipopolysaccharide enhances the production of vascular endothelial growth factor by human pulp cells in culture," *Infection and Immunity*, vol. 67, no. 4, pp. 1633–1639, 1999.

[117] T. M. Botero, M. G. Mantellini, W. Song, C. T. Hanks, and J. E. Nör, "Effect of lipopolysaccharides on vascular endothelial growth factor expression in mouse pulp cells and macrophages," *European Journal of Oral Sciences*, vol. 111, no. 3, pp. 228–234, 2003.

[118] H. Motohira, J. Hayashi, J. Tatsumi, M. Tajima, H. Sakagami, and K. Shin, "Hypoxia and reoxygenation augment bone-resorbing factor production from human periodontal ligament cells," *Journal of Periodontology*, vol. 78, no. 9, pp. 1803–1809, 2007.

[119] H. Agis, G. Watzek, and R. Gruber, "Prolyl hydroxylase inhibitors increase the production of vascular endothelial growth factor by periodontal fibroblasts," *Journal of Periodontal Research*, vol. 47, no. 2, pp. 165–173, 2012.

[120] T. Oyama, T. Sakuta, K. Matsushita, I. Maruyama, S. Nagaoka, and M. Torii, "Effects of roxithromycin on tumor necrosis factor-alpha-induced vascular endothelial growth factor expression in human periodontal ligament cells in culture," *Journal of Periodontology*, vol. 71, no. 10, pp. 1546–1553, 2000.

[121] M. Nokhbehsaim, S. Keser, A. V. B. Nogueira et al., "Beneficial effects of adiponectin on periodontal ligament cells under normal and regenerative conditions," *Journal of Diabetes Research*, vol. 2014, Article ID 796565, 11 pages, 2014.

[122] K. Chatzivasileiou, C. A. Lux, G. Steinhoff, and H. Lang, "Dental follicle progenitor cells responses to Porphyromonas gingivalis LPS," *Journal of Cellular and Molecular Medicine*, vol. 17, no. 6, pp. 766–773, 2013.

[123] C. Yuan, P. Wang, L. Zhu et al., "Coculture of stem cells from apical papilla and human umbilical vein endothelial cell under hypoxia increases the formation of three-dimensional vessel-like structures in vitro," *Tissue Engineering—Part A*, vol. 21, no. 5-6, pp. 1163–1172, 2015.

[124] P. R. Baraniak and T. C. McDevitt, "Stem cell paracrine actions and tissue regeneration," *Regenerative Medicine*, vol. 5, no. 1, pp. 121–143, 2010.

[125] I. Rosová, M. Dao, B. Capoccia, D. Link, and J. A. Nolta, "Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells," *Stem Cells*, vol. 26, no. 8, pp. 2173–2182, 2008.

[126] M. Kubo, T.-S. Li, R. Suzuki et al., "Hypoxic preconditioning increases survival and angiogenic potency of peripheral blood mononuclear cells via oxidative stress resistance," *American Journal of Physiology—Heart and Circulatory Physiology*, vol. 294, no. 2, pp. H590–H595, 2008.

[127] E. Potier, E. Ferreira, R. Andriamanalijaona et al., "Hypoxia affects mesenchymal stromal cell osteogenic differentiation and angiogenic factor expression," *Bone*, vol. 40, no. 4, pp. 1078–1087, 2007.

[128] G. T. Huang, M. Al-Habib, and P. Gauthier, "Challenges of stem cell-based pulp and dentin regeneration: a clinical perspective," *Endodontic Topics*, vol. 28, no. 1, pp. 51–60, 2013.

[129] M. J. Lee, J. W. Kim, and E. G. Yang, "Hinokitiol activates the hypoxia-inducible factor (HIF) pathway through inhibition of HIF hydroxylases," *Biochemical and Biophysical Research Communications*, vol. 396, no. 2, pp. 370–375, 2010.

[130] K. Trimmel, B. Cvikl, H.-D. Müller et al., "L-mimosine increases the production of vascular endothelial growth factor in human tooth slice organ culture model," *International Endodontic Journal*, vol. 48, no. 3, pp. 252–260, 2015.

[131] L. Wei, J. L. Fraser, Z.-Y. Lu, X. Hu, and S. P. Yu, "Transplantation of hypoxia preconditioned bone marrow mesenchymal stem cells enhances angiogenesis and neurogenesis after cerebral ischemia in rats," *Neurobiology of Disease*, vol. 46, no. 3, pp. 635–645, 2012.

[132] F. Wei, T. Song, G. Ding et al., "Functional tooth restoration by allogeneic mesenchymal stem cell-based bio-root regeneration in swine," *Stem Cells and Development*, vol. 22, no. 12, pp. 1752–1762, 2013.

[133] Y. S. Kim, M. Y. Noh, K. A. Cho et al., "Hypoxia/reoxygenation-preconditioned human bone marrow-derived mesenchymal stromal cells rescue ischemic rat cortical neurons by enhancing trophic factor release," *Molecular Neurobiology*, vol. 52, no. 1, pp. 792–803, 2015.

[134] M. H. Theus, L. Wei, L. Cui et al., "In vitro hypoxic preconditioning of embryonic stem cells as a strategy of promoting cell survival and functional benefits after transplantation into the ischemic rat brain," *Experimental Neurology*, vol. 210, no. 2, pp. 656–670, 2008.

[135] K. M. Hargreaves, A. Diogenes, and F. B. Teixeira, "Treatment options: biological basis of regenerative endodontic procedures," *Journal of Endodontics*, vol. 39, no. 3, pp. S30–S43, 2013.

[136] G. T.-J. Huang, "A paradigm shift in endodontic management of immature teeth: conservation of stem cells for regeneration," *Journal of Dentistry*, vol. 36, no. 6, pp. 379–386, 2008.

[137] G. Schmalz and A. J. Smith, "Pulp development, repair, and regeneration: challenges of the transition from traditional dentistry to biologically based therapies," *Journal of Endodontics*, vol. 40, no. 4, supplement, pp. S2–S5, 2014.

[138] D. J. Mooney, C. Powell, J. Piana, and B. Rutherford, "Engineering dental pulp-like tissue in vitro," *Biotechnology Progress*, vol. 12, no. 6, pp. 865–868, 1996.

[139] B. Buurma, K. Gu, and R. B. Rutherford, "Transplantation of human pulpal and gingival fibroblasts attached to synthetic scaffolds," *European Journal of Oral Sciences*, vol. 107, no. 4, pp. 282–289, 1999.

[140] S. Batouli, M. Miura, J. Brahim et al., "Comparison of stem-cell-mediated osteogenesis and dentinogenesis," *Journal of Dental Research*, vol. 82, no. 12, pp. 976–981, 2003.

[141] V. T. Sakai, M. M. Cordeiro, Z. Dong, Z. Zhang, B. D. Zeitlin, and J. E. Nör, "Tooth slice/scaffold model of dental pulp tissue engineering," *Advances in Dental Research*, vol. 23, no. 3, pp. 325–332, 2011.

[142] R. S. Prescott, R. Alsanea, M. I. Fayad et al., "In vivo generation of dental pulp-like tissue by using dental pulp stem cells, a collagen scaffold, and dentin matrix protein 1 after subcutaneous transplantation in mice," *Journal of Endodontics*, vol. 34, no. 4, pp. 421–426, 2008.

[143] G. T.-J. Huang, T. Yamaza, L. D. Shea et al., "Stem/progenitor cell-mediated de novo regeneration of dental pulp with newly deposited continuous layer of dentin in an *in vivo* model," *Tissue Engineering Part A*, vol. 16, no. 2, pp. 605–615, 2010.

[144] V. Rosa, Z. Zhang, R. H. M. Grande, and J. E. Nör, "Dental pulp tissue engineering in full-length human root canals," *Journal of Dental Research*, vol. 92, no. 11, pp. 970–975, 2013.

[145] M. Murakami, H. Horibe, K. Iohara et al., "The use of granulocyte-colony stimulating factor induced mobilization for isolation of dental pulp stem cells with high regenerative potential," *Biomaterials*, vol. 34, no. 36, pp. 9036–9047, 2013.

[146] R. Kuang, Z. Zhang, X. Jin et al., "Nanofibrous spongy microspheres for the delivery of hypoxia-primed human dental pulp stem cells to regenerate vascularized dental pulp," *Acta Biomaterialia*, vol. 33, pp. 225–234, 2016.

[147] K. Iohara, M. Nakashima, M. Ito, M. Ishikawa, A. Nakasima, and A. Akamine, "Dentin regeneration by dental pulp stem cell therapy with recombinant human bone morphogenetic protein 2," *Journal of Dental Research*, vol. 83, no. 8, pp. 590–595, 2004.

[148] K. Iohara, L. Zheng, M. Ito, A. Tomokiyo, K. Matsushita, and M. Nakashima, "Side population cells isolated from porcine dental pulp tissue with self-renewal and multipotency for dentinogenesis, chondrogenesis, adipogenesis, and neurogenesis," *Stem Cells*, vol. 24, no. 11, pp. 2493–2503, 2006.

[149] K. Iohara, M. Fujita, Y. Ariji et al., "Assessment of pulp regeneration induced by stem cell therapy by magnetic resonance imaging," *Journal of Endodontics*, vol. 42, no. 3, pp. 397–401, 2016.

[150] K. Iohara, K. Imabayashi, R. Ishizaka et al., "Complete pulp regeneration after pulpectomy by transplantation of $CD105^+$ stem cells with stromal cell-derived factor-1," *Tissue Engineering Part A*, vol. 17, no. 15-16, pp. 1911–1920, 2011.

[151] K. Iohara, M. Murakami, K. Nakata, and M. Nakashima, "Age-dependent decline in dental pulp regeneration after pulpectomy in dogs," *Experimental Gerontology*, vol. 52, pp. 39–45, 2014.

[152] K. Iohara, M. Murakami, N. Takeuchi et al., "A novel combinatorial therapy with pulp stem cells and granulocyte colony-stimulating factor for total pulp regeneration," *Stem Cells Translational Medicine*, vol. 2, no. 7, pp. 521–533, 2013.

[153] K. Iohara, L. Zheng, M. Ito et al., "Regeneration of dental pulp after pulpotomy by transplantation of $CD31^-/CD146^-$ side population cells from a canine tooth," *Regenerative Medicine*, vol. 4, no. 3, pp. 377–385, 2009.

[154] Y. Wang, Y. Zhao, W. Jia, J. Yang, and L. Ge, "Preliminary study on dental pulp stem cell-mediated pulp regeneration in canine immature permanent teeth," *Journal of Endodontics*, vol. 39, no. 2, pp. 195–201, 2013.

[155] J.-W. Yang, Y.-F. Zhang, C.-Y. Wan et al., "Autophagy in SDF-1α-mediated DPSC migration and pulp regeneration," *Biomaterials*, vol. 44, pp. 11–23, 2015.

[156] R. Ishizaka, K. Iohara, M. Murakami, O. Fukuta, and M. Nakashima, "Regeneration of dental pulp following pulpectomy by fractionated stem/progenitor cells from bone marrow and adipose tissue," *Biomaterials*, vol. 33, no. 7, pp. 2109–2118, 2012.

[157] M. Nakashima and K. Iohara, "Mobilized dental pulp stem cells for pulp regeneration: initiation of clinical trial," *Journal of Endodontics*, vol. 40, no. 4, supplement, pp. S26–S32, 2014.

[158] W. Sonoyama, Y. Liu, D. Fang et al., "Mesenchymal stem cell-mediated functional tooth regeneration in Swine," *PLoS ONE*, vol. 1, article e79, 2006.

[159] S. Na, H. Zhang, F. Huang et al., "Regeneration of dental pulp/dentine complex with a three-dimensional and scaffold-free stem-cell sheet-derived pellet," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 10, no. 3, pp. 261–270, 2016.

[160] Y. Bai, Y. Bai, K. Matsuzaka et al., "Cementum- and periodontal ligament-like tissue formation by dental follicle cell sheets co-cultured with Hertwig's epithelial root sheath cells," *Bone*, vol. 48, no. 6, pp. 1417–1426, 2011.

[161] S. Guo, W. Guo, D. Yi et al., "Comparative study of human dental follicle cell sheets and periodontal ligament cell sheets for periodontal tissue regeneration," *Cell Transplantation*, vol. 22, no. 6, pp. 1061–1073, 2013.

[162] W. Guo, L. Chen, K. Gong, B. Ding, Y. Duan, and Y. Jin, "Heterogeneous dental follicle cells and the regeneration of complex periodontal tissues," *Tissue Engineering Part A*, vol. 18, no. 5-6, pp. 459–470, 2012.

[163] K. Handa, M. Saito, A. Tsunoda et al., "Progenitor cells from dental follicle are able to form cementum matrix in vivo," *Connective Tissue Research*, vol. 43, no. 2-3, pp. 406–408, 2002.

[164] H.-S. Jung, D.-S. Lee, J.-H. Lee et al., "Directing the differentiation of human dental follicle cells into cementoblasts and/or osteoblasts by a combination of HERS and pulp cells," *Journal of Molecular Histology*, vol. 42, no. 3, pp. 227–235, 2011.

[165] M. Oshima, K. Inoue, K. Nakajima et al., "Functional tooth restoration by next-generation bio-hybrid implant as a bio-hybrid artificial organ replacement therapy," *Scientific Reports*, vol. 4, article 6044, 2014.

[166] S. Sowmya, K. P. Chennazhi, H. Arzate, P. Jayachandran, S. V. Nair, and R. Jayakumar, "Periodontal specific differentiation of dental follicle stem cells into osteoblast, fibroblast, and cementoblast," *Tissue Engineering Part C: Methods*, vol. 21, no. 10, pp. 1044–1058, 2015.

[167] T. Yokoi, M. Saito, T. Kiyono et al., "Establishment of immortalized dental follicle cells for generating periodontal ligament in vivo," *Cell and Tissue Research*, vol. 327, no. 2, pp. 301–311, 2007.

[168] W. Guo, Y. He, X. Zhang et al., "The use of dentin matrix scaffold and dental follicle cells for dentin regeneration," *Biomaterials*, vol. 30, no. 35, pp. 6708–6723, 2009.

[169] W. Guo, K. Gong, H. Shi et al., "Dental follicle cells and treated dentin matrix scaffold for tissue engineering the tooth root," *Biomaterials*, vol. 33, no. 5, pp. 1291–1302, 2012.

[170] B. Yang, G. Chen, J. Li et al., "Tooth root regeneration using dental follicle cell sheets in combination with a dentin matrix—based scaffold," *Biomaterials*, vol. 33, no. 8, pp. 2449–2461, 2012.

[171] L. Jiao, L. Xie, B. Yang et al., "Cryopreserved dentin matrix as a scaffold material for dentin-pulp tissue regeneration," *Biomaterials*, vol. 35, no. 18, pp. 4929–4939, 2014.

[172] M. Ducret, H. Fabre, O. Degoul et al., "Manufacturing of dental pulp cell-based products from human third molars: current strategies and future investigations," *Frontiers in Physiology*, vol. 6, article 213, 2015.

[173] B. C. Perry, D. Zhou, X. Wu et al., "Collection, cryopreservation, and characterization of human dental pulp-derived mesenchymal stem cells for banking and clinical use," *Tissue Engineering—Part C: Methods*, vol. 14, no. 2, pp. 149–156, 2008.

[174] T. Takeda, Y. Tezuka, M. Horiuchi et al., "Characterization of dental pulp stem cells of human tooth germs," *Journal of Dental Research*, vol. 87, no. 7, pp. 676–681, 2008.

[175] S. Eaker, M. Armant, H. Brandwein et al., "Concise review: guidance in developing commercializable autologous/patient-specific cell therapy manufacturing," *Stem Cells Translational Medicine*, vol. 2, no. 11, pp. 871–883, 2013.

[176] S. Gottipamula, M. S. Muttigi, U. Kolkundkar, and R. N. Seetharam, "Serum-free media for the production of human mesenchymal stromal cells: a review," *Cell Proliferation*, vol. 46, no. 6, pp. 608–627, 2013.

[177] D. G. Halme and D. A. Kessler, "FDA regulation of stem-cell-based therapies," *The New England Journal of Medicine*, vol. 355, no. 16, pp. 1730–1735, 2006.

[178] K. Bieback, "Platelet lysate as replacement for fetal bovine serum in mesenchymal stromal cell cultures," *Transfusion Medicine and Hemotherapy*, vol. 40, no. 5, pp. 326–335, 2013.

[179] T. Burnouf, D. Strunk, M. B. Koh, and K. Schallmoser, "Human platelet lysate: replacing fetal bovine serum as a gold standard for human cell propagation?" *Biomaterials*, vol. 76, pp. 371–387, 2016.

[180] H. Hemeda, B. Giebel, and W. Wagner, "Evaluation of human platelet lysate versus fetal bovine serum for culture of mesenchymal stromal cells," *Cytotherapy*, vol. 16, no. 2, pp. 170–180, 2014.

[181] G. Ding, J. Niu, and Y. Liu, "Dental pulp stem cells suppress the proliferation of lymphocytes via transforming growth factor-β1," *Human Cell*, vol. 28, no. 2, pp. 81–90, 2015.

[182] G. Ding, W. Wang, Y. Liu et al., "Effect of cryopreservation on biological and immunological properties of stem cells from apical papilla," *Journal of Cellular Physiology*, vol. 223, no. 2, pp. 415–422, 2010.

[183] G. Ding, Y. Liu, Y. An et al., "Suppression of T cell proliferation by root apical papilla stem cells in vitro," *Cells Tissues Organs*, vol. 191, no. 5, pp. 357–364, 2010.

[184] G. Ding, Y. Liu, W. Wang et al., "Allogeneic periodontal ligament stem cell therapy for periodontitis in swine," *Stem Cells*, vol. 28, no. 10, pp. 1829–1838, 2010.

[185] N. Wada, D. Menicanin, S. Shi, P. M. Bartold, and S. Gronthos, "Immunomodulatory properties of human periodontal ligament stem cells," *Journal of Cellular Physiology*, vol. 219, no. 3, pp. 667–676, 2009.

[186] H.-S. Kim, K.-H. Kim, S.-H. Kim et al., "Immunomodulatory effect of canine periodontal ligament stem cells on allogenic and xenogenic peripheral blood mononuclear cells," *Journal of Periodontal and Implant Science*, vol. 40, no. 6, pp. 265–270, 2010.

[187] D. Liu, J. Xu, O. Liu et al., "Mesenchymal stem cells derived from inflamed periodontal ligaments exhibit impaired immunomodulation," *Journal of Clinical Periodontology*, vol. 39, no. 12, pp. 1174–1182, 2012.

[188] Z. Li, C.-M. Jiang, S. An et al., "Immunomodulatory properties of dental tissue-derived mesenchymal stem cells," *Oral Diseases*, vol. 20, no. 1, pp. 25–34, 2014.

[189] S. Yildirim, N. Zibandeh, D. Genc, E. M. Ozcan, K. Goker, and T. Akkoc, "The comparison of the immunologic properties of stem cells isolated from human exfoliated deciduous teeth, dental pulp, and dental follicles," *Stem Cells International*, vol. 2016, Article ID 4682875, 15 pages, 2016.

[190] S. Tomic, J. Djokic, S. Vasilijic et al., "Immunomodulatory properties of mesenchymal stem cells derived from dental pulp and dental follicle are susceptible to activation by toll-like receptor agonists," *Stem Cells and Development*, vol. 20, no. 4, pp. 695–708, 2011.

[191] Y. Zhao, L. Wang, Y. Jin, and S. Shi, "Fas ligand regulates the immunomodulatory properties of dental pulp stem cells," *Journal of Dental Research*, vol. 91, no. 10, pp. 948–954, 2012.

[192] I. Kerkis, C. E. Ambrosio, A. Kerkis et al., "Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: local or systemic?" *Journal of Translational Medicine*, vol. 6, article 35, 2008.

[193] T. Mita, Y. Furukawa-Hibi, H. Takeuchi et al., "Conditioned medium from the stem cells of human dental pulp improves cognitive function in a mouse model of Alzheimer's disease," *Behavioural Brain Research*, vol. 293, pp. 189–197, 2015.

[194] B.-H. Choi, "Periodontal ligament formation around titanium implants using cultured periodontal ligament cells: a pilot study," *International Journal of Oral and Maxillofacial Implants*, vol. 15, no. 2, pp. 193–196, 2000.

[195] F. Feng, K. Akiyama, Y. Liu et al., "Utility of PDL progenitors for in vivo tissue regeneration: a report of 3 cases," *Oral Diseases*, vol. 16, no. 1, pp. 20–28, 2010.

[196] P. Gault, A. Black, J.-L. Romette et al., "Tissue-engineered ligament: implant constructs for tooth replacement," *Journal of Clinical Periodontology*, vol. 37, no. 8, pp. 750–758, 2010.

[197] Y. Lin, G. O. Gallucci, D. Buser, D. Bosshardt, U. C. Belser, and P. C. Yelick, "Bioengineered periodontal tissue formed on titanium dental implants," *Journal of Dental Research*, vol. 90, no. 2, pp. 251–256, 2011.

[198] P. S. Fleming, D. Koletsi, K. O'Brien, A. Tsichlaki, and N. Pandis, "Are dental researchers asking patient-important questions? a scoping review," *Journal of Dentistry*, vol. 49, pp. 9–13, 2016.

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 8596520, 11 pages
http://dx.doi.org/10.1155/2016/8596520

Research Article

Comparison of Stemness and Gene Expression between Gingiva and Dental Follicles in Children

Chung-Min Kang,[1] Seong-Oh Kim,[1] Mijeong Jeon,[2] Hyung-Jun Choi,[1] Han-Sung Jung,[3] and Jae-Ho Lee[1]

[1]*Department of Pediatric Dentistry, College of Dentistry, Yonsei University, Seoul, Republic of Korea*
[2]*Oral Science Research Center, College of Dentistry, Yonsei University, Seoul, Republic of Korea*
[3]*Department of Oral Biology, Division of Histology, College of Dentistry, Yonsei University, Seoul, Republic of Korea*

Correspondence should be addressed to Jae-Ho Lee; leejh@yuhs.ac

Received 17 March 2016; Accepted 10 August 2016

Academic Editor: Werner Geurtsen

The aim of this study was to compare the differential gene expression and stemness in the human gingiva and dental follicles (DFs) according to their biological characteristics. Gingiva ($n = 9$) and DFs ($n = 9$) were collected from 18 children. Comparative gene expression profiles were collected using cDNA microarray. The expression of development, chemotaxis, mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSs) related genes was assessed by quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Histological analysis was performed using hematoxylin-eosin and immunohistochemical staining. Gingiva had greater expression of genes related to keratinization, ectodermal development, and chemotaxis whereas DFs exhibited higher expression levels of genes related to tooth and embryo development. qRT-PCR analysis showed that the expression levels of iPSc factors including *SOX2*, *KLF4*, and *C-MYC* were 58.5 ± 26.3, 12.4 ± 3.5, and 12.2 ± 1.9 times higher in gingiva and *VCAM1* (CD146) and *ALCAM* (CD166) were 33.5 ± 6.9 and 4.3 ± 0.8 times higher in DFs. Genes related to MSCs markers including *CD13*, *CD34*, *CD73*, *CD90*, and *CD105* were expressed at higher levels in DFs. The results of qRT-PCR and IHC staining supported the microarray analysis results. Interestingly, this study demonstrated transcription factors of iPS cells were expressed at higher levels in the gingiva. Given the minimal surgical discomfort and simple accessibility, gingiva is a good candidate stem cell source in regenerative dentistry.

1. Introduction

Tissue engineering using mesenchymal stem cells (MSCs) is one of the most promising therapeutic strategies because MSCs have a high proliferation potential and may be manipulated to permit differentiation before transplantation [1, 2]. To date, different human dental stem cells have been isolated from dental pulp stem cells (DPSCs) [3], stem cells from exfoliated deciduous teeth (SHED) [4], periodontal ligament (PDL) stem cells [5], stem cells from apical papilla (SCAP) [6], and dental follicle precursor cells (DFPCs) [7]. Recently, mounting evidence suggests that gingiva derived mesenchymal stem cells were isolated and characterized as having multilineage differentiation capacity and immunomodulatory properties [8]. The presence of stem cell populations in dental follicles and the gingiva was revealed recently, and the related gene expression patterns remain unclear.

The dental follicle (DF) tissue is a connective fibrous tissue sac surrounding the enamel organ and the dental papilla of the developing tooth germ [9]. The DF cells have been proposed to have the capacity to differentiate into periodontium consisting of cementum, alveolar bone, and PDL [10, 11]. Despite an ectomesenchymal origin similar to that of the DFs, the gingiva appears to exhibit distinct functional activities during the maintenance of tissue integrity and during inflammatory responses [12]. It possesses a unique scarless healing process after wounding instead of the scar formation that is frequently observed in damaged extraoral tissues. So gingival tissue is postulated to have distinctive characteristics that

accelerate wound closure, suggesting unique stemness with the ability to induce directed differentiation and regeneration.

Although some efforts were made to identify the genes that are differentially expressed in the periodontium [12–14], the genetic differences between the gingiva and DFs remain unknown. Given the anatomical and functional differences between the two tissues, it is reasonable to assume that there are also differences in the gene expression patterns. Thus, genetic investigation related to epithelial-mesenchyme interaction between gingiva and dental follicle can provide critical importance in regulating cell population and signaling system in the regeneration of periodontium. The aim of this study is to compare the gene expression patterns of the gingiva and DFs to enhance our understanding of the distinct regenerative ability in gingiva and tissue differentiation capacity in DFs.

2. Materials and Methods

The Institutional Review Board of the Yonsei University Dental Hospital approved the experimental protocol (approval number 2-2015-0005). All the subjects or their guardians have provided written informed consent. We used procedures similar to that recently applied by Song et al. [15] and Lee et al. [14].

2.1. Tissue Sampling and RNA Isolation. Gingival tissues were collected from children ($n = 9$) (5 males and 4 females, aged 7–12 years) with a healthy gingiva who underwent surgical gingival resection for the extraction of a supernumerary tooth, for odontoma, or for orthodontic reasons. The DF tissues were obtained from children ($n = 9$) (6 males and 3 females, aged 6–8 years), and they were separated from the coronal portion of the tooth during the extraction of supernumerary teeth. In DF, a piece of gingival tissue around the extraction socket was carefully curetted. These samples were immediately frozen and stored in liquid nitrogen. We used fresh tissue instead of cultured cells because, at the tissue level, gene expression reflects simultaneous profiles of many genes and can provide additional insights into the physiological processes or tissue-specific functions that are mediated by the coordinated action of sets of genes. Gingiva and DFs were immediately submerged in RLT buffer, which is a component of the RNeasy Fibrous Mini kit® (Qiagen, CA, USA). Prior to the RNA extraction, the tissues in RLT buffer were homogenized using a Bullet Blender® Bead (Next Advanced, Inc., NY, USA). Total RNA was extracted from gingiva and DFs using the RNeasy Fibrous Mini kit (Qiagen, USA) according to the manufacturer's instructions. The extracted RNA was eluted in 25 μL of sterile water. RNA concentrations were measured from absorbance values at a wavelength of 260 nm using a spectrophotometer (NanoDrop ND-2000, Thermo Scientific, IL, USA). The RNA samples used in this study had 260/280 ratios of at least 1.8.

2.2. cDNA Microarray Construction and Data Analysis. Global gene expression analyses were performed using Affymetrix Gene Chip® Human Gene 1.0 ST oligonucleotide arrays (Affymetrix Inc., CA, USA). The average amount of RNA isolated from the gingiva and DFs was 1 μg. As recommended by the manufacturer's protocol, 300 ng of total RNA from each sample was converted to double-stranded cDNA. The cDNA was regenerated via random-primed reverse transcription using a dNTP mix containing dUTP. The fragmented, end-labeled cDNA was hybridized to the Gene Chip® Human Gene 1.0 ST array for 16 hours at 45°C and 60 rpm with a terminal transferase reaction incorporating a biotinylated dideoxynucleotide. After hybridization, the chips were stained and washed in a Genechip Fluidics Station 450® (Affymetrix) and scanned using a Genechip Array scanner 3000 G7® (Affymetrix). To determine whether genes were differentially expressed between the separated tissue groups, a one-way ANOVA was performed on the Robust Multi-Average (RMA) expression values. A multiple testing correction was applied to the p values of the F-statistics to adjust the false discovery rate. Genes with adjusted F-statistic p values <0.05 were extracted. Genes that were highly expressed in the gingiva or DFs and that exhibited differences greater than 4-fold between the signal value of the control and the test group were selected for further study. These genes were then classified based on the information related to gene function that is available in Gene Ontology from the KEGG Pathway database (http://david.abcc.ncifcrf.gov/home.jsp). This microarray data set was approved by the Gene Expression Omnibus (GEO) (http://www.ncbi.nlm.nih.gov/geo/); the GEO accession numbers of the data set are GSE58480 (gingiva) and GSE51342 (dental follicle).

2.3. Quantitative RT-PCR. The single-stranded cDNA required for the polymerase chain reaction (PCR) analysis was produced using 500 ng of extracted total RNA as a template for reverse transcription (RT) (Superscript III Reverse Transcriptase and random primer, Invitrogen, UK). The RT reaction was incubated at 65°C for 5 minutes, then 25°C (5 min), 50°C (1 hr), and 70°C (15 min) to inactivate the activity of the reverse transcriptase. The synthesized cDNA was diluted 1 : 10 in distilled water and used as a template for quantitative RT-PCR using the ABI7300 RT-PCR system (Applied Biosystems, Warrington, UK). The samples were prepared in triplicate with a volume of 25 μL containing 1x Universal TaqMan Master Mix (4369016, Applied Biosystems), the PCR primers at 0.9 μM, and the diluted cDNA. The amplification conditions were 50°C for 2 minutes and 95°C for 10 minutes, followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The following TaqMan gene expression assay primers (Applied Biosystems) were used: KRT6A, CXCL10, CSTA, AMBN, ADAM12, CXCL12, cMYC, KLF4, SOX2, CD106 (VCAM1), CD166 (ALCAM), and 18S rRNA. We selected known genes that are representative of the two tissues and little-known genes that are involved in their physiological functions.

ABI 7300 SDS 1.3.1 software (Applied Biosystems) recorded the fluorescence intensity of the reporter and quencher dyes, and results are plotted versus time, represented by the cycle number. The amplification plots were examined during the early log phase of product accumulation

Table 1: Specific primer used for quantitative RT-PCR analysis.

Gene symbol	Functions	Assay ID	Product size (bp)
KRT6A	Ectoderm development, positive regulation of cell proliferation, cell differentiation	Hs01699178_g1	83
CXCL10	Positive regulation of leukocyte, chemotaxis	Hs01124251_g1	135
CSTA	Keratinocyte differentiation, negative regulation of peptidase activity	Hs00193257_m1	114
AMBN	Cell proliferation, bone mineralization, odontogenesis of dentin-containing tooth	Hs00212970_m1	61
ADAM12	Cell-cell and cell-matrix interactions, including fertilization, muscle development, neurogenesis	Hs01106101_m1	54
CXCL12	Immune response, positive regulation of monocyte chemotaxis	Hs03676656_mH	88
C-MYC	Regulation of transcription, DNA-dependent	Hs00153408_m1	107
KLF4	Mesodermal cell fate determination, negative regulation of cell proliferation, regulation of transcription	Hs00358836_m1	110
SOX2	Negative regulation of transcription from RNA polymerase II promoter, osteoblast differentiation	Hs01053049_s1	91
CD106	Response to hypoxia, acute inflammatory response, chronic inflammatory response	Hs01003372_m1	62
CD166	Cell adhesion, signal transduction, motor axon guidance	Hs00977641_m1	103
18S rRNA		Hs03003631_g1	69

above background (the threshold cycle number, Ct) to obtain a precise quantification of initial target. The Ct values (the threshold cycle (Ct) number) were subsequently used to determine ΔCt values (ΔCt = Ct of the gene minus Ct of the 18S rRNA control). Relative expressions were expressed as the relative change by applying the equation $2^{-\Delta\Delta Ct}$ (ΔΔCt; differences in ΔCt values). All these quantitative RT-PCR procedures were done obtaining triplicated data. The results were analyzed using SPSS 20 software (SPSS Inc., IL, USA). Statistical differences were calculated by Mann–Whitney U tests, and $p < 0.05$ was considered statistically significant. The specific primer assay ID and product sizes for each gene are listed in Table 1.

2.4. Immunohistochemical Staining. For immunohistochemical staining, gingival tissue and DF tissue were fixed in 10% buffered formalin for 1 day, embedded in paraffin, and then sectioned at a thickness of 3 μm. The specimens were subjected to IHC staining with antibodies specific for CXCL10 (rabbit polyclonal, diluted 1 : 50; Ab9807, Abcam, Cambridge, UK), CSTA (rabbit polyclonal, diluted 1 : 2,000; Ab61223, Abcam), AMBN (rabbit polyclonal, diluted 1 : 200; Ab116347, Abcam), and CXCL12 (rabbit polyclonal, diluted 1 : 50; Ab9797, Abcam). Endogenous peroxidase activity was quenched via addition of 3% hydrogen peroxide. The sections were incubated in 5% bovine serum albumin to block nonspecific binding. The primary antibodies were diluted to facilitate optimal staining, and the sections were incubated overnight. After incubation, EnVision+ System HRP-Labeled Polymer anti-rabbit (K4003, Dako North America, Inc., CA, USA) was applied for 20 min. Color development was performed using labeled streptavidin biotin kits (Dako) according to the manufacturer's instructions.

3. Results

3.1. Gene Expression Profiles of the Gingiva and Dental Follicles. 1,182 out of 33,297 (3.6%) genes exhibited an absolute expression change of at least 4-fold. The expression levels of 555 genes were 4-fold higher in the gingiva than in DFs, while the expression levels of 627 genes were at least 4-fold higher in DFs than in the gingiva. The overall data distribution and frequency were confirmed by density and box plots of the ratio of the standardized log intensity to the average intensity. Ultimately, 829 genes were analyzed further, with the exception of several genes with unknown biological functions. The data were further filtered, and the genes are listed in Tables 2 and 3 according to their relative biological functions. In the gingiva, the expression levels of 387 genes were upregulated by 4-fold or more in comparison to DFs, while the expression levels of 442 genes were upregulated by 4-fold in DFs in comparison to the gingiva.

3.2. Gene Ontology Analysis. To identify the biological functions and features of the selected genes, the expression data sets were organized into Gene Ontology Consortium (GO) groups using the DAVID web-based tool. These genes were then classified based on information regarding gene function in gene ontology from the KEGG Pathway database. Figure 1 shows GO classes for the two data sets analyzed (F-statistic $p < 0.05$).

Table 2: Representative genes differentially expressed with higher expression levels in the gingiva than in dental follicles (absolute fold change > 4.0).

Functional category	Gene symbol	Biological process	Accession number	Absolute fold change	Standard deviation
Metabolism and catabolism	LIPK	Lipid catabolic process	NM_001080518	90.99	11.87
	FMO2	Organic acid metabolic process	NM_001460	34.26	7.05
	ARG1	Arginine catabolic process	NM_000045	18.91	5.06
	LIPN	Lipid catabolic process	NM_001080518	13.27	4.19
Protein modification and maintenance	KLK7	Proteolysis	NM_139277	30.47	6.52
	KLK10	Proteolysis	NM_002776	28.97	6.34
	KLK6	Protein autoprocessing	NM_002774	25.58	6.10
	TGM1	Protein modification process	NM_000359	22.21	5.48
	OCLN	Protein complex assembly	NM_002538	12.48	4.48
Structural process	SPRR2A	Keratinization	NM_005988	207.84	18.61
	KRT1	Keratinization	NM_006121	146.08	15.41
	CNFN	Keratinization	NM_032488	74.92	10.64
	CSTA	Keratinocyte differentiation	NM_005213	69.63	10.22
	KRT4	Cytoskeleton organization	NM_002272	39.48	7.50
	KRT3	Cytoskeleton organization	NM_057088	36.71	7.23
	FLG	Keratinocyte differentiation	NM_002016	24.31	5.75
	DSP	Keratinocyte differentiation	NM_004415	17.15	5.22
Transport activity	CLCA4	Ion transport	NM_012128	48.96	8.48
	AQP3	Water transport	NM_004925	27.74	6.41
	SLC5A1	Transmembrane transport	NM_000343	19.52	5.09
	GLTP	Glycolipid transport	NM_016433	7.56	3.04
Developmental process	KRT10	Epidermis development	NM_000421	152.93	15.74
	SCEL	Epidermis development	NM_144777	134.38	14.68
	KRT6B	Ectoderm development	NM_005555	90.30	12.11
	KRT6A	Ectoderm development	NM_005554	57.61	9.64
	SPINK5	Epidermal cell differentiation	NM_001127698	55.60	9.34
	EHF	Epithelial cell differentiation	NM_012153	14.27	5.50
	SOX2	Embryonic development	NM_003106	8.67	3.34
	TUFT1	Odontogenesis	NM_020127	7.87	3.19
Physiologic process	RHCG	Regulation of pH	NM_016321	51.23	8.68
	ABCA12	Cellular homeostasis	NM_173076	39.33	7.55
	EREG	Angiogenesis	NM_001432	13.04	4.29
	NMU	Gastric acid secretion	NM_006681	12.72	4.05
	SCD	Oxidation reduction	NM_005063	4.35	2.25
Nucleic acid synthesis and modification	MACC1	Regulation of cell division	NM_182762	20.30	5.38
	ESRP1	mRNA processing	NM_017697	17.02	5.85
	HIST1H1B	Nucleosome assembly	NM_005322	6.85	2.91
Signal transduction and regulation	IL1F9	Cell-cell signaling	NM_019618	26.31	6.03
	ARAP2	Signal transduction	NM_015230	9.88	3.89
	DAPP1	Signal transduction	NM_014395	8.90	3.32
Apoptosis	MAL	Induction of apoptosis	NM_002371	49.41	8.48
	ALOX12	Antiapoptosis	NM_000697	31.70	6.69
	FAM3B	Apoptosis	NM_058186	27.28	6.16
	BNIPL	Apoptosis	NM_001159642	18.88	5.01

Table 2: Continued.

Functional category	Gene symbol	Biological process	Accession number	Absolute fold change	Standard deviation
Cell adhesion	CLDN17	Cell-cell adhesion	NM_012131	91.67	11.90
	CRNN	Cell-cell adhesion	NM_016190	71.09	10.39
	DSC3	Homophilic cell adhesion	NM_024423	27.40	6.38
	CDSN	Cell adhesion	NM_001264	26.60	5.80
	DSG3	Cell adhesion	NM_001944	23.82	7.07
Cell cycle and transcriptional regulation	GRHL1	Regulation of transcription	NM_198182	31.32	6.62
	IRF6	Cell cycle arrest	NM_006147	13.05	4.87
	CASZ1	Regulation of transcription	NM_001079843	4.29	2.27
	E2F8	Regulation of transcription	NM_024680	4.20	2.21
Immune and inflammatory process	SERPINB4	Immune response	NM_002974	73.33	10.65
	IL1F6	Inflammatory response	NM_014440	43.13	7.87
	IL1RN	Inflammatory response	NM_173842	26.09	6.48
	IL1A	Inflammatory response	NM_000575	23.93	5.74
	CD1A	Immune response	NM_001763	4.16	2.19
Cytokine and chemokine activity	CXCL17	Chemotaxis	NM_198477	11.34	3.83
	CCL21	Chemotaxis	NM_002989	6.25	2.78
	ANLN	Cytokinesis	NM_018685	5.84	2.63
	CXCL10	Chemotaxis	NM_001565	4.29	2.37

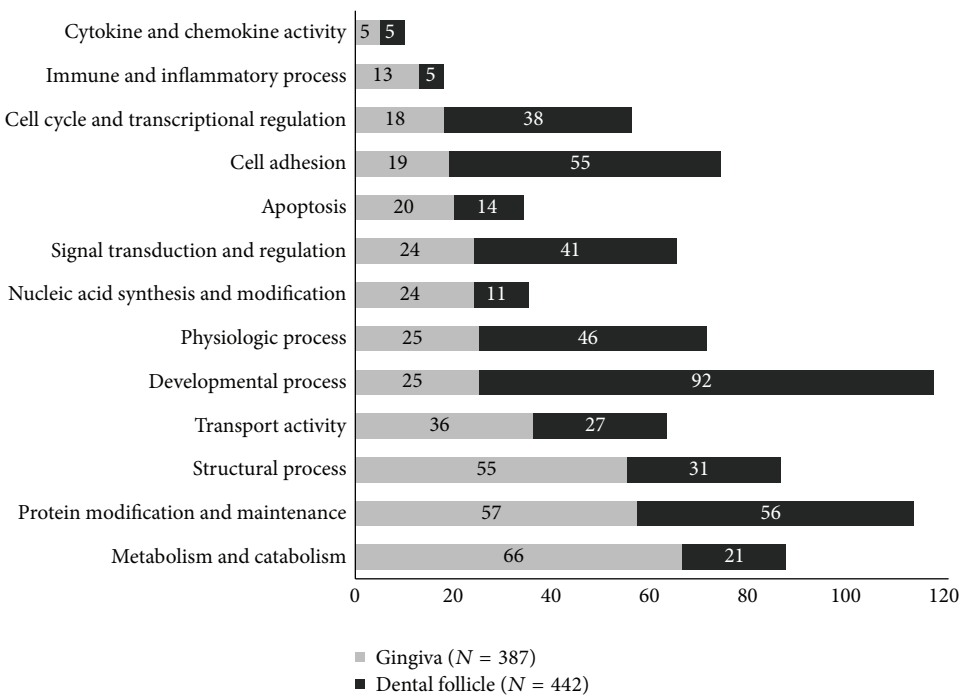


Figure 1: Main categories of genes expressed in the gingiva and dental follicles according to biological process. x-axis: the number of involved genes.

A total of 66 genes encoding metabolic and catabolic process were expressed more abundantly in the gingiva than in the DFs. Fifty-five genes related to structural processes such as keratinization and cytoskeleton organization were expressed at higher levels in the gingiva. On the other hand, 92 developmental process-related genes were highly expressed in DFs as a result of biological processes including odontogenesis, ossification, and bone mineralization. Cell cycle-associated genes and signal transduction- and regulation-related genes were expressed at higher levels in DFs. These results are consistent with the occurrence of higher proliferation rates in DFs.

Table 3: Representative genes differentially expressed with higher expression levels in dental follicles than in the gingiva (absolute fold change > 4.0).

Functional category	Gene symbol	Biological process	Accession number	Absolute fold change	Standard deviation
Metabolism and catabolism	ALDH1L2	Carbon metabolic process	NM_001034173	19.63	5.13
	MOXD1	Histidine catabolic process	NM_015529	17.92	4.91
	ELOVL2	Fatty acid metabolic process	NM_017770	12.62	4.07
	FBXL7	Protein catabolic process	NM_012304	8.58	3.27
Protein modification and maintenance	ADAM12	Metalloendopeptidase activity	NM_003474	37.09	7.25
	MMP16	Metalloendopeptidase activity	NM_005941	24.32	5.82
	MMP2	Metalloendopeptidase activity	NM_004530	19.64	5.17
	MMP8	Metalloendopeptidase activity	NM_002424	11.86	3.89
	MMP13	Metalloendopeptidase activity	NM_002427	7.60	3.16
	ADAM22	Proteolysis	NM_021723	5.97	2.75
Structural process	COL11A1	Extracellular matrix organization	NM_001854	29.15	6.38
	MAP1B	Microtubule bundle formation	NM_005909	10.30	3.61
	FBN2	Anatomical structure morphogenesis	NM_001999	9.02	3.40
	LUM	Collagen fibril organization	NM_002345	8.68	3.32
Transport activity	KCNT2	Ion transport	NM_198503	11.30	3.80
	ABCC9	Potassium ion transport	NM_005691	11.18	3.77
	RHOBTB3	Retrograde transport	NM_014899	10.62	3.72
	SLC4A4	Sodium ion transport	NM_001098484	10.12	3.68
	HEPH	Copper ion transport	NM_138737	8.34	3.28
Developmental process	AMBN	Odontogenesis	NM_016519	117.54	16.99
	CDH11	Ossification	NM_001797	38.12	7.40
	ALPL	Biomineral tissue development	NM_000478	33.21	6.83
	ASPN	Bone mineralization	NM_017680	33.05	6.85
	FGF7	Embryonic development	NM_002009	29.53	6.44
	COL1A2	Skeletal system development	NM_000089	14.50	4.41
	RUNX2	Ossification	NM_001024630	13.85	4.23
	PDGFRB	Embryonic development	NM_002609	11.85	3.93
	WNT2	Mesenchymal cell proliferation	NM_003391	10.28	3.73
	BMP5	Ossification	NM_021073	7.13	3.28
	LEF1	Wnt receptor signaling pathway	NM_016269	5.83	2.66
	PAX3	Organ morphogenesis	NM_181457	4.70	2.38
	MSX1	Organ morphogenesis	NM_002448	4.23	2.24
Physiologic process	VAT1L	Oxidation reduction	NM_020927	12.30	3.98
	TFPI	Blood coagulation	NM_006287	9.49	3.49
	TPM1	Muscle contraction	NM_000366	8.78	3.30
	SOBP	Sensory perception	NM_018013	8.27	3.21
Nucleic acid synthesis and modification	EYA4	DNA repair	NM_004100	24.90	5.86
	NAP1L3	Nucleosome assembly	NM_004538	16.47	4.68
	SNRPN	RNA splicing	BC043194	5.05	1.58
Signal transduction and regulation	PDE7B	Signal transduction	NM_018945	22.99	5.59
	CHN1	Signal transduction	NM_018945	22.98	5.60
	LIFR	Cytokine-mediated signaling pathway	NM_002310	8.78	3.31
	FSTL1	BMP signaling pathway	NM_007085	8.75	3.31
Apoptosis	SEMA3A	Apoptosis	NM_006080	51.87	8.72
	PEG10	Apoptosis	NM_015068	21.89	5.43
	SULF1	Apoptosis	NM_001128205	11.18	3.77
	NELL1	Induction of apoptosis	NM_006157	8.67	3.27

Table 3: Continued.

Functional category	Gene symbol	Biological process	Accession number	Absolute fold change	Standard deviation
Cell adhesion	OMD	Cell adhesion	NM_005014	40.83	7.69
	VCAN	Cell adhesion	NM_004385	35.76	7.25
	SPON1	Cell adhesion	NM_006108	32.63	6.78
Cell cycle and transcriptional regulation	MYEF2	Transcription	NM_016132	6.71	2.88
	SYCP2	Cell cycle	NM_014258	5.41	2.53
	APBB2	Cell cycle arrest	NM_004307	5.25	2.49
Immune and inflammatory process	TPST1	Inflammatory response	NM_003596	9.00	3.34
	PXDN	Immune response	NM_012293	8.89	3.40
	IFI44L	Immune response	NM_006820	6.01	2.79
	PECAM1	Phagocytosis	NM_000442	4.26	2.25
	COLEC12	Phagocytosis, recognition	NM_130386	4.23	2.22
Cytokine and chemokine activity	CXCL12	Chemotaxis	NM_000609	11.04	3.79
	SLIT3	Chemotaxis	NM_003062	8.94	3.34
	CMTM3	Chemotaxis	NM_144601	5.24	2.52
	STX2	Cytokinesis	NM_194356	4.39	2.27
	CCR1	Chemotaxis	NM_001295	4.31	2.36

3.3. Confirmation of Gene Differential Expression Using Quantitative RT-PCR. Quantitative RT-PCR analysis verified the cDNA microarray results. Six genes for which the difference in expression levels between the gingiva and DFs was at least 4-fold were selected. Mann–Whitney "*U*" test was performed to correlate the relative change with differential expression as detected by PCR. The expression levels of *KRT6A*, *CSTA*, and *CXCL10* were 13406.7 ± 14962.8, 1524.4 ± 714.8, and 4.7 ± 2.0 times higher in gingiva, and *AMBN*, *ADAM12*, and *CXCL12* were 20585.4 ± 24267.0, 192.5 ± 66.5, and 66.0 ± 6.5 times higher in DFs (Figure 2). These results were consistent with the microarray results.

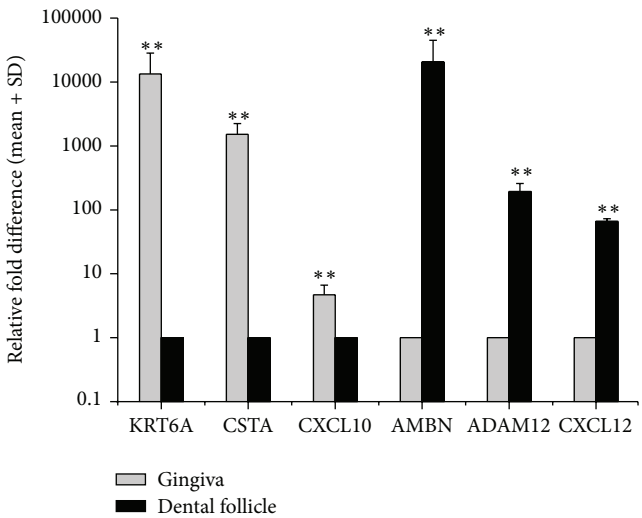


Figure 2: The relative difference in mRNA expression of six differentially expressed genes between the gingiva and dental follicles using quantitative RT-PCR. The data are presented as the mean + standard deviation and expressed as the relative change by applying the equation $2^{-\Delta\Delta Ct}$. y-axis: a log scale measure. $^{**}P < 0.05$.

3.4. Verification of Array Results by Immunohistochemical Staining. The following four proteins were the targets of the IHC study: *CXCL10*, *CSTA*, *AMBN*, and *CXCL12* (Figure 3). *CXCL10* was broadly stained in the epithelial area of the gingiva. *CSTA* was strongly stained in all of the layers of the gingiva. *AMBN* was not stained in the gingiva but stained around the outer area of the DFs. *CXCL12* was stained in a single cellular layer and in the collagenous connective tissue of DFs. The results were consistent with those of the cDNA microarray analysis at the protein level.

3.5. Stemness Characterization by Surface Protein Markers. Based on previous studies, dental stem cells were characterized using surface protein markers [16, 17]. The comparative expression results for stem cell marker genes are listed in Figure 4(a). Our results indicated that DF tissue derived MSCs are a cell population that is more positive for mesenchymal MSC markers (including *CD13*, *CD34*, *CD73*, *CD90*, and *CD105*) according to the International Society for Cell Therapy [18]. The comparative expression of four induced pluripotent stem cells (iPSCs) marker genes (i.e., *OCT-3*, *4*, *SOX2*, *cMYC*, and *KLF4*) were expressed at higher levels in the gingiva. As a result of qRT-PCR, *SOX2*, *KLF4*, and *cMYC* appeared 58.5, 12.43, and 12.23 times higher from the gingiva and *VCAM1 (CD106)* and *ALCAM (CD166)* were 33.54 and 4.27 times higher in DFs (Figure 4(b)). However, OCT-3, 4 did not show a clear difference in comparison to the other markers (0.46-fold difference).

4. Discussion

In this study, a cDNA microarray comparison analysis was performed to focus on differences in the gene expression profiles of gingiva and DFs in children. The majority of

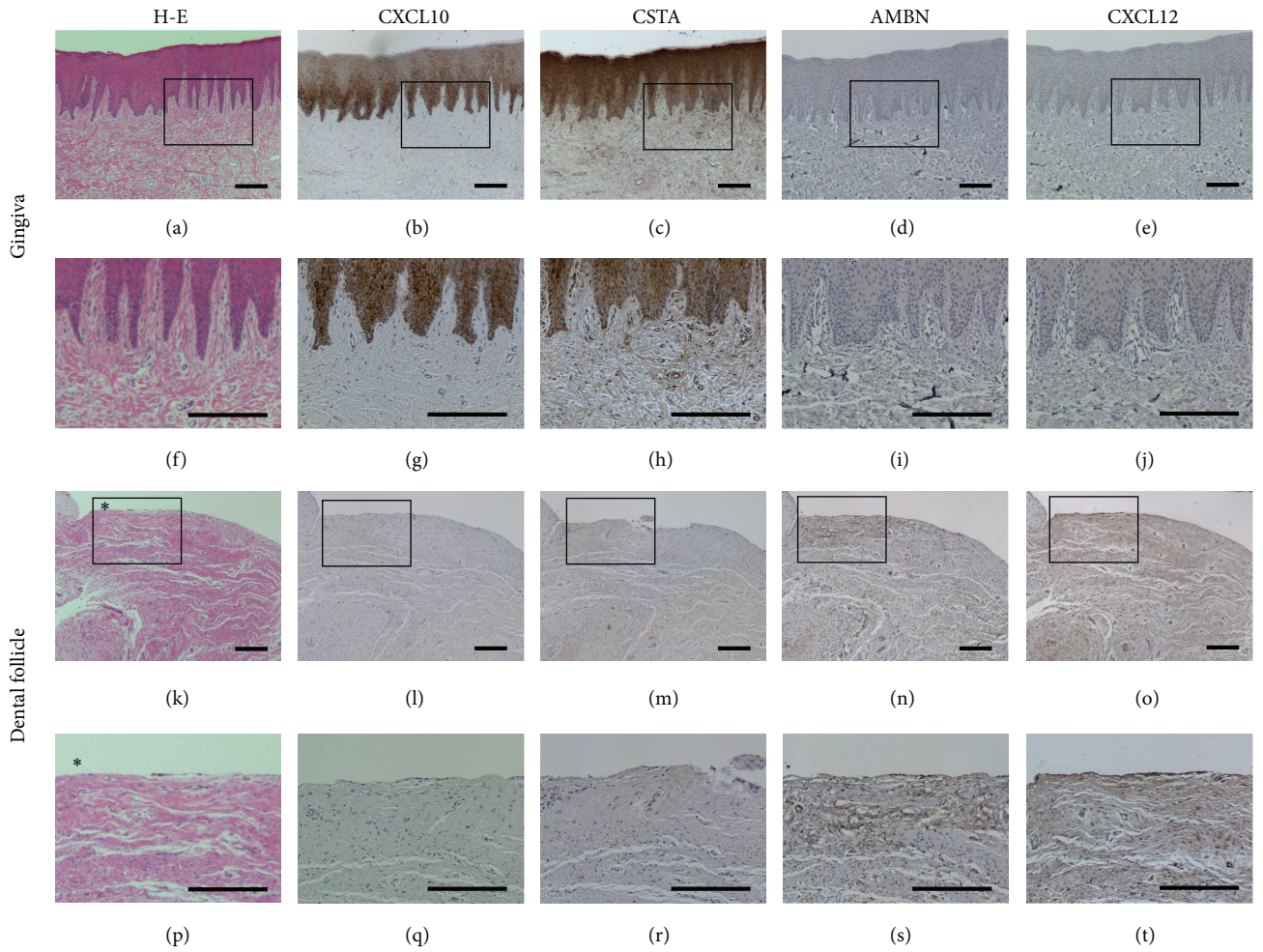


FIGURE 3: Verification of microarray results by immunohistochemical (IHC) staining. Hematoxylin-eosin staining in the gingiva (a, f) and dental follicles (DFs) (k, p) (asterisk: outer border neighboring alveolar bone). IHC staining for *CXCL10* in the gingiva (b, g) and DFs (l, q). IHC staining for *CSTA* in the gingiva (c, h) and DFs (m, r). The expression of *CXCL10* and *CSTA* was stained markedly in the gingival epithelium. The IHC staining for *AMBN* in the gingiva (d, i) and DFs (n, s). AMBN was stained around the outer layer of the DFs. The IHC staining for *CXCL12* in the gingiva (e, j) and dental follicles (o, t). CXCL12 was stained in both a cellular layer and the collagenous connective tissue of DFs (scale bars: 200 μm).

genes (32,115 out of 33,297, 96.5%) showed similar expression level between the gingiva and DFs when using a 4-fold absolute change cutoff value. Most of those genes encoded cell adhesion proteins, proteins involved in structural processes, or proteins related to signal transduction and regulation. This finding suggests that the gingiva and DFs differentiate into different tissue later although they originate from an ectomesenchymal cell. This is likely due to the regulation of comparable intracellular signaling pathways. In contrast, approximately 4% of genes were differentially expressed above the selected threshold. While accounting for only a small portion of the whole gene array, these genes might contribute to the distinct biological functions and distinguish each other phenotypically and morphologically. To investigate this assumption, comparative gene expression was analyzed with respect to the biological functions of the genes.

In the gingiva, *KRT1*, *CSTA*, and *FLG* were expressed at significantly higher levels. The gingival epithelium is a stratified squamous keratinizing tissue, and these genes are related to keratinization or keratinocyte differentiation. *KRT1* marks the cornification pathway of differentiation and is expressed in keratinized areas [19]. *CSTA* is one of the precursor proteins of the cornified cell envelope in keratinocytes and plays a role in epidermal development and maintenance [20]. *FLG* is essential for the regulation of epidermal homeostasis and interacts with keratin intermediate filaments [21]. Epidermis and ectoderm development-related genes were strongly upregulated in the gingiva versus DFs. *KRT6B* and *KRT6A* were markedly upregulated in the gingiva, with 90.30- and 57.61-fold differential expression, respectively. These proteins are rapidly induced in wound-proximal epidermal keratinocytes after skin injury

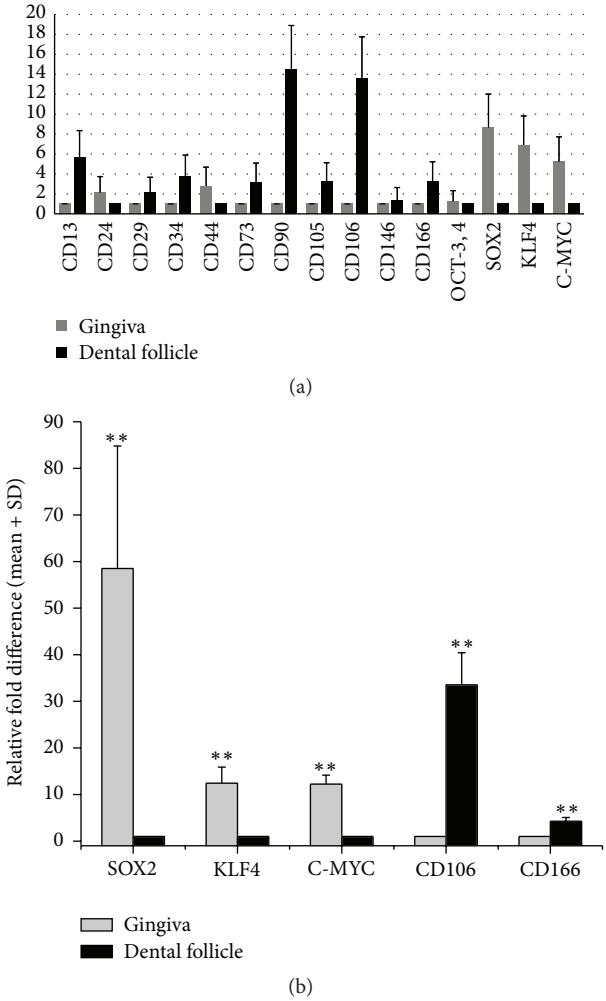


Figure 4: The relative gene expression of dental-derived stem cell and induced pluripotent stem cell markers using cDNA microarray (a). The relative fold difference in the expression of five stem cell marker genes between the gingiva and dental follicles using quantitative RT-PCR (b). The data are presented as the mean + standard deviation (a, b) and expressed as the relative change by applying the equation $2^{-\Delta\Delta Ct}$ (b). $^{**}P < 0.05$.

and regulate the migratory potential of skin keratinocytes during wound repair [22]. *SCEL* may function in the assembly or regulation of proteins in the keratinized envelope [23]. The upregulation of these genes may indicate the existence of a fast turnover rate in the gingiva and may facilitate fibroblast proliferation, which is an important event for tissue repair.

The oral mucosa is affected by exposure to various extrinsic factors such as chemicals and microorganisms. Genes related to apoptosis and chemotaxis such as *CXCL10*, *CXCL17*, *ANLN*, and *CCL21* were strongly expressed in the gingiva. *CXCL10* is secreted by the keratinocytes and is a marker of the host immune response [24]. This chemokine plays an important role in the infiltration of Th1 cells and affects the gingiva by exacerbating periodontal disease [25]. The overexpression of these chemokines might be associated with the generation and delivery of immune and inflammatory responses in the gingiva.

On the other hand, genes related to tooth and embryo development exhibited significantly higher expression in DFs. These results are consistent with those of a previous DF gene expression study that compared DFs to the PDL [14]. The increased expression of *AMBN* indicates that DFs play an important role in enamel matrix formation and mineralization [26]. In this study, *WNT2* and *LEF1* were upregulated in DFs suggesting that DFs are involved in the complex interplay of signaling factors that regulate tooth initiation and morphogenesis [27, 28]. *Runx2* is a key regulator of osteoblast marker genes and promotes the differentiation of mesenchymal stem cells into osteoblasts. The literature indicates that *Runx2* functions in the dental mesenchyme and mediates transduction signals from the dental epithelium to the mesenchyme during tooth development [29]. It also influences the molecular events that regulate tooth eruption—the most important physiologic role is likely being at the eruptive site [30]. Given the adaptive role of DFs, the presence of these genes suggests a central role of DFs in tooth formation.

Genes encoding protein modification- and signal transduction-related proteins tend to be expressed at higher levels in DFs than in the gingiva. The metalloprotease *ADAM 12* has been implicated in biological processes including fertilization and neurogenesis in DFs [11]. MMP-13 may be a major collagenolytic enzyme that degrades the extracellular matrix during tooth eruption. The upexpression of MMP-13 means DFs have important functions for the coordination of tooth eruption [31]. CXCL12 is a chemotactic factor for mesenchymal stem cells and mediates the suppressive effect of those cells on osteoclastogenesis. This factor can be expressed in DFs during tooth development including the epithelium surrounding the developing tooth bud [32].

To verify cDNA microarray results, six genes of different functions were selected for quantitative RT-PCR analyses. The expression levels of *KRT6A*, *CSTA*, and *CXCL10* were upregulated in the gingiva; *AMBN*, *ADAM12*, and *CXCL12* were upregulated in DFs. These results were consistent with the microarray results. To better understand the roles of the differentially expressed genes, IHC analysis was performed to identify their functions at tissue level. *CXCL10* and *CSTA* were strongly stained in all of the layers of the gingival tissue but were not stained in DFs. The genes that are highly expressed in the gingiva are stained in the epithelium because the prominent difference in structure between the gingiva and DFs is in the keratinized epithelium. *AMBN* and *CXCL12* were broadly stained in the outer area of DFs especially in the reduced enamel epithelium.

Several cell populations with stem cells properties have been isolated from different parts of dental tissue. Their participation in tissue repair and maintenance has been proposed [1]. Although it is difficult to characterize dental stem cells using surface protein markers, our results indicate the relative overexpression of important markers including CD13, CD34, CD73, and CD105 in DFs. These are ubiquitously expressed by all dental stem or precursor cells [6, 16]. With the exception of CD90, CD13, and CD34 which were frequently cited as dental-derived stem cell markers in

previous studies, we selected CD106 *(VCAM1)* and CD166 *(ALCAM)*, which are expressed more strongly in dental follicles. Other dental-derived stem marker genes including CD29, CD90, and CD73 were expressed at higher levels indicating self-renewing and differentiation capacities in DFs [33].

Interestingly, the gingiva expressed high levels of iPS-associated markers *(OCT4*, *cMYC*, SOX2, and *KLK4)* versus DFs [34]. These proteins are transcription factors that are essential for maintaining the self-renewal capacity or pluripotency [35]. The iPS cells offer an advantage over traditional MSCs because they display an unlimited growth capacity that can serve as an inexhaustible source of stem cells [36]. A similar comparable report analyzed that dental tissue derived mesenchymal-like stem cells can be reprogrammed into iPSCs more efficiently, when compared to other mature somatic cells from human body such as adult MSCs and adult dermal fibroblasts [37].

The accessibility of dental tissue, including MSCs, might still be limited because these cells can only be isolated under specific circumstances, such as during the extraction of teeth. However, the gingiva is one of the most convenient tissues to collect by biopsy, with less scar formation and less postsurgical donor discomfort. In addition, gingival tissues are routinely resected during dental procedures in children, such as surgical extraction of impacted teeth and surgical opening for teeth with delayed eruption, and these tissues are generally treated as biomedical waste. In the laboratory, it is also feasible to isolate stem cells from gingival tissue based on their highly proliferative nature. Thus, the gingiva can be an important alternative source of stem cells in regenerative dentistry. If stem cells isolated from gingival tissue can be utilized similar to the storage of umbilical cord blood, the dynamic features of these cells reveal much potential for their use. Although this study is limited to monitoring expression patterns without a clinical link, comparative gene expression analysis of different tissues might provide genetic information concerning functions, such as tissue repair and tooth development. Further investigations are needed to evaluate the neurogenesis capacity, mineralization potential, and cell proliferation capacity of stem cells from gingiva and dental follicles based on of this study.

5. Conclusion

For the first time, this study profiles differential gene expression between the gingiva and DFs. cDNA microarray was performed to characterize and compare the molecular fingerprints of stemness. The DFs have been considered a multipotent tissue based on their ability to generate cementum, bone, and PDL. While the gingiva was not noticed for pluripotent stemness before, this study demonstrated transcription factors of iPS cells were expressed at higher levels in the gingiva and most dental-derived stem cell markers were strongly upregulated in the DFs. Given the minimal postsurgical discomfort and simple accessibility of gingival tissue, the gingiva is a good candidate stem cell source in regenerative dentistry.

Disclosure

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests

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

Acknowledgments

The authors gratefully acknowledge all participants for their collaboration. This research was supported by the Basic Science Research Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology (2011-0022160 and 2012R1A1A2041910).

References

[1] T. I. Mitrano, M. S. Grob, F. Carrion et al., "Culture and characterization of mesenchymal stem cells from human gingival tissue," *Journal of Periodontology*, vol. 81, no. 6, pp. 917–925, 2010.

[2] P. M. Bartold, S. Shi, and S. Gronthos, "Stem cells and periodontal regeneration," *Periodontology 2000*, vol. 40, no. 1, pp. 164–172, 2006.

[3] S. Gronthos, M. Mankani, J. Brahim, P. G. Robey, and S. Shi, "Postnatal human dental pulp stem cells (DPSCs) in vitro and in vivo," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 97, no. 25, pp. 13625–13630, 2000.

[4] M. Miura, S. Gronthos, M. Zhao et al., "SHED: stem cells from human exfoliated deciduous teeth," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 100, no. 10, pp. 5807–5812, 2003.

[5] B. M. Seo, M. Miura, S. Gronthos et al., "Investigation of multipotent postnatal stem cells from human periodontal ligament," *The Lancet*, vol. 364, no. 9429, pp. 149–155, 2004.

[6] W. Sonoyama, Y. Liu, D. Fang et al., "Mesenchymal stem cell-mediated functional tooth regeneration in Swine," *PLoS ONE*, vol. 1, no. 1, article e79, 2006.

[7] C. Morsczeck, W. Götz, J. Schierholz et al., "Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth," *Matrix Biology*, vol. 24, no. 2, pp. 155–165, 2005.

[8] X. Xu, C. Chen, K. Akiyama et al., "Gingivae contain neural-crest- and mesoderm-derived mesenchymal stem cells," *Journal of Dental Research*, vol. 92, no. 9, pp. 825–832, 2013.

[9] S. Yao, F. Pan, V. Prpic, and G. E. Wise, "Differentiation of stem cells in the dental follicle," *Journal of Dental Research*, vol. 87, no. 8, pp. 767–771, 2008.

[10] A. R. Ten Cate, "The development of the periodontium—a largely ectomesenchymally derived unit," *Periodontology 2000*, vol. 14, no. 1, pp. 9–19, 1997.

[11] C. Morsczeck, G. Schmalz, T. E. Reichert et al., "Gene expression profiles of dental follicle cells before and after osteogenic differentiation in vitro," *Clinical Oral Investigations*, vol. 13, no. 4, pp. 383–391, 2009.

[12] X. Han and S. Amar, "Identification of genes differentially expressed in cultured human periodontal ligament fibroblasts

vs. human gingival fibroblasts by DNA microarray analysis," *Journal of Dental Research*, vol. 81, no. 6, pp. 399–405, 2002.

[13] T. Fujita, T. Iwata, H. Shiba et al., "Identification of marker genes distinguishing human periodontal ligament cells from human mesenchymal stem cells and human gingival fibroblasts," *Journal of Periodontal Research*, vol. 42, no. 3, pp. 283–286, 2007.

[14] H.-S. Lee, J. Lee, S.-O. Kim et al., "Comparative gene-expression analysis of the dental follicle and periodontal ligament in humans," *PLoS ONE*, vol. 8, no. 12, article e84201, 2013.

[15] J. S. Song, D. H. Hwang, S.-O. Kim et al., "Comparative gene expression analysis of the human periodontal ligament in deciduous and permanent teeth," *PLoS ONE*, vol. 8, no. 4, Article ID e61231, 2013.

[16] C. Morsczeck, G. Schmalz, T. E. Reichert, F. Völlner, K. Galler, and O. Driemel, "Somatic stem cells for regenerative dentistry," *Clinical Oral Investigations*, vol. 12, no. 2, pp. 113–118, 2008.

[17] K. Ranganathan and V. Lakshminarayanan, "Stem cells of the dental pulp," *Indian Journal of Dental Research*, vol. 23, no. 4, p. 558, 2012.

[18] M. Dominici, K. Le Blanc, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.

[19] R. P. Carmichael, C. A. McCulloch, and G. A. Zarb, "Quantitative immunohistochemical analysis of keratins and desmoplakins in human gingiva and peri-implant mucosa," *Journal of Dental Research*, vol. 70, no. 5, pp. 899–905, 1991.

[20] Š. Magister and J. Kos, "Cystatins in immune system," *Journal of Cancer*, vol. 4, no. 1, pp. 45–56, 2013.

[21] P. Ovaere, S. Lippens, P. Vandenabeele, and W. Declercq, "The emerging roles of serine protease cascades in the epidermis," *Trends in Biochemical Sciences*, vol. 34, no. 9, pp. 453–463, 2009.

[22] J. D. Rotty and P. A. Coulombe, "A wound-induced keratin inhibits Src activity during keratinocyte migration and tissue repair," *Journal of Cell Biology*, vol. 197, no. 3, pp. 381–389, 2012.

[23] A. E. Kalinin, A. V. Kajava, and P. M. Steinert, "Epithelial barrier function: assembly and structural features of the cornified cell envelope," *BioEssays*, vol. 24, no. 9, pp. 789–800, 2002.

[24] A. Antonelli, S. M. Ferrari, D. Giuggioli, E. Ferrannini, C. Ferri, and P. Fallahi, "Chemokine (C-X-C motif) ligand (CXCL)10 in autoimmune diseases," *Autoimmunity Reviews*, vol. 13, no. 3, pp. 272–280, 2014.

[25] Y. Hosokawa, I. Hosokawa, S. Shindo et al., "Black tea polyphenol inhibits CXCL10 production in oncostatin M-stimulated human gingival fibroblasts," *International Immunopharmacology*, vol. 11, no. 6, pp. 670–674, 2011.

[26] M. MacDougall, B. R. Dupont, D. Simmons et al., "Ameloblastin gene (AMBN) maps within the critical region for autosomal dominant amelogenesis imperfecta at chromosome 4q21," *Genomics*, vol. 41, no. 1, pp. 115–118, 1997.

[27] Y. D. Zhang, Z. Chen, Y. Q. Song, C. Liu, and Y. P. Chen, "Making a tooth: growth factors, transcription factors, and stem cells," *Cell Research*, vol. 15, no. 5, pp. 301–316, 2005.

[28] F. Liu, E. Y. Chu, B. Watt et al., "Wnt/β-catenin signaling directs multiple stages of tooth morphogenesis," *Developmental Biology*, vol. 313, no. 1, pp. 210–224, 2008.

[29] T. Åberg, X.-P. Wang, J.-H. Kim et al., "Runx2 mediates FGF signaling from epithelium to mesenchyme during tooth morphogenesis," *Developmental Biology*, vol. 270, no. 1, pp. 76–93, 2004.

[30] G. E. Wise, S. Frazier-Bowers, and R. N. D'Souza, "Cellular, molecular, and genetic determinants of tooth eruption," *Critical Reviews in Oral Biology and Medicine*, vol. 13, no. 4, pp. 323–334, 2002.

[31] I. Takahashi, M. Nishimura, K. Onodera et al., "Expression of MMP-8 and MMP-13 genes in the periodontal ligament during tooth movement in rats," *Journal of Dental Research*, vol. 82, no. 8, pp. 646–651, 2003.

[32] A. M. Havens, E. Chiu, M. Taba Jr. et al., "Stromal-derived factor-lα (CXCL 12) leveis increase in periodontal disease," *Journal of Periodontology*, vol. 79, no. 5, pp. 845–853, 2008.

[33] D. G. Phinney and D. J. Prockop, "Concise review: mesenchymal stem/multipotent stromal cells: the state of transdifferentiation and modes of tissue repair—current views," *Stem Cells*, vol. 25, no. 11, pp. 2896–2902, 2007.

[34] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[35] N. Wada, B. Wang, N.-H. Lin, A. L. Laslett, S. Gronthos, and P. M. Bartold, "Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts," *Journal of Periodontal Research*, vol. 46, no. 4, pp. 438–447, 2011.

[36] K. Hynes, D. Menicanin, J. Han et al., "Mesenchymal stem cells from iPS cells facilitate periodontal regeneration," *Journal of Dental Research*, vol. 92, no. 9, pp. 833–839, 2013.

[37] X. Yan, H. Qin, C. Qu, R. S. Tuan, S. Shi, and G. T.-J. Huang, "iPS cells reprogrammed from human mesenchymal-like stem/progenitor cells of dental tissue origin," *Stem Cells and Development*, vol. 19, no. 4, pp. 469–480, 2010.

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 1947157, 13 pages
http://dx.doi.org/10.1155/2016/1947157

Research Article

Investigation of the Cell Surface Proteome of Human Periodontal Ligament Stem Cells

Jimin Xiong,[1,2] Danijela Menicanin,[3] Peter S. Zilm,[1,4] Victor Marino,[1] P. Mark Bartold,[1] and Stan Gronthos[5,6]

[1]*Colgate Australian Clinical Dental Research Centre, School of Dentistry, University of Adelaide, Adelaide, SA 50052, Australia*
[2]*Laboratory of Tissue Regeneration and Immunology and Department of Periodontics, Beijing Key Laboratory of Tooth Regeneration and Function Reconstruction, School of Stomatology, Capital Medical University, Beijing 1000443, China*
[3]*School of Medicine, Faculty of Health Sciences, University of Adelaide, Adelaide, SA 50054, Australia*
[4]*Oral Microbiology, School of Dentistry, University of Adelaide, Adelaide, SA 50055, Australia*
[5]*Mesenchymal Stem Cell Laboratory, School of Medicine, Faculty of Health Sciences, University of Adelaide, Adelaide, SA 50056, Australia*
[6]*South Australian Health and Medical Research Institute, Adelaide, SA 5000, Australia*

Correspondence should be addressed to Stan Gronthos; stan.gronthos@adelaide.edu.au

Received 27 May 2016; Accepted 3 July 2016

Academic Editor: Athina Bakopoulou

The present study examined the cell surface proteome of human periodontal ligament stem cells (PDLSC) compared to human fibroblasts. Cell surface proteins were prelabelled with CyDye before processing to extract the membrane lysates, which were separated using 2D electrophoresis. Selected differentially expressed protein "spots" were identified using Mass spectrometry. Four proteins were selected for validation: CD73, CD90, Annexin A2, and sphingosine kinase 1 previously associated with mesenchymal stem cells. Flow cytometric analysis found that CD73 and CD90 were highly expressed by human PDLSC and gingival fibroblasts but not by keratinocytes, indicating that these antigens could be used as potential markers for distinguishing between mesenchymal cells and epithelial cell populations. Annexin A2 was also found to be expressed at low copy number on the cell surface of human PDLSC and gingival fibroblasts, while human keratinocytes lacked any cell surface expression of Annexin A2. In contrast, sphingosine kinase 1 expression was detected in all the cell types examined using immunocytochemical analysis. These proteomic studies form the foundation to further define the cell surface protein expression profile of PDLSC in order to better characterise this cell population and help develop novel strategies for the purification of this stem cell population.

1. Introduction

Despite encouraging outcomes, therapeutic utilization of mesenchymal stem cells (MSC) is constrained by the lack of understanding and definition of their properties and developmental status following *ex vivo* expansion. Heterogeneity inherent within progenitor populations presents as one of the major limitations to their clinical application in regenerative medicine. The variability and inconsistencies in cellular properties allude to a hierarchical order within stem cell populations and result in the coexistence of subsets of distinct morphologies, phenotypes, proliferation rates, and biological functions [1–3]. Currently, there is a lack of individual or a set of markers that can distinguish different subsets within MSC-like populations of different origins from more differentiated fibroblastic cells in any tissue.

Identification of stem/progenitor cells residing in the periodontium [4–6] has offered a potential novel therapeutic avenue for treating periodontal tissues damaged due to trauma, injury, and disease. Periodontal diseases are highly prevalent among all human populations and if untreated cause the destruction of periodontal supporting tissues and can potentially result in tooth loss. Predictable regeneration of periodontal tissues as a result of advanced periodontal diseases is beyond the scope of current technologies and, therefore, alternative strategies are being investigated.

In addition to periodontal ligament stem cells (PDLSC), the periodontium contains multiple cell types including fibroblasts, endothelial cells, epithelial cell rests of Malassez (ERM), osteoblasts, and cementoblasts [7]. This array of specialised cell types is integrated into and cofunctions to provide the periodontium with its essential and unique structural and mechanical properties. This biological complexity and cellular heterogeneity highlights the need for identification of surface markers specific to each cell subset within the periodontium to enable identification and discriminant isolation of desired and required cell populations.

It has been demonstrated that PDLSC share a phenotypic profile characteristic of bone marrow derived mesenchymal stem cells (BMSC) including expression of BMSC markers CD29, CD44, CD90, and CD105 [8]. Furthermore, PDLSC express the early BMSC and perivascular cell surface markers STRO-1 and CD146/MUC18 [4], with a subset of progenitors presenting with other antigens associated with perivascular tissues (alpha-smooth muscle actin and pericyte-associated antigen, 3G5) [9]. Together, these findings designate a possible perivascular origin of PDLSC, in accord with earlier findings by McCulloch and colleagues [10, 11]. In conjunction, comparative genomic analyses identified unique features exhibited by PDLSC when compared to BMSC and dental pulp stem cells (DPSC). These studies demonstrated increased levels of scleraxis (a tendon-specific transcription factor) [4] and PLAP-1 (periodontal ligament associated protein-1/asporin) expression in PDLSC [12]. A panel of markers, proposed for the current identification of PDLSC, includes alkaline phosphatase, type I collagen, periostin, runt-related transcription factor-2 (Runx2), and epithelial growth factor receptor, which are also expressed by BMSC, considering that both cell populations commonly hold the innate capacity for formation of mineralized matrix in the form of cementum and bone, respectively [13]. Since the cell surface markers described above are ubiquitously expressed by MSC-like populations derived from all dental tissues, specific cell surface antigens, capable of distinguishing between individual dental stem cell population subsets, are yet to be identified [14]. Therefore, our understanding of the cell surface phenotype of PDLSC falls short when considering the need to isolate and purify stem/progenitor cell subsets from the heterogeneous PDL population. This has driven the use of proteomics, the technology investigating global protein expression, to characterise the cell surface phenotype of PDLSC.

Proteomic studies investigating dental tissues have been summarized by McCulloch [15]. While the majority of studies focused on protein expression by periodontal microbiota [16–18], a limited number of papers examined proteomic profiles of periodontal ligament cells and tissues [15]. In this study, we provide an insight into the cell surface proteome of PDLSC to identify potential discriminatory PDLSC markers not expressed by other cells residing in the periodontium.

2. Methods and Materials

2.1. Isolation of Human PDLSC and Gingival Fibroblasts. Human PDLSC and gingival fibroblasts (GF) were isolated from three donors and cultured as previously described (Human Research Ethics Committee of the University of Adelaide, Approval Number H-112-2008) [4, 19]. Briefly, gingival and periodontal ligament tissues were collected from excised gingiva and middle third of the root, respectively. The tissues were digested in equal volumes of collagenase type I (3 mg/mL; Worthington Biochemical, Lakewood, NJ) and dispase type II (4 mg/mL; Roche Diagnostics, Indianapolis, IN) for 2 hours at 37°C. Isolated cells were maintained and cultured in modified α-MEM media (α-MEM; Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% FCS (Thermo Electron, Melbourne, VIC, Australia), 50 U/mL and 50 μg/mL penicillin and streptomycin (Sigma-Aldrich), 1 mM sodium pyruvate, 2 mM L-glutamine (SAFC, Lenexa, KS, USA), and 100 μM L-ascorbate-2-phosphate (Novachem, Melbourne, VIC, Australia) at 37°C and 5% CO_2 in a humidified environment, with a twice-weekly medium change. Cells were harvested and further expanded once upon reaching confluence. This process was repeated when cells reached 80% confluence until desired cell numbers were obtained.

Human neonatal foreskins, collected from routine circumcisions, were used to isolate epithelial sheets after overnight incubation with 4 mg/mL dispase at 4°C, followed by trypsinization for 5 min at 37°C to obtain basal keratinocytes. Keratinocytes were cultured in DMEM containing 10% fetal calf serum, 20 ng/mL epidermal growth factor (Sigma-Aldrich), and 0.4 μg/mL hydrocortisone (Sigma-Aldrich), at 37°C and 5% CO_2 in a humidified environment, with a twice-weekly medium change.

2.2. Immunohistochemistry. Chamber slides (Nalge-Nunc Lab-Tek, Rochester, NY, USA) were seeded with 8×10^3 cells per cm^2, in media with additives for 2 days. The slides were fixed with 4% paraformaldehyde (PFA) and endogenous peroxidase activity was inhibited using 0.5% H_2O_2 in methanol. The sections were incubated with primary antibodies or isotype control antibodies overnight at 4°C, secondary antibodies for 1 hour at room temperature, Vectastain ABC Reagents (Vector Laboratories, Burlingame, CA, USA) according to the manufacturer's recommendations, or horseradish-peroxidase-labelled streptavidin (Promega, Madison, WI, USA) at 1 in 1000 dilution and then developed with diaminobenzidine (Dako, Campbellfield, VIC, Australia). The slides were counterstained briefly with haematoxylin (ProSciTech, Thuringowa Central, QLD, Australia). Antibodies used in this study are 1B5, mouse IgG1 isotype control (1 : 25; Professor L. K. Ashman, University of Newcastle, NSW, Australia); mouse IgG1 anti-human CD73 (1 : 25; BD Pharmingen, Sparks, MD, USA); mouse IgG1 anti-human CD90 (1 : 25; BD Pharmingen); mouse IgG1 anti-human Annexin A2 (1 : 12.5; Invitrogen, Waltham, MA, USA); rabbit anti-human sphingosine kinase 1 (1 : 20; Cayman Chemical, Ann Arbor, MI, USA); normal rabbit Ig (1 : 20; Vector Laboratories, Burlingame, CA, USA); goat anti-mouse IgG biotin secondary antibody (1 : 200; Southern Biotechnology Associates, Birmingham, AL); and goat anti-rabbit IgG biotin secondary antibody (1 : 150; Vector Laboratories).

2.3. Immunophenotypic Profiling. Single cell suspensions of 2×10^5 cells were blocked with 5% FCS, 1% bovine serum albumin (BSA, SAFC), 50 U/mL penicillin, 50 μg/mL streptomycin, and 5% normal human serum (Red Cross, SA, Australia) in HBSS on ice. Cells were treated with primary or isotype control antibodies (CD73, CD90, and Annexin A2) at a concentration of 20 μg/mL, followed by incubation with phycoerythrin (PE) conjugated goat anti-mouse IgG1 (1 : 50; Southern Biotechnology Associates). Samples were fixed in PBS with 0.1% formalin and 20 mg/mL glucose. Analysis was performed on a fluorescence-activated cell sorter fitted with 250 MW argon laser (Beckman Coulter Cytomics FC500, using CXP Cytometry List Mode Data Acquisition and Analysis Software version 2.2; Beckman Coulter, Miami, FL, USA).

2.4. Proteomic Analysis. All equipment and reagents were purchased from Bio-Rad Laboratories (Hercules, CA, USA) unless stated otherwise. CyDye DIGE Fluor minimal dye was purchased from GE Healthcare (Buckinghamshire, UK).

2.5. Cell Surface Labelling Using CyDye DIGE Fluor Minimal Dye. CyDye fluorescent cell surface protein labelling was performed as previously reported [4, 20]. Briefly, approximately 20 million subconfluent PDLSC or GF were detached with either 1 mM PUCK's EDTA or 3 mg/mL type I collagenase and aliquoted into ~5 million cells per tube. Cells were washed in ice cold HBSS (pH 7.4) followed by ice cold HBSS (pH 8.5) and centrifuged at 800 ×g for 2 minutes. The cell pellets were resuspended in 200 μL labelling buffer containing HBSS (pH 8.5) and 1 M urea. Cells were then labelled with 600 pmol of either Cy2, Cy3, or Cy5 or CyDye DIGE Fluor minimal dyes on ice in the dark for 20 minutes. Staining was quenched by adding 20 μL lysine (10 mM) for 10 minutes. Surface-labelled cells were pelleted by centrifugation and resuspended in 202 μL HBSS (pH 7.4). An aliquot (2 μL) was taken prior to and after labelling to check for labelling efficiency using flow cytometry.

2.6. Membrane Protein Enrichment. Proteins were isolated and fractionated using a phase separation kit (Mem-PER, Pierce, Rockford, IL, USA) according to the manufacturer's instructions. Briefly, 150 μL reagent A containing 1 μL protease inhibitor (Sigma) was added to cell pellets containing PDLSC or GF. Following 10-minute incubation, 450 μL mixture of reagents B and C was added to cell lysates and tubes were kept on ice for 30 minutes. The preparation was centrifuged at 10,000 ×g for 3 minutes, at 4°C, and the supernatant was incubated at 37°C for 20 minutes. Following centrifugation at 10,000 ×g and phase separation, the hydrophobic fraction containing membrane proteins was carefully removed and purified using ReadyPrep 2-D Cleanup Kit. Membrane protein enrichment efficiency was assessed and cells were subjected to up to three membrane fractionation steps.

2.7. Membrane Protein Separation by Two-Dimensional Gel Electrophoresis (2DE). Membrane proteins were solubilised with ReadyPrep reagent 3 buffer for 1 hour at room temperature. The protein was solubilised by gentle aspiration through a fine-gauge needle, as previously described by Zilm et al. [21]. The protein concentration was determined using RC/DC Protein Assay Kit according to the manufacturer's instructions. Proteins were separated in the first dimension using 11 cm immobilised pH gradient (IPG) strips (pH 3–10) which had been passively rehydrated for 24 hours in 330 μL rehydration/extraction buffer #3, containing 0.2% (w/v) pH 3–10 ampholytes and 1.2% (v/v) De-Streak Reagent (GE Healthcare, Buckinghamshire, UK). IEF was performed using a Protean IEF cell. Briefly, membrane protein preparations containing 150 μg protein were cup-loaded onto the anode end of the IPG strip. The IEF cycle consisted of 8 steps outlined in Table 1, with a 50 μA/strip current limit, and the temperature was maintained at 20°C. Duplicate IPG strips were run concurrently. Following IEF, the IPG strips were equilibrated as previously described [22]. Polyacrylamide gels (18 × 18 cm) containing 8% T, 3.3% C, 0.1% SDS, and 375 mM Tris/HCl (pH 8.8) were cast without stacking gels using a Protean II XL casting chamber. Proteins were separated in the second dimension using a Protean II XL Multicell (Bio-Rad Laboratories) in tris-glycine tank buffer (25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS) and resolved at 7 mA/gel.

Table 1: IEF conditions for 11 cm IPG (3–10).

Step number	Voltage	Voltage ramping mode	Time
Step 1	150 V	Linear	1 hour
Step 2	300 V	Linear	2.30 hours
Step 3	600 V	Linear	1.50 hours
Step 4	1200 V	Linear	1.50 hours
Step 5	4000 V	Slow	1.50 hours
Step 6	8000 V	Slow	1 hour
Step 7	8000 V	Linear	30000 volt-hours
Step 8	500 V	Slow	0.15 hours

2.8. Gel Visualisation. Gels were scanned using a Typhoon Trio Variable Mode Imager (Molecular Dynamics Inc., Sunnyvale, CA) with a pixel resolution of 100 μm at the CyDye excitation and emission wavelengths described by the manufacturer. Image analysis was performed using PDQuest software (version 7.2, Bio-Rad Laboratories). Replicate groups, each containing four gels, were used for analysis. Protein spots were automatically detected and manually edited. Gel staining was normalized using the total density in gels.

2.9. Flamingo Fluorescent Staining. To visualise all proteins, gels were fixed in 40% ethanol (v/v)/10% acetic acid (v/v) in Milli-Q water and stained with Flamingo Fluorescent Stain (Bio-Rad Laboratories) according to the manufacturer's instructions. Gels were destained in 0.1% (v/v) Tween 20 in Milli-Q water for 10 minutes prior to imaging. Gels were scanned using a Typhoon Trio Variable Mode Imager using a green laser (532 nm) excitation source and 610 ± 30 nm bandpass emission filter.

2.10. Automated Spot Picking. Gel images were scanned using a Typhoon Trio Variable Mode Imager and imported into DeCyder software (version 6.5, GE Healthcare) and spots were detected using the automated method. Spots of interest were selected to generate a pick-list. The pick-list was exported from DeCyder and imported into Spot Picker software (version 1.2, GE Healthcare). Spots were excised using the Ettan Spot Cutting Robot (GE Healthcare) according to the manufacturer's instructions. Gel plugs were washed twice with 0.1 M ammonium bicarbonate buffer (NH_4HCO_3), followed by Milli-Q water, then dehydrated in acetonitrile (ACN), and dried.

2.11. Protein Identification by Liquid Chromatography-Electrospray Ionisation-Ion Trap (LC-ESI-IT) Mass Spectrometry (MS). Each gel plug was digested with 10 μL of 5 mM ammonium bicarbonate with 10% ACN containing 100 ng trypsin (Promega) for 16 hours at 37°C. Peptides were extracted sequentially with 1% formic acid (FA), 50% ACN/0.1% FA, and ACN, and the combined extracts were concentrated by centrifugal evaporation and diluted in 6 μL 3% ACN/0.1% FA. Vacuum concentrated samples were resuspended in 0.1% FA in 2% ACN to a total volume of ~8 μL. LC-ESI-IT MS/MS was performed using an online 1100 series HPLC system (Agilent Technologies) and HCT Ultra 3D-Ion Trap mass spectrometer (Bruker Daltonics). The LC system was interfaced to the MS using an Agilent Technologies Chip Cube operating with a ProtID-Chip-150 (II), which integrates the enrichment column (Zorbax 300SB-C18, 4 mm, 40 nL), analytical column (Zorbax 300 SB-C18, 150 mm × 75 μm), and nanospray emitter. 5 μL samples were loaded onto the enrichment column set at a flow rate of 4 μL/min in Mobile Phase A (0.1% FA in 2% v/v ACN) and resolved with 1–30% gradient of Mobile Phase B (0.1% FA in 98% w/v ACN) over 32 minutes at 300 nL/min. Ionizable species ($300 < m/z < 3{,}000$) were trapped and the two most intense ions eluting at the time were fragmented by collision-induced dissociation. Active exclusion was used to exclude a precursor ion for 30 seconds following the acquisition of two spectra.

2.12. Protein Identification Using Web-Based Bioinformatics Tools. MS and MS/MS spectra were subjected to peak detection and deconvolution using Data Analysis (version 3.4, Bruker Daltonics, Billerica, MA, USA). Compound lists were exported into BioTools (version 3.1, Bruker Daltonics) and then submitted to Mascot (version 2.2, Boston, MA, USA) using the following parameters: fixed modification = carbamidomethyl (C), variable modification = oxidation (M), MS mass tolerance = 1.5 Da, MS/MS mass tolerance = 0.8 Da, peptide charge = 1+, 2+, or 3+, and missed cleavages = 3. Data were matched to the Swiss-Prot protein database.

3. Results

3.1. Membrane Protein Expression of Ex Vivo Expanded Human PDLSC. CyDye-tagged membrane-associated proteins derived from human PDLSC following *ex vivo* expansion were separated by 2-dimensional electrophoresis. Based on the CyDye imaging, a total of 80 well-resolved proteins spots with a molecular weight range of 10–110 kDa were detected after automatic exclusion of pseudospots and the locations of the identified protein spots on the representative raw image are shown in Figure 1.

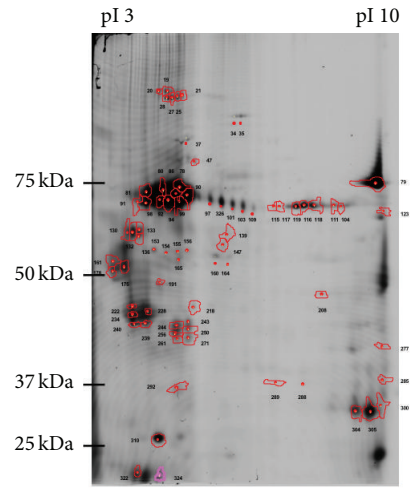


Figure 1: Representative raw 2DE gel of CyDye labelled proteins of *ex vivo* expanded human periodontal ligament stem cells and the location of proteins identified on the raw 2DE image. Following cell surface labelling with CyDye and membrane protein enrichment, proteins were separated by 2DE using a pI range of pH 3–10 and a molecular weight range of 10–110 kDa. Following image analysis, 80 well-resolved protein spots were detected. A total of 32 membrane-associated protein spots were consistently found on replicate gels.

3.2. Identification of Proteins Expressed by Human PDLSC. Following spot excision and analysis by mass spectrometry, a total of 32 protein spots were identified as membrane-associated proteins (Figure 1). Table 2 outlines the details of the membrane-associated proteins, including the protein name, spot number, predicted molecular weight and pI values, ID/total queries, combined ion scores, and coverage. Some proteins were identified in multiple spots (e.g., 5′-nucleotidase, Annexin A2, and sphingosine kinase 1) suggesting the presence of isoforms, possibly as a result of posttranslational modifications. Differences in the observed molecular weight/pI and the expected values were observed in some proteins (e.g., sphingosine kinase 1), possibly due to posttranslational modifications, proteolysis, or protein aggregation. Importantly, this approach was validated by the identification of MSC-associated stem cell surface proteins, 5′-nucleotidase (CD73) and Thy-1 membrane glycoprotein (CD90), previously shown to be expressed by PDLSC [8]. Furthermore, MS identified other membrane-associated markers, such as Annexin A2 and sphingosine kinase 1, the expression of which had not previously been reported by human PDLSC. All four proteins were chosen for further confirmatory analyses.

3.3. Validation of the Expression of 5′-Nucleotidase, Thy-1 Membrane Glycoprotein, Annexin A2, and Sphingosine Kinase 1. To confirm the expression of selected proteins including

Table 2: Membrane-associated proteins on human PDLSC.

Swiss-Prot 57.7 Accession number	Protein name	Spot number	Predicted MW (kDa)/pI	ID/total queries	Combined ion scores	Coverage (%)
1A01_HUMAN	HLA class I histocompatibility antigen, A-1 alpha chain	243	41.1/6.1	2/612	66	10
1C06_HUMAN	HLA class I histocompatibility antigen, Cw-6 alpha chain	239	41.4/5.7	4/638	175	18
5NTD_HUMAN	5′-Nucleotidase	78	63.9/6.6	13/518	287	17
		80	63.9/6.6	8/513	206	11
		90	63.9/6.6	41/546	872	32
		99	63.9/6.6	13/535	191	15
		101	63.9/6.6	39/517	945	38
		103	63.9/6.6	42/539	933	33
		109	63.9/6.6	25/536	606	22
		111	63.9/6.6	2/558	34	6
		326	63.9/6.6	53/544	961	35
		86	63.9/6.6	25/356	521	31
		97	63.9/6.6	45/414	1044	39
AMPB_HUMAN	Aminopeptidase B	94	73.2/5.5	5/559	172	10
ANXA2_HUMAN	Annexin A2	285	38.8/7.6	36/581	489	47
		288	38.8/7.6	87/585	1907	67
		289	38.8/7.6	56/600	950	55
		292	38.8/7.6	3/594	76	11
		304	38.8/7.6	14/536	241	28
CAP2_HUMAN	Adenylyl cyclase-associated protein 2	154	53.1/6.0	2/576	67	6
		156	53.1/6.0	4/557	102	8
		165	53.1/6.0	3/619	102	11
CO1A1_HUMAN	Collagen alpha-1(I) chain	292	139.9/5.6	7/594	103	4
CO6A3_HUMAN	Collagen alpha-3(VI) chain	20	345.2/6.3	9/568	159	3
DNJA1_HUMAN	DnaJ homolog subfamily A member 1	208	45.6/6.6	4/521	95	12
EHD3_HUMAN	EH domain-containing protein 3	139	62.0/6.1	4/600	71	8
EZRI_HUMAN	Ezrin	65	69.5/5.9	28/558	455	26
FLNC_HUMAN	Filamin-C	29	293.4/5.6	4/492	49	2
		78	293.4/5.6	3/518	65	1
		139	293.4/5.6	14/600	363	6
		147	293.4/5.6	13/525	237	4
		191	293.4/5.6	4/586	147	2
GELS_HUMAN	Gelsolin	46	86.0/5.9	46/636	1011	35
K2C1_HUMAN	Keratin, type II cytoskeletal 1	25	66.2/8.2	5/578	128	9
		46	66.2/8.2	11/636	456	17
		47	66.2/8.2	7/491	170	7
		80	66.2/8.2	11/513	227	13
		91	66.2/8.2	3/666	94	12
		94	66.2/8.2	4/559	146	8
		98	66.2/8.2	2/639	57	7
		104	66.2/8.2	2/520	80	3
		111	66.2/8.2	5/558	79	10
		130	66.2/8.2	3/614	60	8
		147	66.2/8.2	20/525	256	13
		153	66.2/8.2	2/615	126	7
		154	66.2/8.2	3/576	107	8
		228	66.2/8.2	10/664	466	22
		289	66.2/8.2	14/600	386	15
		292	66.2/8.2	2/594	55	3
		324	66.2/8.2	5/570	127	10

Table 2: Continued.

Swiss-Prot 57.7 Accession number	Protein name	Spot number	Predicted MW (kDa)/pI	ID/total queries	Combined ion scores	Coverage (%)
KAP2_HUMAN	cAMP-dependent protein kinase type II-alpha regulatory subunit	178	45.8/5.0	13/621	360	22
NOMO2_HUMAN	Nodal modulator 2	19	140.4/5.5	9/599	244	8
		20	140.4/5.5	10/568	189	12
		25	140.4/5.5	10/578	226	8
		27	140.4/5.5	11/583	224	10
		28	140.4/5.5	11/436	244	12
NUCB2_HUMAN	Nucleobindin-2	178	50.3/5.0	3/621	100	5
PDIA6_HUMAN	Protein disulfide-isomerase A6	175	48.5/5.0	6/592	209	12
		178	48.5/5.0	7/621	278	10
RUVB2_HUMAN	RuvB-like 2	191	51.3/5.5	25/586	614	34
SBP1_HUMAN	Selenium-binding protein 1	165	52.9/5.9	7/619	126	8
SNX4_HUMAN	Sorting nexin-4	155	52.2/5.7	5/637	157	14
SPHK1_HUMAN	Sphingosine kinase 1	19	42.9/6.6	12/599	253	18
		20	42.9/6.6	5/568	188	16
		21	42.9/6.6	4/518	138	11
		34	42.9/6.6	3/517	200	11
STML2_HUMAN	Stomatin-like protein 2	239	38.6/6.9	13/638	584	37
		240	38.6/6.9	6/656	266	27
STXB3_HUMAN	Syntaxin-binding protein 3	111	68.6/8.0	5/558	97	7
		118	68.6/8.0	6/479	103	7
SWP70_HUMAN	Switch-associated protein 70	94	69.4/5.7	3/559	80	5
		99	69.4/5.7	6/535	136	7
THY1_HUMAN	Thy-1 membrane glycoprotein	304	18.2/9.0	3/536	55	16
UBP14_HUMAN	Ubiquitin carboxyl-terminal hydrolase 14	130	56.5/5.2	6/614	176	15
		132	56.5/5.2	8/611	184	18
		133	56.5/5.2	3/635	100	5
		136	56.5/5.2	15/642	372	24
ULA1_HUMAN	NEDD8-activating enzyme E1 regulatory subunit	133	60.7/5.2	6/635	127	8
VATB2_HUMAN	V-type proton ATPase subunit B, brain isoform	153	56.8/5.6	4/615	191	12
		154	56.8/5.6	9/576	252	14
		155	56.8/5.6	7/637	300	16
VDAC1_HUMAN	Voltage-dependent anion-selective channel protein 1	300	30.9/8.6	7/601	128	18
		304	30.9/8.6	34/536	902	59
		305	30.9/8.6	19/524	319	38
VIME_HUMAN	Vimentin	130	53.7/5.1	2/614	68	6
		161	53.7/5.1	28/626	710	46
		175	53.7/5.1	44/592	1026	63
		178	53.7/5.1	46/621	1306	58
		222	53.7/5.1	3/630	87	6
		228	53.7/5.1	5/664	139	9
		239	53.7/5.1	4/638	139	9
		240	53.7/5.1	5/656	169	12
VINC_HUMAN	Vinculin	25	124.3/5.5	14/578	226	16
		27	124.3/5.5	30/583	643	25

CD73, CD90, Annexin A2, and sphingosine kinase 1 (SPK1), additional studies were performed to investigate their expression in human PDLSC, GF, and keratinocytes (epithelial cell population). Flow cytometric analysis demonstrated high surface expression of CD73 and CD90 and low cell surface levels of Annexin A2 expression in human PDLSC and GF populations (Figure 2). In contrast, human keratinocytes showed a lack of cell surface expression for CD73, CD90, and Annexin A2 (Figure 2). Table 3 summarizes levels of surface expression of these four antigens on assessed cell types. In summary, CD73 and CD90 were expressed by human PDLSC and GF, but not by human keratinocytes, confirming that they are MSC-associated markers. Annexin A2 was demonstrated to be expressed at low levels by human

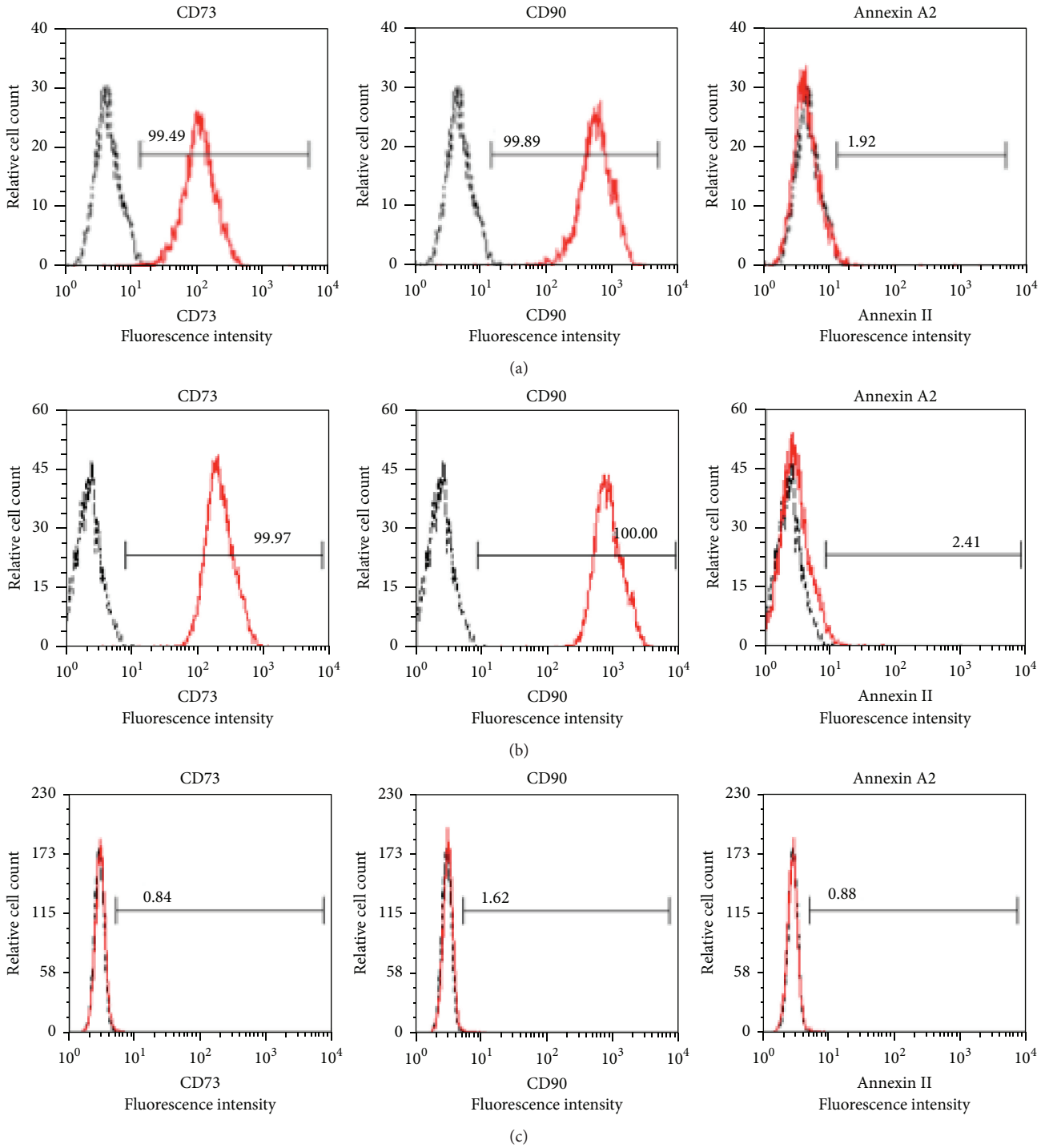


Figure 2: Validation of surface expression of CD73, CD90, and Annexin A2 using flow cytometric analysis in human (a) periodontal ligament stem cells, (b) gingival fibroblasts, and (c) skin keratinocytes.

PDLSC (1.92–7.83%) and human GF (2.41–4.66%), while human keratinocytes were largely negative for Annexin A2 expression (0.88–1.64%). Previous studies have shown that SPK1 can translocate to the plasma membrane upon cell stimulation by cytokines [23–29]. No positive expression was detected with the anti-SPK1 antibody to the human cell types by flow cytometric analysis (data not shown), most likely because the available antibody reagent did not react with the extracellular domain of SPK1.

Additional studies were performed to investigate the expression of Annexin A2 and SPK1 in human PDLSC, GF, and keratinocytes, using immunocytochemistry. All cell types studied were positive to anti-Annexin A2 and anti-SPK1 antibodies (Figure 3). Of note, no reactivity was observed

Table 3: Flow cytometric analysis of cell surface expression of CD73, CD90, and Annexin A2. Data represent median% (range); $n = 3$ replicate experiments.

Antigen	PDLSC	GF	Keratinocytes
CD73	**99.90** (99.4–99.9)	**99.97** (99.9–100)	**0.82** (0.78–0.84)
CD90	**99.98** (99.8–100)	**99.98** (99.9–100)	**1.58** (1.55–1.62)
Annexin A2	**4.48** (1.92–7.83)	**2.92** (2.41–4.66)	**0.86** (0.86–1.64)

with the anti-CD73 or anti-CD90 antibodies to all cell types examined (data not shown), indicating that the specific epitopes identified by these antibodies were compromised following processing for immunocytochemical analysis.

4. Discussion

Initially, the first proteomic reference map of undifferentiated periodontal ligament fibroblasts identified 117 proteins, consistently expressed across three clones, which included a variety of expected cytoskeleton- and metabolism-related proteins [30]. This comparative analysis of the proteome revealed that the percentage of total cytoskeleton-related proteins identified in periodontal ligament fibroblasts (26.5%) was higher than that in dermal fibroblasts (15%). It was proposed that this difference is assigned to mechanical loading and rapid remodelling associated with periodontal ligament tissue [30].

Assessment of protein expression during differentiation of PDLSC identified 29 proteins, differentially expressed during early cementoblastic/osteogenic differentiation [31], and demonstrated a reduction in expression of cytoskeletal proteins and their binding partners, potentially attributed to cytoskeletal rearrangements during differentiation processes [32]. Interestingly, higher expression of the calcium-binding protein Annexin A4 was noted following osteogenic differentiation. Annexins are thought to play an important role in osteogenic development including Annexin A2 and Annexin A5 which are highly expressed in skeletal tissues and upregulated in osteogenic cultures of MSC [33, 34].

A direct comparison of protein expression profiles between ovine PDLSC, DPSC, and BMSC identified 58 differentially expressed proteins between at least two MSC populations, with the expression of 6 proteins upregulated in PDLSC relative to both DPSC and BMSC, 5 proteins upregulated in DPSC relative to both PDLSC and BMSC, and 1 protein upregulated in BMSC relative to both PDLSC and DPSC [35]. An increase in PDLSC expression of heat-shock protein beta 1, Annexin A3, and Annexin A4 compared to DPSC and BMSC was thought to relate to high turnover of periodontal tissues.

The aim of the present study was to determine the surface expression profile of human PDLSC and to compare the expression of prospective cell surface markers in human PDLSC, GF, and keratinocytes (as a source of epithelial cells). Our findings identified 80 proteins expressed on the surface of human PDLSC, 32 of which were membrane associated and four of which were selected for further validation due to their known association with other MSC-like populations as a proof-of-principal analysis. These include CD73 and CD90, well known MSC-associated markers, and Annexin A2 and SPK1. Annexin A2 is calcium dependent [36–42] and has been reported to be associated with the stem cell niche [43–49] and SPK1 has recently been demonstrated to be associated with the progenitor phenotype of endothelial cells [50]. CD73 and CD90 were highly expressed by human PDLSC and GF but not by human keratinocytes, indicating that these antigens could be used as potential markers for distinguishing mesenchymal from epithelial cell populations. Annexin A2 was demonstratively expressed at the cell surface at low copy number by human PDLSC and GF, using flow cytometric analysis, while human keratinocytes lacked any cell surface expression of Annexin A2. Expression of SPK1 was detected in all analysed cell types using immunocytochemical analysis.

CD73, originally defined as a lymphocyte differentiation antigen, functions as a cosignalling molecule on T lymphocytes and is required for lymphocyte binding to endothelium [51]. Expression of CD73 has been demonstrated on various cell types including lymphocytes, endothelial cells, and MSC. It is thought to play physiological roles in epithelial ion and fluid transport, maintaining barrier functions, mediating endothelial permeability, adapting to hypoxia, and contributing to microbial responses [52]. CD73 is an extracellular enzyme that catalyzes the formation of immunosuppressive adenosine by converting adenosine 5′-monophosphate (AMP) to its bioactive intermediate, adenosine, which in turn activates adenosine receptors, when released into the extracellular space, and regulates various physiological functions [52, 53]. Adenosine signalling, modulated by CD39 and CD73 expression, has been highlighted as a novel modulator in the immunosuppression of T-cell proliferation by MSC [54, 55]. As such, this may be contributory to immunomodulatory properties of PDLSC [8] and may highlight an avenue for anti-inflammatory therapy in periodontal disease [4, 56].

The expression of CD90 on PDLSC has been well documented [56–59]; however, its role in PDLSC function remains largely unknown. CD90, also known as Thy-1 (thymocyte differentiation antigen-1), is found to be expressed in various cell types such as hematopoietic stem/progenitor cells [60], hepatic stem cells in human fetal liver [61], liver cancer stem cells [62], neurons, fibroblasts, vascular pericytes, and MSC [63]. Its expression is developmentally regulated [64] and remains one of the minimal criteria for defining human MSC, proposed by the committee for the International Society for Cellular Therapy (ISCT) [63, 65]. While the biological role of CD90 is unclear, a number of associated immunological and nonimmunological functions have been previously addressed [64]. In addition to its involvement in T-cell activation [64], it is believed to be associated with many cellular processes and pathological conditions in a context-dependent manner [63], including cell-cell and cell-matrix interactions, cell motility, and thymocyte adhesion to epithelium [64]. Moreover, CD90 expression is also associated with fibroblast phenotypes relevant to wound healing and fibrosis. The differential

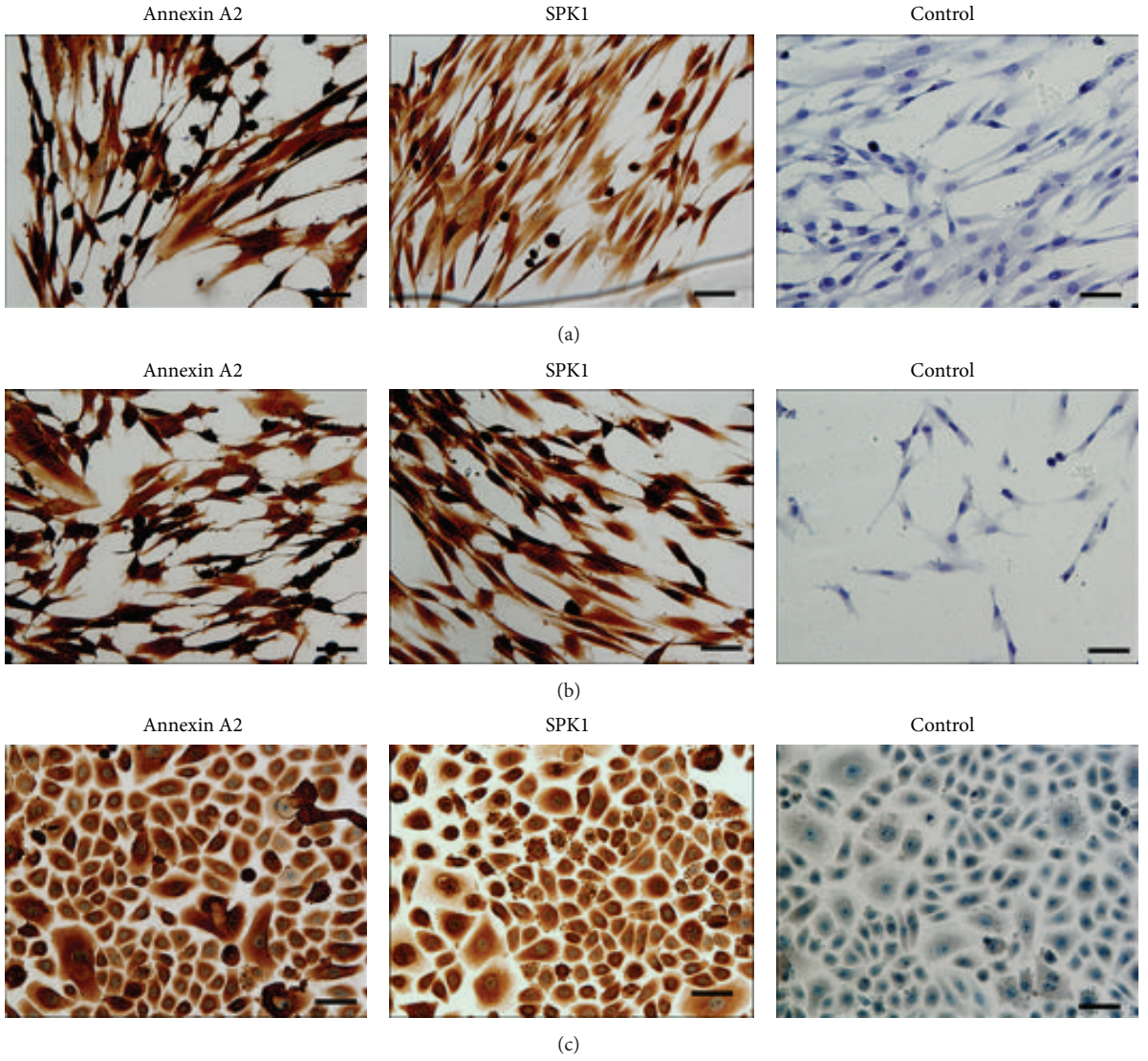


Figure 3: The expression of Annexin A2 and sphingosine kinase 1 (SPK1) in fixed and permeabilized (a) human periodontal ligament stem cells (PDLSC), (b) gingival fibroblasts (GF), and (c) keratinocytes using immunohistochemistry. All cell types studied were positive to anti-Annexin A2 and anti-SPK1 antibodies. Scale bar = 50 μm.

CD90 expression is associated with cell-extracellular matrix interactions and cell migration and, as such, is correlated with distinct cellular morphology [64].

Annexin A2, firstly identified as an intracellular protein, has since been found extracellularly both in secreted and in membrane bound form [66]. While Annexin A2 monomer is largely present in the cytoplasm, the formation of the heterotetramer allows its binding to the plasma membrane [67–70]. Potential roles of extracellular Annexin A2 include plasminogen activation, cell-cell adhesion, and immunoglobulin transport [66]. Increasing evidence has highlighted the roles of the Annexin family of calcium-dependent, phopholipid-binding proteins in the mineralization process [36–42] and found them to be highly expressed in calcifying cartilage and bone and to serve to initiate mineralization of extracellular matrix [71]. Previously, it has been suggested that Annexin members also have the capacity to function in a compensatory manner of each other during skeletal development [72]. In a study investigating intracellular processes involved in mineralization, the overexpression of Annexin A2 has been shown to increase ALP activity and cartilage and bone formation, while diminished Annexin A2 expression resulted in decreased mineralization [38]. Collectively, previous findings related to Annexins in dental tissues are consistent with their roles in support of osteogenic differentiation and formation of minerals [31, 33, 35, 73, 74].

We identified Annexin A2 as one of the cell surface proteins expressed by human PDLSC. Further flow cytometric analysis showed the surface expression of Annexin A2 at low copy number by human GF. As a member of the Annexin family which plays important roles in the mineralization process [36–42], the low expression of Annexin A2 in

human GF may be correlated to the fact that human GF demonstrated limited osteogenic potential when cultured in osteogenic conditions (data not shown). A recent study [75] suggested that Annexin A2 regulates adhesion, homing, and engraftment within stem cell niches at endosteal [43–47] and vascular [48, 49] sites; hence, we propose that it may be a potential marker of the PDLSC niche in periodontal tissues.

SPK1, the more characterised of the two SPK isoforms, enhances cell growth and proliferation and is involved in immune regulation and tumorigenesis [76]. This highly conserved lipid kinase catalyzes the phosphorylation of proapoptotic sphingosine to form antiapoptotic sphingosine-1-phosphate (S1P) [77] and, as such, is an important cell fate determinant [78]. S1P is a key sphingolipid metabolite that regulates various physiological and pathological processes such as cell proliferation, differentiation, apoptosis, migration, invasion, and angiogenesis [78] and is thought to promote cell growth and proliferation and suppresses apoptosis [79]. In addition to their roles in regulating cell proliferation and apoptosis, SPK-S1P-S1P receptors have been shown to be involved in immune regulation such as immune cell trafficking, activation, and T-cell differentiation [77].

Multiple studies have demonstrated that SPK and S1P play important roles in the maintenance of stem cells [76] including endothelial progenitor cells, rate of endothelial progenitor cell differentiation [50, 80, 81], neural progenitors [82], human embryonic stem cells [76, 83], hematopoietic stem cell [76], and muscle progenitors [84]. Furthermore, SPK1 is a maker of oncogenic potential, tumour progression, and cancer prognosis in numerous tissue types [85, 86]. It is predominantly a cytosolic enzyme, which lacks an obvious membrane anchoring sequence. However, considerable evidence has suggested that SPK1 can be translocated to the plasma membrane upon cell stimulation by growth factors and cytokines [23–25, 27–29, 83]. The present study initially identified SPK1 in human PDLSC by proteomic analysis and SPK1 expression was demonstrated in all cell types examined by immunocytochemical analysis. However, we were unable to demonstrate the cell surface expression of this enzyme, limited by the availability of an SPK1 antibody suitable for flow cytometry.

5. Conclusion

In summary, this study is the first, to date, to investigate the cell surface proteome of *ex vivo* expanded human PDLSC. In addition to the expression of recognised MSC-associated cell surface antigens CD73 and CD90, PDLSC were also found to express two novel cell surface proteins, Annexin A2 and sphingosine kinase 1. Interestingly, previous studies have implicated CD73, CD90, Annexin A2, and sphingosine kinase 1 expression in the maintenance of various stem cell populations. Importantly, this study found that human skin epithelial cells lacked the expression of CD73, CD90, and Annexin A2. These proteomic findings provide the platform to further define the cell surface protein expression profile of PDLSC in order to further characterise this cell population and support development of novel isolation and purification strategies.

Disclosure

P. Mark Bartold and Stan Gronthos are co-senior authors.

Competing Interests

The authors declare that there are no competing interests regarding the publication of this paper.

Acknowledgments

This study was partly funded by NHMRC project Grant APP1043994, fellowship APP1042677, and ADRF Grant 37.2013.

References

[1] T. Graf and M. Stadtfeld, "Heterogeneity of embryonic and adult stem cells," *Cell Stem Cell*, vol. 3, no. 5, pp. 480–483, 2008.

[2] M. Owen and A. J. Friedenstein, "Stromal stem cells: marrow-derived osteogenic precursors," *Ciba Foundation Symposium*, vol. 136, pp. 42–60, 1988.

[3] S. Gronthos, A. C. W. Zannettino, S. E. Graves, S. Ohta, S. J. Hay, and P. J. Simmons, "Differential cell surface expression of the STRO-1 and alkaline phosphatase antigens on discrete developmental stages in primary cultures of human bone cells," *Journal of Bone and Mineral Research*, vol. 14, no. 1, pp. 47–56, 1999.

[4] B.-M. Seo, M. Miura, S. Gronthos et al., "Investigation of multipotent postnatal stem cells from human periodontal ligament," *The Lancet*, vol. 364, no. 9429, pp. 149–155, 2004.

[5] D. Menicanin, K. M. Mrozik, N. Wada et al., "Periodontal-ligament-derived stem cells exhibit the capacity for long-term survival, self-renewal, and regeneration of multiple tissue types in vivo," *Stem Cells and Development*, vol. 23, no. 9, pp. 1001–1011, 2014.

[6] S. C. Chen, V. Marino, S. Gronthos, and P. M. Bartold, "Location of putative stem cells in human periodontal ligament," *Journal of Periodontal Research*, vol. 41, no. 6, pp. 547–553, 2006.

[7] J. C. Rincon, W. G. Young, and P. M. Bartold, "The epithelial cell rests of Malassez—a role in periodontal regeneration?" *Journal of Periodontal Research*, vol. 41, no. 4, pp. 245–252, 2006.

[8] T. Iwata, M. Yamato, H. Tsuchioka et al., "Periodontal regeneration with multi-layered periodontal ligament-derived cell sheets in a canine model," *Biomaterials*, vol. 30, no. 14, pp. 2716–2723, 2009.

[9] P. M. Bartold, S. Shi, and S. Gronthos, "Stem cells and periodontal regeneration," *Periodontology 2000*, vol. 40, no. 1, pp. 164–172, 2006.

[10] C. A. G. McCulloch, "Progenitor cell populations in the periodontal ligament of mice," *Anatomical Record*, vol. 211, no. 3, pp. 258–262, 1985.

[11] C. A. G. McCulloch, E. Nemeth, B. Lowenberg, and A. H. Melcher, "Paravascular cells in endosteal spaces of alveolar bone contribute to periodontal ligament cell populations," *Anatomical Record*, vol. 219, no. 3, pp. 233–242, 1987.

[12] S. Yamada, S. Murakami, R. Matoba et al., "Expression profile of active genes in human periodontal ligament and isolation of PLAP-1, a novel SLRP family gene," *Gene*, vol. 275, no. 2, pp. 279–286, 2001.

[13] Y. Saito, T. Yoshizawa, F. Takizawa et al., "A cell line with characteristics of the periodontal ligament fibroblasts is negatively regulated for mineralization and Runx2/Cbfa1/Osf2 activity, part of which can be overcome by bone morphogenetic protein-2," *Journal of Cell Science*, vol. 115, no. 21, pp. 4191–4200, 2002.

[14] C. Morsczeck, G. Schmalz, T. E. Reichert, F. Völlner, K. Galler, and O. Driemel, "Somatic stem cells for regenerative dentistry," *Clinical Oral Investigations*, vol. 12, no. 2, pp. 113–118, 2008.

[15] C. A. McCulloch, "Proteomics for the periodontium: current strategies and future promise," *Periodontology 2000*, vol. 40, no. 1, pp. 173–183, 2006.

[16] Y. Zhang, T. Wang, W. Chen et al., "Differential protein expression by *Porphyromonas gingivalis* in response to secreted epithelial cell components," *Proteomics*, vol. 5, no. 1, pp. 198–211, 2005.

[17] Q. Xia, T. Wang, F. Taub et al., "Quantitative proteomics of intracellular *Porphyromonas gingivalis*," *Proteomics*, vol. 7, no. 23, pp. 4323–4337, 2007.

[18] P. D. Veith, G. H. Talbo, N. Slakeski, and E. C. Reynolds, "Identification of a novel heterodimeric outer membrane protein of *Porphyromonas gingivalis* by two-dimensional gel electrophoresis and peptide mass fingerprinting," *European Journal of Biochemistry*, vol. 268, no. 17, pp. 4748–4757, 2001.

[19] M. J. Somerman, S. Y. Archer, G. R. Imm, and R. A. Foster, "A comparative study of human periodontal ligament cells and gingival fibroblasts in vitro," *Journal of Dental Research*, vol. 67, no. 1, pp. 66–70, 1988.

[20] C. Mayrhofer, S. Krieger, G. Allmaier, and D. Kerjaschki, "DIGE compatible labelling of surface proteins on vital cells in vitro and in vivo," *Proteomics*, vol. 6, no. 2, pp. 579–585, 2006.

[21] P. S. Zilm, A. Mira, C. J. Bagley, and A. H. Rogers, "Effect of alkaline growth pH on the expression of cell envelope proteins in *Fusobacterium nucleatum*," *Microbiology*, vol. 156, part 6, pp. 1783–1794, 2010.

[22] A. Gorg, G. Boguth, C. Obermaier, and W. Weiss, "Two-dimensional electrophoresis of proteins in an immobilized pH 4-12 gradient," *Electrophoresis*, vol. 19, no. 8-9, pp. 1516–1519, 1998.

[23] N. Ancellin, C. Colmont, J. Su et al., "Extracellular export of sphingosine kinase-1 enzyme. Sphingosine 1-phosphate generation and the induction of angiogenic vascular maturation," *The Journal of Biological Chemistry*, vol. 277, no. 8, pp. 6667–6675, 2002.

[24] J. A. Hengst, J. M. Guilford, T. E. Fox, X. Wang, E. J. Conroy, and J. K. Yun, "Sphingosine kinase 1 localized to the plasma membrane lipid raft microdomain overcomes serum deprivation induced growth inhibition," *Archives of Biochemistry and Biophysics*, vol. 492, no. 1-2, pp. 62–73, 2009.

[25] K. E. Jarman, P. A. B. Moretti, J. R. Zebol, and S. M. Pitson, "Translocation of sphingosine kinase 1 to the plasma membrane is mediated by calcium- and integrin-binding protein 1," *Journal of Biological Chemistry*, vol. 285, no. 1, pp. 483–492, 2010.

[26] S. M. Pitson, P. Xia, T. M. Leclercq et al., "Phosphorylation-dependent translocation of sphingosine kinase to the plasma membrane drives its oncogenic signalling," *The Journal of Experimental Medicine*, vol. 201, no. 1, pp. 49–54, 2005.

[27] R. V. Stahelin, J. H. Hwang, J.-H. Kim et al., "The mechanism of membrane targeting of human sphingosine kinase 1," *The Journal of Biological Chemistry*, vol. 280, no. 52, pp. 43030–43038, 2005.

[28] B. W. Wattenberg, "Role of sphingosine kinase localization in sphingolipid signaling," *World Journal of Biological Chemistry*, vol. 1, no. 12, pp. 362–368, 2010.

[29] K. W. Young, J. M. Willets, M. J. Parkinson et al., "Ca^{2+}/calmodulin-dependent translocation of sphingosine kinase: role in plasma membrane relocation but not activation," *Cell Calcium*, vol. 33, no. 2, pp. 119–128, 2003.

[30] E. Reichenberg, M. Redlich, P. Cancemi et al., "Proteomic analysis of protein components in periodontal ligament fibroblasts," *Journal of Periodontology*, vol. 76, no. 10, pp. 1645–1653, 2005.

[31] L. Wu, X. Wei, J. Ling et al., "Early osteogenic differential protein profile detected by proteomic analysis in human periodontal ligament cells," *Journal of Periodontal Research*, vol. 44, no. 5, pp. 645–656, 2009.

[32] L. J. Foster, P. A. Zeemann, C. Li, M. Mann, O. N. Jensen, and M. Kassem, "Differential expression profiling of membrane proteins by quantitative proteomics in a human mesenchymal stem cell line undergoing osteoblast differentiation," *Stem Cells*, vol. 23, no. 9, pp. 1367–1377, 2005.

[33] A.-X. Zhang, W.-H. Yu, B.-F. Ma et al., "Proteomic identification of differently expressed proteins responsible for osteoblast differentiation from human mesenchymal stem cells," *Molecular and Cellular Biochemistry*, vol. 304, no. 1-2, pp. 167–179, 2007.

[34] H. J. Sun, Y. Y. Bahk, Y. R. Choi, J. H. Shim, S. H. Han, and J. W. Lee, "A proteomic analysis during serial subculture and osteogenic differentiation of human mesenchymal stem cell," *Journal of Orthopaedic Research*, vol. 24, no. 11, pp. 2059–2071, 2006.

[35] K. M. Mrozik, P. S. Zilm, C. J. Bagley et al., "Proteomic characterization of mesenchymal stem cell-like populations derived from ovine periodontal ligament, dental pulp, and bone marrow: analysis of differentially expressed proteins," *Stem Cells and Development*, vol. 19, no. 10, pp. 1485–1499, 2010.

[36] M. Balcerzak, E. Hamade, L. Zhang et al., "The roles of annexins and alkaline phosphatase in mineralization process," *Acta Biochimica Polonica*, vol. 50, no. 4, pp. 1019–1038, 2003.

[37] D. C. Genetos, A. Wong, S. Watari, and C. E. Yellowley, "Hypoxia increases Annexin A2 expression in osteoblastic cells via VEGF and ERK," *Bone*, vol. 47, no. 6, pp. 1013–1019, 2010.

[38] J. M. Gillette and S. M. Nielsen-Preiss, "The role of annexin 2 in osteoblastic mineralization," *Journal of Cell Science*, vol. 117, no. 3, pp. 441–449, 2004.

[39] T. L. Haut Donahue, D. C. Genetos, C. R. Jacobs, H. J. Donahue, and C. E. Yellowley, "Annexin V disruption impairs mechanically induced calcium signaling in osteoblastic cells," *Bone*, vol. 35, no. 3, pp. 656–663, 2004.

[40] T. Kirsch, B. Swoboda, and H.-D. Nah, "Activation of annexin II and V expression, terminal differentiation, mineralization and apoptosis in human osteoarthritic cartilage," *Osteoarthritis and Cartilage*, vol. 8, no. 4, pp. 294–302, 2000.

[41] D. Pfander, B. Swoboda, and T. Kirsch, "Expression of early and late differentiation markers (proliferating cell nuclear antigen, syndecan-3, annexin VI, and alkaline phosphatase) by human osteoarthritic chondrocytes," *American Journal of Pathology*, vol. 159, no. 5, pp. 1777–1783, 2001.

[42] W. Wang and T. Kirsch, "Retinoic acid stimulates annexin-mediated growth plate chondrocyte mineralization," *The Journal of Cell Biology*, vol. 157, no. 6, pp. 1061–1069, 2002.

[43] J. Zhang, C. Niu, L. Ye et al., "Identification of the haematopoietic stem cell niche and control of the niche size," *Nature*, vol. 425, no. 6960, pp. 836–841, 2003.

[44] S. Stier, Y. Ko, R. Forkert et al., "Osteopontin is a hematopoietic stem cell niche component that negatively regulates stem cell pool size," *The Journal of Experimental Medicine*, vol. 201, no. 11, pp. 1781–1791, 2005.

[45] S. K. Nilsson, H. M. Johnston, G. A. Whitty et al., "Osteopontin, a key component of the hematopoietic stem cell niche and regulator of primitive hematopoietic progenitor cells," *Blood*, vol. 106, no. 4, pp. 1232–1239, 2005.

[46] L. M. Calvi, G. B. Adams, K. W. Weibrecht et al., "Osteoblastic cells regulate the haematopoietic stem cell niche," *Nature*, vol. 425, no. 6960, pp. 841–846, 2003.

[47] F. Arai, A. Hirao, M. Ohmura et al., "Tie2/angiopoietin-1 signaling regulates hematopoietic stem cell quiescence in the bone marrow niche," *Cell*, vol. 118, no. 2, pp. 149–161, 2004.

[48] S. T. Avecilla, K. Hattori, B. Heissig et al., "Chemokine-mediated interaction of hematopoietic progenitors with the bone marrow vascular niche is required for thrombopoiesis," *Nature Medicine*, vol. 10, no. 1, pp. 64–71, 2004.

[49] M. J. Kiel, Ö. H. Yilmaz, T. Iwashita, O. H. Yilmaz, C. Terhorst, and S. J. Morrison, "SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells," *Cell*, vol. 121, no. 7, pp. 1109–1121, 2005.

[50] J. M. Barrett, K. A. Parham, J. B. Pippal et al., "Over-expression of sphingosine kinase-1 enhances a progenitor phenotype in human endothelial cells," *Microcirculation*, vol. 18, no. 7, pp. 583–597, 2011.

[51] B. Zhang, "CD73: a novel target for cancer immunotherapy," *Cancer Research*, vol. 70, no. 16, pp. 6407–6411, 2010.

[52] S. P. Colgan, H. K. Eltzschig, T. Eckle, and L. F. Thompson, "Physiological roles for ecto-5′-nucleotidase (CD73)," *Purinergic Signalling*, vol. 2, no. 2, pp. 351–360, 2006.

[53] P. A. Beavis, J. Stagg, P. K. Darcy, and M. J. Smyth, "CD73: a potent suppressor of antitumor immune responses," *Trends in Immunology*, vol. 33, no. 5, pp. 231–237, 2012.

[54] C. Sattler, M. Steinsdoerfer, M. Offers et al., "Inhibition of T-cell proliferation by murine multipotent mesenchymal stromal cells is mediated by CD39 expression and adenosine generation," *Cell Transplantation*, vol. 20, no. 8, pp. 1221–1230, 2011.

[55] F. Saldanha-Araujo, F. I. S. Ferreira, P. V. Palma et al., "Mesenchymal stromal cells up-regulate CD39 and increase adenosine production to suppress activated T-lymphocytes," *Stem Cell Research*, vol. 7, no. 1, pp. 66–74, 2011.

[56] N. Wada, D. Menicanin, S. Shi, P. M. Bartold, and S. Gronthos, "Immunomodulatory properties of human periodontal ligament stem cells," *Journal of Cellular Physiology*, vol. 219, no. 3, pp. 667–676, 2009.

[57] T. Kato, K. Hattori, T. Deguchi et al., "Osteogenic potential of rat stromal cells derived from periodontal ligament," *Journal of Tissue Engineering and Regenerative Medicine*, vol. 5, no. 10, pp. 798–805, 2011.

[58] A. Tomokiyo, H. Maeda, S. Fujii et al., "A multipotent clonal human periodontal ligament cell line with neural crest cell phenotypes promotes neurocytic differentiation, migration, and survival," *Journal of Cellular Physiology*, vol. 227, no. 5, pp. 2040–2050, 2012.

[59] O. Trubiani, S. F. Zalzal, R. Paganelli et al., "Expression profile of the embryonic markers nanog, OCT-4, SSEA-1, SSEA-4, and frizzled-9 receptor in human periodontal ligament mesenchymal stem cells," *Journal of Cellular Physiology*, vol. 225, no. 1, pp. 123–131, 2010.

[60] C. M. Baum, I. L. Weissman, A. S. Tsukamoto, A.-M. Buckle, and B. Peault, "Isolation of a candidate human hematopoietic stem-cell population," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 89, no. 7, pp. 2804–2808, 1992.

[61] N. M. Masson, I. S. Currie, J. D. Terrace, O. J. Garden, R. W. Parks, and J. A. Ross, "Hepatic progenitor cells in human fetal liver express the oval cell marker Thy-1," *American Journal of Physiology—Gastrointestinal and Liver Physiology*, vol. 291, no. 1, pp. G45–G54, 2006.

[62] Z. F. Yang, D. W. Ho, M. N. Ng et al., "Significance of CD90+ cancer stem cells in human liver cancer," *Cancer Cell*, vol. 13, no. 2, pp. 153–166, 2008.

[63] J. E. Bradley, G. Ramirez, and J. S. Hagood, "Roles and regulation of Thy-1, a context-dependent modulator of cell phenotype," *BioFactors*, vol. 35, no. 3, pp. 258–265, 2009.

[64] S. M. M. Haeryfar and D. W. Hoskin, "Thy-1: more than a mouse pan-T cell marker," *Journal of Immunology*, vol. 173, no. 6, pp. 3581–3588, 2004.

[65] M. Dominici, K. Le Blanc, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.

[66] D. A. Siever and H. P. Erickson, "Extracellular annexin II," *International Journal of Biochemistry and Cell Biology*, vol. 29, no. 11, pp. 1219–1223, 1997.

[67] N. Zobiack, V. Gerke, and U. Rescher, "Complex formation and submembranous localization of annexin 2 and S100A10 in live HepG2 cells," *FEBS Letters*, vol. 500, no. 3, pp. 137–140, 2001.

[68] J. R. Glenney Jr., "Co-precipitation of intestinal p36 with a 73K protein and a high molecular weight factor," *Experimental Cell Research*, vol. 162, no. 1, pp. 183–190, 1986.

[69] V. Gerke and K. Weber, "Identity of p36K phosphorylated upon *Rous sarcoma* virus transformation with a protein purified from brush borders; calcium-dependent binding to non-erythroid spectrin and F-actin," *The EMBO Journal*, vol. 3, no. 1, pp. 227–233, 1984.

[70] C. Thiel, M. Osborn, and V. Gerke, "The tight association of the tyrosine kinase substrate annexin II with the submembranous cytoskeleton depends on intact p11- and Ca2+-binding sites," *Journal of Cell Science*, vol. 103, no. 3, pp. 733–742, 1992.

[71] H. C. Anderson, "Molecular biology of matrix vesicles," *Clinical Orthopaedics and Related Research*, no. 314, pp. 266–280, 1995.

[72] B. Brachvogel, J. Dikschas, H. Moch et al., "Annexin A5 is not essential for skeletal development," *Molecular and Cellular Biology*, vol. 23, no. 8, pp. 2907–2913, 2003.

[73] K. Yuan, J.-S. Huang, C.-W. Hsu, and I.-J. Hung, "A mineralization-associated membrane protein plays a role in the biological functions of the peptide-coated bovine hydroxyapatite," *Journal of Periodontal Research*, vol. 42, no. 5, pp. 420–428, 2007.

[74] D. Bozic, L. Grgurevic, I. Erjavec et al., "The proteome and gene expression profile of cementoblastic cells treated by bone morphogenetic protein-7 in vitro," *Journal of Clinical Periodontology*, vol. 39, no. 1, pp. 80–90, 2012.

[75] Y. Jung, J. Wang, J. Song et al., "Annexin II expressed by osteoblasts and endothelial cells regulates stem cell adhesion, homing, and engraftment following transplantation," *Blood*, vol. 110, no. 1, pp. 82–90, 2007.

[76] A. Pébay, C. S. Bonder, and S. M. Pitson, "Stem cell regulation by lysophospholipids," *Prostaglandins and Other Lipid Mediators*, vol. 84, no. 3-4, pp. 83–97, 2007.

[77] H. Chi, "Sphingosine-1-phosphate and immune regulation: trafficking and beyond," *Trends in Pharmacological Sciences*, vol. 32, no. 1, pp. 16–24, 2011.

[78] A. Olivera and S. Spiegel, "Sphingosine kinase: a mediator of vital cellular functions," *Prostaglandins and Other Lipid Mediators*, vol. 64, no. 1–4, pp. 123–134, 2001.

[79] S. Spiegel, O. Cuvillier, L. C. Edsall et al., "Sphingosine-1-phosphate in cell growth and cell death," *Annals of the New York Academy of Sciences*, vol. 845, pp. 11–8, 1998.

[80] V. Limaye, X. Li, C. Hahn et al., "Sphingosine kinase-1 enhances endothelial cell survival through a PECAM-1-dependent activation of PI-3K/Akt and regulation of Bcl-2 family members," *Blood*, vol. 105, no. 8, pp. 3169–3177, 2005.

[81] C. S. Bonder, W. Y. Sun, T. Matthews et al., "Sphingosine kinase regulates the rate of endothelial progenitor cell differentiation," *Blood*, vol. 113, no. 9, pp. 2108–2117, 2009.

[82] H. Meng, Y. Yuan, and V. M. Lee, "Loss of Sphingosine kinase 1/S1P signaling impairs cell growth and survival of neurons and progenitor cells in the developing sensory ganglia," *PLoS ONE*, vol. 6, no. 11, Article ID e27150, 2011.

[83] A. Pébay, R. C. B. Wong, S. M. Pitson et al., "Essential roles of sphingosine-1-phosphate and platelet-derived growth factor in the maintenance of human embryonic stem cells," *Stem Cells*, vol. 23, no. 10, pp. 1541–1548, 2005.

[84] Y. Nagata, T. A. Partridge, R. Matsuda, and P. S. Zammit, "Entry of muscle satellite cells into the cell cycle requires sphingolipid signaling," *The Journal of Cell Biology*, vol. 174, no. 2, pp. 245–253, 2006.

[85] P. Xia, J. R. Gamble, L. Wang et al., "An oncogenic role of sphingosine kinase," *Current Biology*, vol. 10, no. 23, pp. 1527–1530, 2000.

[86] D. Shida, K. Takabe, D. Kapitonov, S. Milstien, and S. Spiegel, "Targeting SphK1 as a new strategy against cancer," *Current Drug Targets*, vol. 9, no. 8, pp. 662–673, 2008.

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 7154327, 16 pages
http://dx.doi.org/10.1155/2016/7154327

Review Article

Gingival Mesenchymal Stem/Progenitor Cells: A Unique Tissue Engineering Gem

Karim M. Fawzy El-Sayed[1,2] and Christof E. Dörfer[1]

[1] *Clinic of Conservative Dentistry and Periodontology, Christian Albrechts University of Kiel, Arnold Heller Straße 3, Haus 26, 24105 Kiel, Germany*
[2] *Oral Medicine and Periodontology Department, Faculty of Oral and Dental Medicine, Cairo University, Egypt*

Correspondence should be addressed to Karim M. Fawzy El-Sayed; karim.fawzy@gmail.com

Received 19 January 2016; Revised 28 March 2016; Accepted 28 April 2016

Academic Editor: Mahmoud Rouabhia

The human gingiva, characterized by its outstanding scarless wound healing properties, is a unique tissue and a pivotal component of the periodontal apparatus, investing and surrounding the teeth in their sockets in the alveolar bone. In the last years gingival mesenchymal stem/progenitor cells (G-MSCs), with promising regenerative and immunomodulatory properties, have been isolated and characterized from the gingival lamina propria. These cells, in contrast to other mesenchymal stem/progenitor cell sources, are abundant, readily accessible, and easily obtainable via minimally invasive cell isolation techniques. The present review summarizes the current scientific evidence on G-MSCs' isolation, their characterization, the investigated subpopulations, the generated induced pluripotent stem cells- (iPSC-) like G-MSCs, their regenerative properties, and current approaches for G-MSCs' delivery. The review further demonstrates their immunomodulatory properties, the transplantation preconditioning attempts via multiple biomolecules to enhance their attributes, and the experimental therapeutic applications conducted to treat multiple diseases in experimental animal models in vivo. G-MSCs show remarkable tissue reparative/regenerative potential, noteworthy immunomodulatory properties, and primary experimental therapeutic applications of G-MSCs are very promising, pointing at future biologically based therapeutic techniques, being potentially superior to conventional clinical treatment modalities.

1. Introduction

The human periodontium, the tooth supporting and investing organ, comprising the alveolar bone, the periodontal ligament, the root cementum, and the gingiva develops and functions as one unit. The majority of the periodontal tissues originate embryonically from the neural crest ectomesenchyme [1]. The gingiva, histologically composed of epithelium and connective tissue, constitutes a distinctive as well as a pivotal component of the human periodontium developmentally and anatomically, surrounding the necks of the teeth and investing the tooth-bearing alveolar bone. One of the gingiva's renowned characteristics is its notable wound healing and regenerative aptitude, with a fast reconstitution of tissue architecture following injury or excision with little, if any, evidence of scarring [2]. This tissue is easily accessible and is often resected during standard surgical procedures, including dental crown lengthening and multiple periodontal surgeries, with minimal discomfort to the patient [3].

Developmentally, the craniofacial ectomesenchyme is derived from the neural crest and the mesoderm. The multipotent cranial neural crest cells (CNCCs) migrate ventrolaterally to reside in the first branchial arches, starting from the four-somite stage, giving rise to mesenchymal structures in the craniofacial region, including neural tissues, cartilage, bone, and teeth [4, 5]. In addition to a common neural crest ectomesenchymal origin, lined by ectoderm for all oral soft tissues, the tooth-investing gingival connective tissue shows a unique developmental origin, arising partly from the perifollicular mesenchyme (the outer layer of the dental follicle) [1], as well as partly from the dental follicle proper (the inner layer of the dental follicle) [6], from which dental follicle stem/progenitor cells (DFSCs) were isolated [7]. Periodontal ligament cells [8], originating themselves

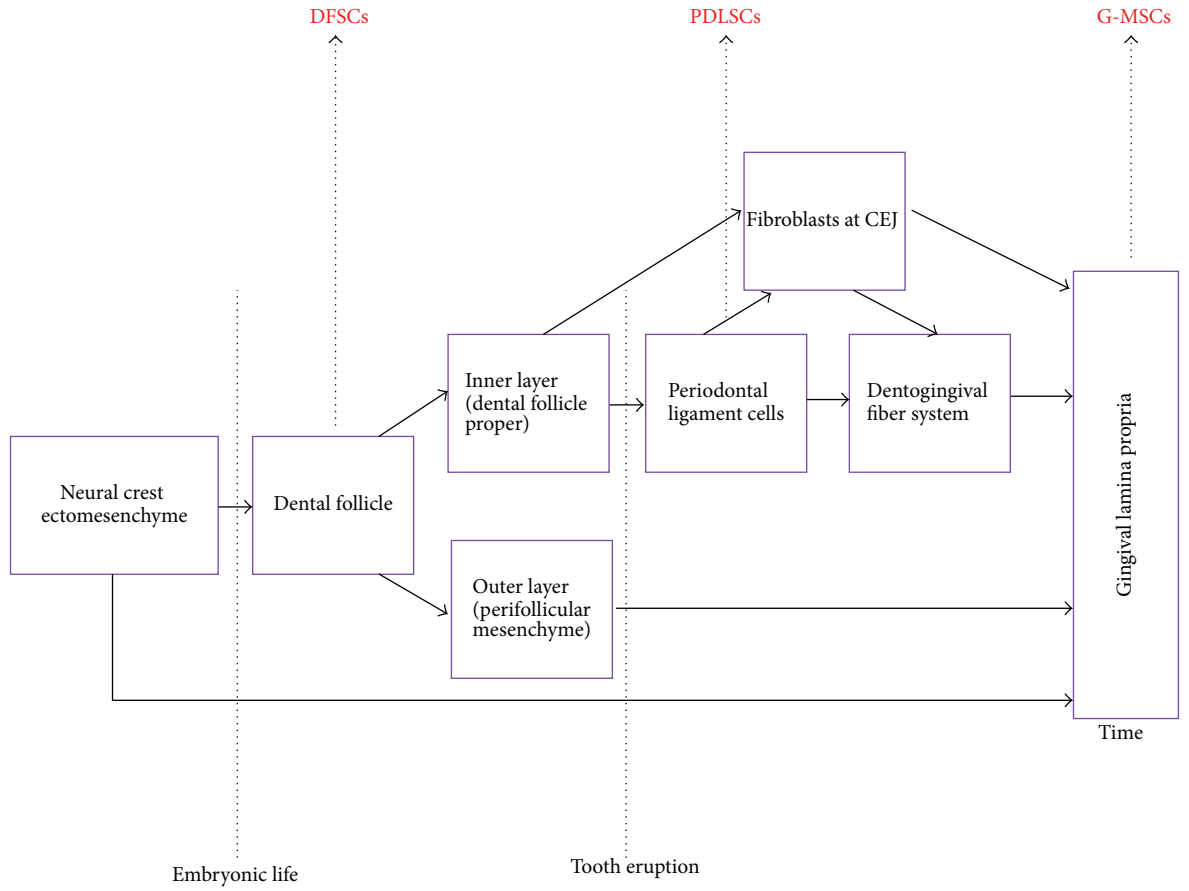


Figure 1: Schematic drawing of the oral tissues contributing to the developmental origin of human gingival lamina propria. DFSCs: dental follicle stem cells, G-MSCs: gingival mesenchymal stem/progenitor cells, PDLSCs: periodontal ligament stem cells.

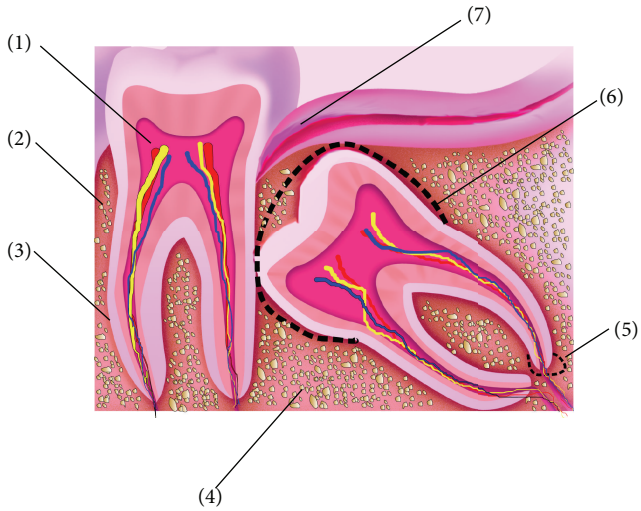
from the dental follicle proper [1] and from which a subpopulation of periodontal ligament stem/progenitor cells (PDLSCs) has been characterized [9], further contribute to its development. In addition, earlier studies demonstrated the presence of fibroblasts stemming from the inner layer of the dental follicle in the free gingival lamina propria at the cementoenamel junction [6] and further suggested that the dentogingival fiber system originates in part from the periodontal ligament cells [8] (Figure 1). This developmental contribution, provided by the dental follicle proper and the periodontal ligament cells to the perifollicular mesenchyme, accounts for an anatomical distinctiveness of the tooth-investing gingival connective tissue compared to other oral mucosal tissues [3].

The numerous functions of adult gingival wound fibroblasts and their variance in responsiveness to growth factors as well as their capacity to produce particular extracellular matrix proteins during healing validated an earlier hypothesis that gingival connective tissue fibroblasts embody a heterogeneous cell population [8, 10–13]. It further implied the existence of a resident population of adult mesenchymal stem/progenitor cells, giving rise to these heterogeneous cells. Previous studies described the isolation of progenitors from oral soft tissues, including the incisive papillae and rugae area of the palate [14], the maxillary tuberosity [15], the oral mucosa [16], the whole [17], the attached and free [3, 18, 19], and the hyperplastic gingiva [20]. Clinically, special attention is placed on the gingiva as a source mesenchymal stem/progenitor cells, representing the most abundant, accessible, and conservative minimally invasive source for stem/progenitor cells' isolation from the oral cavity [21] (Figure 2).

2. Gingival Mesenchymal Stem/Progenitor Cells' (G-MSCs) Isolation

A wide array of designations currently exist for mesenchymal stem/progenitor cells isolated from the gingival lamina propria, including gingiva-derived mesenchymal stem/stromal cells (G-MSCs) [22], gingival-tissue-derived stem cells (GT-MSCs) [18], gingival multipotent progenitor cells (GMPCs) [17], and gingival margin-derived stem/progenitor cells [3]. For clarity, the term gingival mesenchymal stem/progenitor cells (G-MSCs) will be used to uniformly designate these cells in the present review.

Studies reporting on techniques for G-MSCs' isolation surgically obtained gingival tissue samples from human subjects or animals via gingivectomy techniques and deep-ithelialized them, leaving only the connective tissue. The connective tissue biopsies were minced and digested to obtain



(1) DPSCs and SHEDs
(2) Alveolar bone proper-derived stem cells
(3) PDLSCs
(4) BM-MSCs
(5) SCAP
(6) DFSCs
(7) G-MSCs

Figure 2: Sources of oral stem/progenitor cells isolated. DFSCs: dental follicle stem cells, G-MSCs: gingival mesenchymal stem/progenitor cells, PDLSCs: periodontal ligament stem cells, SHEDs: stem cells from the human exfoliated deciduous teeth, DPSCs: dental pulp stem cells, BM-MSCs: bone marrow mesenchymal stem cells, and SCAP: stem cells from the apical papilla.

single-cell suspensions [18, 20, 23–25] or kept intact and the tissue explants culture method was used to grow out the adherent connective tissue cells [3, 15, 26, 27]. The obtained cells were subsequently cultured and expanded in vitro for 3-4 weeks.

Diverse G-MSCs' isolation and expansion protocols were proposed (Table 1). Some of the outlined protocols, except for I, III, IV, and V, did not attempt to select stem/progenitor cells' population from the heterogeneous gingival connective tissue cells via single-cell cloning [23, 28, 29] or magnetic activated cell sorting (MACS) techniques [3, 30]. This raises a question about whether thereafter characterized cultures would represent enriched mesenchymal stem/progenitor cell cultures or merely mixed gingival connective tissue cell cultures, encompassing stem/progenitor cells in their original low percentages, usually present in the gingival lamina propria. A recent study relying on a STRO-1/MACS scheme for G-MSCs' isolation underlined the importance of the utilization of a cell selection/sorting technique for G-MSCs' isolation, pointing out that two cell populations, a STRO-1/MACS$^+$ and a STRO-1/MACS$^-$, with distinctive properties and marker expression profiles exist in the human gingival connective tissue. The study demonstrated that the STRO-1/MACS$^+$-cell population, in contrast to the STRO-1/MACS$^-$ one, harbored the cells with stem/progenitor cells' characteristics and distinctive osteogenic marker expression and validated thereby the effectiveness of the STRO-1/MACS technique in the field of G-MSCs' isolation [3].

3. G-MSCs' Characterization

To characterize G-MSCs and compare their properties to bone marrow mesenchymal stromal cells (BM-MSCs), the forerunner and gold standard in the field mesenchymal stromal cells' (MSCs) isolation, characterization, and research [31], most studies referenced the minimal criteria proposed by the International Society for Cellular Therapy (ISCT) for MSCs' characterization [32]. MSCs should show self-renewal capabilities and plastic adherence under standard culture conditions. More than 95% of the alleged MSCs' population should express the surface markers CD73, CD90, and CD105, as measured by flow cytometry, and these cells must lack the expression (less than 2%) of the surface markers CD11b, CD14, CD19, CD34, CD45, CD79α, and HLA-DR. Finally, the cells should show the ability to differentiate into at least three tissue lineages (e.g., osteoblastic, adipocytic, and chondroblastic) under standard in vitro inductive conditions.

3.1. Self-Renewal. Self-renewal ability is one of the basic cellular characteristics of stem/progenitor cells. MSCs may divide asymmetrically, giving rise to two distinct daughter cells, one MSC and a second daughter programmed to differentiate into a committed lineage, or divide symmetrically, producing two identical copies of the original MSC [44]. Similarly, human G-MSCs demonstrated this ability through the formation of colony forming units (CFUs) [3, 15, 17, 18, 20, 22, 25].

As compared to BM-MSCs, G-MSCs show a faster proliferation rate (the population doubling time remaining constant in the range of 30–50 hours from primary to long-term cultures, whereas in BM-MSCs it increases from 50–60 hours in primary to up to 160–180 hours in long-term cultures) [18, 20, 25]. This significant property was primarily ascribed to a continuous activation of the telomerase enzyme even in long-term cultures [25]. Unlike BM-MSCs, which demonstrate abnormalities typical of the Hayflick model of cellular aging [45] at 8–10 passages, G-MSCs retain a stable morphology, maintain normal karyotype, do not lose MSCs' characteristics at higher passages, and are not tumorigenic [15, 18], despite their origin from healthy [3] or hyperplastic/inflamed gingival tissue [20, 23].

3.2. Multilineage Differentiation Potential. Similar to previous investigations on MSCs from other tissue sources, several studies reported on a multilineage differentiation ability of G-MSCs into osteoblastic, adipocytic, chondrocytic, endothelial, and neural directions, when incubated in in vitro inductive culture conditions (Table 2) [15, 17, 20, 22, 25, 40].

Osteogenic differentiation was demonstrated by the formation of calcified Alizarin-Red positive deposits [3, 15, 17, 18, 20, 22, 25] and through transmission electron microscopic (TEM) ultrastructural examinations, showing cellular features of mature osteoblasts, including the presence of two or three extended nucleoli, mitochondria with extended morphology, vacuoles in the process of exocytosis, extracellular granular and nongranular matrix, collagen

Table 1: Human G-MSCs isolation protocols.

Protocol number	Tissue culture method	Study
(I)	(1) Collected tissue incubated overnight with 2 mg/mL dispase at 4°C overnight to separate epithelium (2) The minced tissues are digested in 4 mg/mL collagenase IV for 2 h at 37°C (3) Cell filtered through 70 μm strainer (4) Cells seeded out (5) Single-cell cloning	[25]
(II)	(1) Tissue mincing (2) Tissue digestion in 0.1% collagenase and 0.2% dispase for 15 min at 37°C (3) Discarding of the first cell fraction containing some epithelial cells (4) Tissues are that further incubated with enzyme solution for 5, 10, and 15 min and all cell fractions that are pooled (5) Cells seeded out in tissue culture flasks	[18]
(III)	(1) Tissues digested with 0.4% dispase for 30 min at 37°C followed by collagenase type I (0.66 mg/mL) for 50 min (2) Cell filtered through 70 μm strainer to single-cell suspensions (3) Single-cell cloning	[20]
(IV)	(1) The minced tissues are digested in 3 mg/mL collagenase and 4 mg/mL dispase for 2 hours at 37°C (2) Cell filtered through 70 μm strainer (3) Single-cell suspension plated at a concentration of 60 cells/cm^2 (4) Selection of single-cell-derived colonies	[23]
(V)	(1) Tissue deepithelized under magnification and cut in small pieces (2 × 2 mm) and rinsed (2) Tissue placed in dry culture flasks to adhere for 30 min then medium slowly added (3) Flasks incubated for cells to grow out (4) STRO-1 magnetic cell sorting	[19]
(VI)	(1) The minced tissues are digested in 2 mg/mL collagenase and 1 mg/mL dispase for 30 min (2) Discarding of the first cell fraction containing some epithelial cells (3) Tissues that are further incubated with same enzyme solution for 90 min at 37°C (4) Cell filtered through 70 μm strainer (5) Cells seeded out	[24]

Table 2: Multilineage induction protocols.

Differentiation direction	Inductive medium composition
Osteogenic	α-MEM, 15% FCS, 100 μg/mL streptomycin, 1% amphotericin, 0.1 μM dexamethasone, 10 mM β-glycerophosphate, and 50 μg/mL ascorbic acid
Adipogenic	α-MEM, 15% FCS, 100 μg/mL streptomycin and 1% amphotericin, 1 μM dexamethasone, 10 μg/mL insulin, 100 μg/mL 1-methyl-3-isobutylxanthin, 60 μM indomethacin, and 4 mM L-glutamine
Chondrogenic	α-MEM, 100 μg/mL streptomycin and 1% amphotericin, 10 ng/mL TgF-β, 0.1 μM dexamethasone, 50 μg/mL ascorbic acid, 10 μg/mL insulin, and 1% ITS 100x
Neuronal	(I) Cells cultured on chamber slides coated with poly-D-lysine/laminin, cultured in DMEM/F12 with 10% FBS, 1 × N-2 supplement, 100 U/mL penicillin and 100 μg/mL streptomycin, 10 ng/mL fibroblast growth factor 2, and 10 ng/mL epidermal growth factor (II) Cells cultured on chamber slides coated with poly-D-lysine/laminin, cultured in DMEM/F12 with 125 ng/mL basic fibroblast growth factor (bFGF), 1000 unit/mL leukemia inhibitory factor, and 4 mM forskolin
Endothelial	Cells cultured in 8-well chamber slides precoated with fibronectin and cultivated in the presence or absence of endothelial growth medium 2

fibers, and areas of early mineralization [46]. Osteogenic differentiation was further demonstrated on the mRNA level through the expression of bone specific markers, including Runx2, collagen I, collagen III, alkaline phosphatase (ALP), osteonectin (ON), osteopontin (OP), and osterix [3, 25, 28, 39]. G-MSCs with suitable carriers implanted subcutaneously into immunocompromised mice generated connective tissue-like structures [20, 25], bone matrix [17, 22, 47], or mineralized tissues that exhibited certain similarities to cementum and bone, positively staining for collagen (Col), Ca, cementum attachment protein (CAP), cementum protein 1 (CP-1), bone sialoprotein (BSP), ALP, and osteocalcin (OC) [48].

Adipogenic differentiation was demonstrated by Oil-Red-O staining and the expression of the adipogenic markers peroxisome proliferator-activated receptor gamma (PPARγ), fatty acid synthase, and lipoprotein lipase (LPL) [3, 18, 25, 28]. Alginate-encapsulated G-MSCs were able to be differentiated into osteogenic and adipogenic tissues in vitro and through scanning electron microscopic (SEM) examinations

demonstrated the formation of hydroxyapatite-like crystalline structures [47].

Chondrogenic differentiation was evident by Toluidine-Blue staining and the expression of Sox-9, aggrecan, and Col-II [18] or by Alcian blue staining and aggrecan expression [3, 49] in 3D micromasses of G-MSCs. In a study, G-MSCs cultured for 3 weeks in chondrogenic inductive medium, followed by 2 weeks of hypoxic conditioning to induce cellular hypertrophy, further demonstrated Sox-9-dependent differentiation into chondrocyte and synoviocyte lineages in self-organized distinct areas that resembled native cartilage templates. Nonhypoxic conditions induced the expression of Sox-9, aggrecan, and Col-IIA1. With hypoxia cellular hypertrophy was induced, with downregulation of Sox-9, aggrecan, and Col-IIA1 and upregulation of Indian Hedgehog (IHH), Col-XA1, vascular endothelial growth factor a (VEGFA), matrix metalloproteinase 13 (MMP13), Runx2, and Col-IA1. Peripheral cells in the micromass cultures were organized in layers of cuboidal cells with villous structures facing the inductive medium and were strongly positive for cadherin-11, a marker of synoviocytes. Inhibition of cadherin-11 by siRNA transfection showed inhibition of the formation of this peripheral cell lining [49].

A further study reported on the ability of G-MSCs for neuronal and endothelial differentiation [25]. This remains however to be a controversial issue in the scientific community, regarding the minimal evidence provided to support the differentiation results. The study reported that neuronal differentiation was evident by the immunohistochemical staining of glial fibrillary acidic protein (GFAP), neurofilament 160/200 (NF-M), and β-tubulin III in the neuronal induced cultures. Here it should be noted that GFAP is not specific for neuronal differentiation, as the protein filament, aside of being expressed by astrocytes [50] and ependymal cells [51], is present in many cell types including glomeruli and peritubular fibroblasts in rat kidneys [52], Leydig cells of the testis in humans [53], and human osteocytes and chondrocytes [54]. For endothelial differentiation, the study relied solely on the expression of CD31 [25]. Apart from the immunohistochemical staining, no quantification of specific gene expressions for neuronal or endothelial differentiation was undertaken.

3.3. MSCs' Associated Markers. Currently, no explicit surface marker constellation exists for MSCs' characterization. For standardization purposes, studies commonly refer to the marker arrangement proposed by the ISCT [32] for G-MSCs' identification (see the above). Many studies further augmented the ISCT's list by additional markers, including CD13, CD38, CD44, CD54, CD117, CD144, CD146, CD166, Sca-1, STRO-1, SSEA-4, Oct-3/4, Oct-4A, Nanog, nestin, integrin β1, and vimentin [3, 24, 26, 36, 37, 43] (most commonly explored markers listed in Table 3).

Marker expression was shown to be altered by culturing conditions, where G-MSCs cultured as 3D spheroids demonstrated elevated expression Stro-1, CXC chemokine receptor 4 (CXCR-4), Oct-4, and Nanog, important transcriptional factors relevant to stem cell properties, and decreased expression of other MSCs-associated markers, including CD29, CD90, and CD105 [34]. Ascorbic acid (vitamin C) primed G-MSCs significantly elevated the expression of SSEA-3, Sox-2, Oct-3/4, Nanog, and TRA-1-60 [27]. Oct-3/4, Nanog, and Sox-2 expression are vital for maintaining a progenitor status with an unlimited stem cells' division, without affecting their self-renewal or differentiation capacity [55, 56]. Nanog is further a key gene for maintaining the cells' pluripotency [55, 57]. The expression of pluripotency markers, including Oct-3/4, Nanog, and Sox-2, by G-MSCs, similar to the expression described in a population of dental pulp pluripotent-like stem cells (DPPSCs) [55], presents an interesting finding and questions the true potential of G-MSCs. A proposed explanation, similar to previously described stem/progenitor cell sources [58, 59] could be that the human gingiva harbors subpopulations of stem/progenitor cells with pluripotent characteristics. However, another and in our view very interesting explanation is that pluripotency could be maintained/induced through specific culture conditions or biomolecules. DPPSCs cultured in a cell culture medium containing LIF (leukemia inhibitory factor), EGF (epidermal growth factor), and PDGF (platelets derived growth factor) expressed the pluripotency markers [55]. Similarly, G-MSCs' incubation in ascorbic acid (see the following) significantly elevated their pluripotency markers [27].

This varied expression of multi- as well as pluripotent markers by G-MSCs under different settings/culturing conditions, their remarkable differentiation potential (even apparently breaching endodermal and neuroectodermal barriers), and their long-term telomerase expression [25], similar to embryonic stem cells, raise the question about whether the true potential of G-MSCs has been elucidated yet. Further extensive research is needed in this area to precisely define the genuine potential of G-MSCs, the possible presence of subpopulations with diverse differentiation potentials, and the development of culture techniques and settings that could positively influence/direct their cellular properties prior to transplantation.

4. G-MSCs' Subpopulations

A study demonstrated the existence of G-MSCs in inflamed gingival tissues, exhibiting a phenotypic profile, an in vitro differentiation capacity and an in vivo developmental potential similar to G-MSCs obtained from healthy gingival tissues [23]. This finding is of prime importance, as G-MSCs isolated from the gingival tissues usually reside in a field of constant bacterial challenge, with resultant tissue inflammatory changes, in the oral cavity. It further underlines their positive attributes. Their resistance to inflammatory stimuli while retaining their MSCs' properties makes them a promising cellular source for tissue engineering therapeutic applications in vivo, where they could be exposed to similar inflammatory conditions.

It was further demonstrated that the gingival lamina propria contains two subpopulations of G-MSCs: 90% neural-crest-derived G-MSCs (N-GMSCs) and 10% mesoderm-derived G-MSCs (M-GMSCs) with distinctive stem cell properties. Compared to M-GMSCs, N-GMSCs showed an elevated aptitude to differentiate into neural cells, as was

Table 3: Major surface markers expressed on G-MSCs.

Study	CD13 %	CD14 %	CD29 %	CD31 %	CD34 %	CD38 %	CD44 %	CD45 %	CD54 %	CD73 %	CD90 %	CD105 %	CD117 %	CD146 %	CD166 %	SSEA-4 %	STRO-1 %	HLA-DR %	Oct-4	Nanog
Zhang et al., 2009 [25]			99.8					0.1		99.9	100	29.9		7.1		36.9	18.3		+	
Tomar et al., 2010 [18]			78.74		3.37		95.25	3.21		98.03	98.32	97.16								
Fournier et al., 2010 [17]			100		0		100	0		100	100	100		3–17			35	0		
Mitrano et al., 2010 [15]	99.48				0.95	0.25	99.4	0.85	0.80	98.98	99.52	96.1								
Tang et al., 2011 [20]			82.4		0.3		90	0.5			76.4	92.5		93.3			75.6			
Wang et al., 2011 [22]			99.98		0.01			0.41			92.87	34.75					17.89			
Zhang et al., 2012 [33]			94.7		1.23		79.0			80.4	98.3	41.1		10.8		14.2	13		+	+
Zhang et al., 2012 *Spheriod* [34]			67.5		0.6		44.8			65.4	80.0	10.8		9.5		14.7	25.2		+	+
Yang et al., 2013 [35]			100	1.3				1.9			99.9	97		55.2			16.3			
Xu et al., 2013 [36]					0.03		98.03	0.14		47.47	90.06	73.59	0.08							
El-Bialy et al., 2014 [26]		~2			~1			~1		~65	~50	~45								
El-Sayed et al., 2015 [19]		0.1			0.07			0.12		95.11	99.33	97.71		8.47			31.64			
Gao et al., 2014 [28]			13.4		1.8			1.6			99.4	99.5		8.5			10.0			
Gay et al., 2014 [37]												+				+			+	+
Moshaverinia et al., 2014 [38]					–							+		+						
Moshaverinia et al., 2014 [39]					–									+	+					
Wu et al., 2014 [40]				2.28	1.67			0.53		99.76	99.44	99.02					8.05	0.19		
Xu et al., 2014 [41]			98.17				99.43			96.71		90.85					20.31			
Jiang et al., 2015 [42]			94.5					3.9			92	25.5								
Jin et al., 2015 [24]		0.05			0.07		99.94	0.02		99.47	99.84	94.96		+	+	+	+			
van Pham et al., 2016 [27]		–			–		+	–		+	+	+						–		
Yin et al., 2016 [43]																+			+	

evident by an increase in nestin, neurofilament M (NF-09), and β-tubulin III expression, as well as chondrocytes, as was evident by Col-II and Sox-9 expression, and demonstrated enhanced immunomodulatory properties, inducing activated T-cell apoptosis, elevation of Tregs, and downregulation of Th-17. It appeared that the N-GMSCs mediated immunomodulation is associated with an elevated expression of Fas Ligand (FasL). However, both subpopulations showed no difference in their aptitude for osteogenic and adipogenic differentiation [36]. Further studies are needed to investigate the presence and properties of additional G-MSCs' subpopulations.

5. Gingiva-Derived iPSCs

The encouraging therapeutic prospective/potential of MSCs in the field of tissue engineering and regenerative approaches has highlighted the need for identifying easily accessible sources to obtain them in large quantities. A proposed source for obtaining large populations of MSCs is through the controlled induction of pluripotent stem cells (iPSCs) from the abundant and readily accessible human gingival fibroblasts (GFs).

Initially, iPSCs were generated from human and mouse GFs via genomic insertion of reprogramming factors carried on retroviral vectors [60, 61]. Although currently retroviral vectors provide the highest transfection efficiency, the technique harbors a high risk of cellular genetic mutation and viral genomic transmission [62, 63]. Retroviral-induced iPSCs from the GFs showed fast proliferation with a typical fibroblastic morphology and, unlike classical iPSCs cultures, the capacity to proliferate on standard culture flasks in the absence of a feeder cell layer. The gingival iPSCs, generated through transduction of Oct-3/4, Sox-2, Klf4, and c-myc and subsequently cultured for two weeks and passaged (up to 5–10 passages) in a MSCs' medium, consisting of minimum essential medium eagle-alpha modified (α-MEM) with 10% fetal calf serum (FCS), penicillin/streptomycin, sodium pyruvate, L-ascorbate-2-phosphate, L-glutamine, nonessential amino acids and HEPES, expressed MSCs-associated markers (CD73, CD90, CD105, CD146, and CD166), lacked the expression of the pluripotent (TRA160, TRA181, and ALP) and hematopoietic markers (CD14, CD34, and CD45), and showed a multilineage differentiation potential into osteoblastic, adipocytic, and chondrocytic directions [64]. The lack of pluripotent markers' expression however questions whether the described cells are true iPSCs or if they have undergone differentiation under the MSCs' culture conditions into a mesenchymal stromal cell type and qualify them, therefore, to be designated "iPSCs-like G-MSCs."

In a second study, true iPSCs were generated from GFs through a virus/integration-free and feeder-free approach, delivering the reprogramming factors of Oct-4, Sox-2, Klf4, L-myc, Lin28, and TP53 shRNA on episomal plasmid vectors. The generated gingival iPSCs presented morphology and proliferation characteristics similar to embryonic stem cells (ESCs), expressed, in contrast to the earlier study [64], pluripotent markers including Oct-4, Tra181, Nanog, and SSEA-4, maintained a normal karyotype, and showed decreased CpG methylation ratio in the promoter regions of Oct-4 and Nanog. In vivo teratoma formation assay demonstrated the development of tissues representative of the three germ layers, confirming their pluripotency [43].

A further study demonstrated in an opposite direction the successful differentiation of GFs integration-free episomal plasmid vectors-derived iPSCs into $CD44^{+}CD73^{+}CD90^{+}CD105^{+}$ G-MSCs-like cells, with osteogenic, adipogenic, and chondrogenic differentiation capabilities [65]. A recent study tested the osteogenic differentiation of iPSCs from GFs seeded on a nanohydroxyapatite/chitosan gelatin (nHA/CG) porous scaffold with two shapes (rod and sphere) in vitro and in vivo. Results revealed that sphere-nHA/CG significantly increased iPSCs proliferation and their osteogenic differentiation aptitude in vitro. iPSCs which were cultured on sphere-nHA/CG produced large, while iPSCs which were grown on rod-nHA/CG showed tiny bone in-vivo [66]. These results point clearly again at the influential effect of culturing conditions/matrix properties on the cellular differentiation potential.

6. Immunomodulatory Properties of G-MSCs

Besides the well-established self-renewal, multipotent differentiation, and tissue regeneration capabilities, G-MSCs, similar to other MSCs sources, possess outstanding immunomodulatory properties, which could be of great therapeutic interest. Generally, MSCs are nonimmunogenic and hold immunomodulatory capability, allowing for their allogenic transplantation without host immunosuppression. The interaction that occurs between G-MSCs and the surrounding inflammatory cells is thereby very complex (Figure 3). These immunomodulatory properties allow G-MSCs to ameliorate inflammatory diseases therapeutically, through their influence on the local microenvironment [33]. The cellular and molecular mechanisms, by which G-MSCs exert their immunomodulatory effects, are currently a matter of intense research, representing a potentially promising tool in cellular therapy [15].

7. Effects of G-MSCs on the Innate Immune System

The innate immune system is the first line of the host's defense and is comprised of several types of immune molecules and cells [67], particularly toll-like receptors (TLRs), dendritic cells (DCs), macrophages, and mast cells (MCs). Multiple studies revealed how G-MSCs exhibit potent immunomodulatory effects on these cells [29, 68].

7.1. Toll-Like Receptors (TLRs). Toll-like receptors (TLRs), major molecules linking the innate and adaptive immunity, are germ line-encoded pattern-recognition receptors (PRRs), detecting specific pathogen-associated molecular patterns (PAMPs) and thereby promoting immune cells' activation [69, 70]. G-MSCs may interact with their inflammatory environment via toll-like receptors (TLRs). A recent study outlined a distinctive G-MSCs' TLRs expression profile [71].

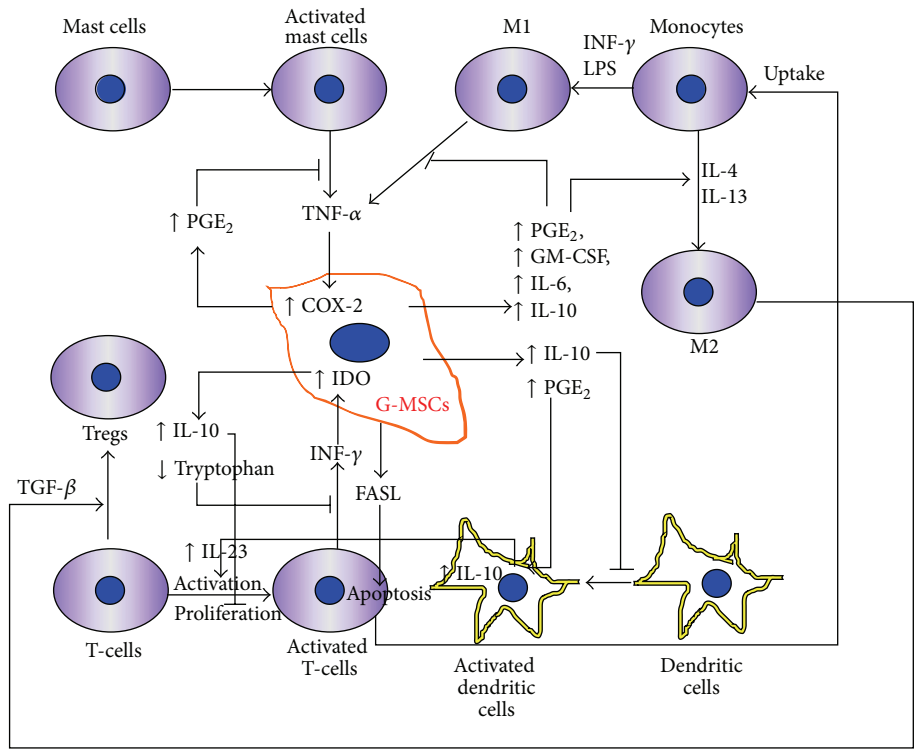


Figure 3: Immunomodulatory "crosstalk" between G-MSCs and mast cells, macrophages (with their M1 and M2 phenotypes), dendritic cells, and T-cells. COX-2: cyclooxygenase-2; PGE_2: prostaglandin E_2; GM-CSF: granulocyte-macrophage colony-stimulating factor; INF: interferon; IL: interleukin; TGF: transforming growth factor; IDO: indoleamine 2,3-dioxygenase; LPS: lipopolysaccharide.

In basic medium, G-MSCs expressed TLRs 1, 2, 3, 4, 5, 6, 7, and 10. The inflammatory medium significantly upregulated TLRs 1, 2, 4, 5, 7, and 10 and diminished TLR 6 expression. Whether this differential up/downregulation of the TLRs is reflective of an increased/decreased ability to respond to the respective ligands remains to be explored. The described TLRs' expression profile of G-MSCs in inflamed and uninflamed conditions could impact their therapeutic potential in inflammatory environments in vivo [72].

7.2. Dendritic Cells. Dendritic cells (DCs) are major antigen-presenting cells, linking the innate and adaptive immunity [73]. Prostaglandin E_2 (PGE_2), a lipid mediator produced from arachidonic acid by cyclooxygenase (COX), acts on four cellular receptor subtypes (EP1–EP4), encoded by Ptger1–Ptger4 genes, causing diverse physiological actions, including pyrexia, pain sensation, and inflammation. PGE_2 may further exert an anti-inflammatory effect, especially when binding with EP3 receptors usually present on mast cells (discussed in detail below) [74]. DCs express EP4 and its binding to PGE_2 normally induces an IL-23 mediated proinflammatory reaction with Th-17 activation [74]. However, through PGE_2 production, G-MSCs were reported to significantly arrest the maturation and activation of DCs, reducing their antigen presentation capacity and attenuating the inflammatory response [68]. This could be explained by an elevation/activation of the anti-inflammatory cytokine IL-10, through a PGE_2-mediated activation of the system E prostanoid (EP) receptor/cAMP/protein-kinase-A (PKA), which phosphorylates S133-cAMP response element-binding protein (CREB), to create a docking site for the coactivator CREB-binding protein and the initiation of IL-10 transactivation. PKA activity also inhibits salt-induced kinases (SIKs), which allow the cytoplasmic retention of CREB coactivators transducer of regulated CREB activity, (TORC)/CREB-regulated transcriptional coactivator (CRTC) 2, and TORC/CRTC3, and thereby elevates IL-10 levels [75]. PGE_2 further represses the TLR-induced cytokine induction in DCs in the absence of IL-10 [75], thereby contributing to the anti-inflammatory effect. This PGE_2-mediated attenuation effect may be reversed through indomethacin, an inhibitor of cyclooxygenases [68].

7.3. Macrophages. Macrophages, essential cellular components of the innate immune response [73], can generally be categorized into M1 (proinflammatory) and M2 (anti-inflammatory) subpopulations. M2 macrophages are considered to possess anti-inflammatory properties in light of their increased production of anti-inflammatory cytokines, including IL-10, and TGF-β [76], which could affect T-cells (see the following). G-MSCs demonstrated an ability for the polarization of macrophages into the M2 phenotype via enhanced secretion of IL-6, IL-10, GM-CSF, and PGE_2 [29, 33]. The immunomodulatory effect exerted by PGE_2 is expected to be the same as described above. This in turn reduces the inflammatory response in the tissues.

7.4. Mast Cells. Mast cells (MCs), key cells of the innate immunity, are critical in allergic and inflammatory disorders [77]. G-MSCs demonstrate suppressive effects on specific functions of MCs in vitro and in vivo, including de novo production of the major proinflammatory cytokine TNF-α, from activated human mast cells (HMC-1) in a cell-cell contact-independent manner. The outlined G-MSCs-induced blockage of the de novo production of proinflammatory cytokines by MCs is alleged to be partly mediated by the tumor necrosis factor-alpha/prostaglandin E_2 (TNF-α/PGE_2) feedback axis. However, G-MSCs demonstrated no obvious inhibitory effects on MCs' degranulation in vitro. In vivo, however, G-MSCs' administration suppressed MCs' degranulation. The described inhibitory effects were dependent on the COX_2/PGE_2 pathway and mediated by PGE_2-EP3 receptors [78], suggesting collectively that the TNF-α/COX_2/PGE_2 axis constitutes a negative feedback loop in the crosstalk between G-MSCs and MCs [68].

8. Effects of G-MSCs on the Acquired Immune System

Effects of G-MSCs on T-Cells. G-MSCs have been shown to exhibit a powerful dose dependent suppressive effect on the cellular proliferation and activation of human peripheral blood mononuclear cells (PBMC) stimulated either by phytohemagglutinin (PHA) [25] or by allogenic lymphocytes in a mixed lymphocyte reaction (MLR) [15, 20]. G-MSCs appear to possess the ability to suppress the proliferation of mitogen-activated lymphocytes in vitro [18, 20, 29]. The G-MSCs' suppressed PHA-dependent T-lymphocyte proliferation and activation occur via upregulation in IL-10 and downregulation tryptophan secretion in a cell-cell contact dependent and independent manner, seemingly mediated via indoleamine 2, 3-dioxygenase. (IDO) [25, 33]. The inflammatory cytokine INF-γ, secreted by activated T-lymphocytes in the coculture system, is assumed to act hereby as a feedback signal between G-MSCs and T-cells [25]. Additionally, findings from both in vitro and in vivo studies showed that G-MSCs could significantly inhibit Th17 cells and simultaneously promote the expansion of $CD4^+CD25^+FoxP3^+$ regulatory T-cells (Tregs), a cell type that has been recognized to play an important role in controlling autoimmunity [79–82]. The mechanism underlying it is believed to be mediated through a TGF-β dependent mechanism, involving M2 macrophages, following the uptake of apoptotic T-cells. The latter effect is induced through the Fas-Ligand (FasL) secreted by the G-MSCs, a type-II transmembrane protein, belonging to the TNF family, which through binding with its receptor induces T-cell apoptosis [42].

Collectively, G-MSCs' induced immunomodulation [20, 29, 68, 83, 84], through a complex interplay with various inflammatory cells and molecules, represents a promising and an effective treatment perspective for various inflammatory and autoimmune diseases.

9. G-MSCs' Cell Delivery Strategies

Providing a suitable microenvironment for MSCs' delivery, proliferation, and differentiation in the presence of exogenous stimuli and growth factors is a critical step toward successful clinical applications [47, 85]. As a fundamental part of the tissue engineering triad, consisting of cells, biomolecules, and scaffolds, cell delivery vehicles or scaffolds play an important role in the in vivo performance of MSCs and could influence the outcome of any regenerative therapy [86]. A variety of cell delivery approaches currently exist for G-MSCs' application, including scaffold-free direct local or systemic injection for homing [30, 34, 41], cell sheet engineering [87], and scaffold-augmented G-MSCs' transplantation [22, 38, 39, 47, 88, 89].

For mandibular and calvarial critical size defect reconstruction, G-MSCs were seeded in a collagen gel scaffold [22]. A periodontal regeneration study, seeding G-MSCs on collagen and inorganic bovine bone matrix, demonstrated that the cells attached and spread on both scaffold types prior to their transplantation into the experimental animals [89]. Multiple studies outlined the positive regenerative effect of a RGD (arginine-glycine-aspartic acid) tripeptide, vital peptides for cellular recognition, and attachment via integrins, enclosing alginate scaffold. The scaffold provided inward flux of nutrients and sufficient levels of oxygen, mimicked the natural cell-interactive function of the extracellular matrix (ECM), and provided a favorable physiochemical microenvironment with ligands, which specifically bind with G-MSCs' receptors. Encapsulated G-MSCs differentiated into osteogenic and adipogenic tissues in vitro, demonstrating that the encapsulation process did not negatively affect their stem/progenitor cells properties [38, 39, 47].

A study incorporated G-MSCs together with interleukin-1 receptor antagonist (IL-1ra) in a hyaluronic acid based synthetic hydrogel extracellular matrix (HA-sECM) and demonstrated successful cell inclusion, via SEM examination, as well as a controlled short-term IL-1ra release prior to transplantation into an experimental periodontitis model in vivo. On transplantation G-MSCs/HA-sECM construct demonstrated a remarkable periodontal regenerative potential [88].

Recently, G-MSCs were seeded on tetracycline-loaded silk fibroin membranes (TC-SFMs). Significantly higher cell viability was noted with 1% and 5% TC-SFMs. The morphology of G-MSCs on 0% and 1% TC-SFMs showed spindle shaped cells and at 10% TC-SFMs G-MSCs appeared spheroidal. G-MSCs cultured on 1% and 5% TC-SFMs showed higher proliferation and osteogenic potential and osteogenic gene expression for Runx2, Col-I, and BSP than G-MSCs on 10% TC-SFM [90].

The further developments of suitable G-MSCs' delivery vehicles/scaffolds, of their mechanical properties, their consistency, and their controlled resorption/tissue replacement, and of the incorporation and controlled release of biological molecules in a biomimetic manner remain all aspects for vital future improvement and research in the field of G-MSCs' transplantation.

10. G-MSCs' In Vitro Preconditioning

Numerous innovative and traditional biological agents as well as culturing conditions, including enamel matrix derivative (EMD), traditional oriental herbal medicines, vitamin C, Risedronate, and hypoxia, have recently been tested for their preconditioning effect in vitro in an attempt to improve the cellular properties and regenerative treatment outcome of G-MSCs in vivo.

10.1. Enamel Matrix Derivative (EMD). Emdogain is a commercially available enamel matrix derivative (EMD) [91], comprised of a mixture of hydrophobic enamel matrix proteins, nearly 90% of which is amelogenin, along with other enamel matrix proteins, such as amelin, ameloblastin, enamelin, and tuftelin [92], in a antimicrobial propyleneglycol-alginate (PGA) carrier. During tooth germ development, EMD is produced by the epithelial root sheath of Hertwig and plays a crucial role during root cementogenesis and during the development of the periodontal apparatus anchoring the root cementum to the surrounding alveolar bone via Sharpey's fibers [93]. In vitro studies reported on the aptitude of EMD to induce proliferation, migration, adhesion, mineralization, and differentiation as well as the increased collagen and protein production in periodontal ligament, dental follicle, and alveolar bone proper-derived stem/progenitor cells [94–97]. In vitro EMD preconditioning enhanced G-MSCs' proliferation. EMD further induced their osteogenic differentiation, with an amplified mRNA expression of Cbfα-l (a transcription factor of the runt-domain gene family), ALP (the early marker of osteogenic differentiation), and OC (the specific late marker of osteogenic differentiation and the major noncollagenic protein of the bone matrix) as well as an increased calcified nodule formation [40].

10.2. Traditional Oriental Herbal Medicines. Traditional oriental herbal medicines used in China, Japan, and Korea as *Asiasari radix* (*A. radix*), *Cimicifugae rhizoma,* and *Angelicae dahuricae* radix have been tested for their effect on G-MSCs in vitro. *A. radix*, commonly used in the treatment of dental diseases, including toothache and aphthous stomatitis, negatively influenced the viability and altered the morphology of G-MSCs in vitro [98]. Similarly, *Cimicifugae rhizoma*, commonly used as an anti-inflammatory, analgesic, and antipyretic remedy, negatively influenced the viability of the G-MSCs, especially at high concentrations, reducing cell number and CCK-8 values as well as altering their morphology from spindle to round shaped [99]. In contrast, *Angelicae dahuricae* radix, also an anti-inflammatory, analgesic, antipyretic, and antioxidant remedy, showed no effect on cell viability or morphology of G-MSCs [100]. Studies on these agents are still at an early stage, making it hard to draw a conclusion on the mechanism of action, the feasibility, and value of these herbal remedies in G-MSCs' preconditioning.

10.3. Vitamin C (Ascorbic Acid). Vitamin C (ascorbic acid (AA)) is a commonly used vitamin with antioxidant properties. Earlier studies confirmed that AA, an essential agent in stem/progenitor cells' proliferation, is characterized by its ability to trigger pluripotent markers' expression in both adult and embryonic stem cells [101, 102]. G-MSCs cultured in various concentrations of AA (10–250 μM) showed increased cell proliferation, significantly reducing the S and G2/M cell cycle time in a dose dependent manner. However, with AA concentrations higher than 250 μM (the cell-toxicity threshold), AA could intoxicate G-MSCs and drive them to apoptosis [27]. The increased cell proliferation effect could be attributed to the fact that AA upregulates the expression of multiple proliferation-related genes, comprising Fos, E2F2, Ier2, Mybl1, Cdc45, JunB, FosB, and Cdca5 as well as the mRNA expression of HGF, IGFBP6, VEGF, bFGF, and KGF [101].

AA-treated G-MSCs at concentrations below the defined cell-toxicity threshold showed significantly higher expression of the regenerative markers SSEA-3, Sox-2, Oct-3/4, Nanog, and TRA-1-60 and maintained the G-MSCs' phenotype, their marker expression, and their cell differentiation capacity [27]. Similar reports showed that AA plays a crucial role in inducing a pluripotent state in mouse embryonic stem cells through the modulation of micro-RNA expression [103]. Further reports suggested that AA can enhance somatic reprogramming to produce pluripotent stem cells [102]. The underlying mechanism is postulated to be related to the increase of promoter activity of pluripotent genes and enhancer protein levels [28].

Interestingly, despite the demonstrated pluripotency-inductive effect in vitro, AA preconditioned G-MSCs showed no tumor formation when transplanted in athymic mice in vivo [27]. The potential of AA and other biomolecules to affect the MSCs' potency opens a new perspective in G-MSCs' research.

10.4. Risedronate. G-MSCs were cultured in the presence of Risedronate (1–10 μM), a nitrogen-containing bisphosphonate commonly used for the prevention and treatment of postmenopausal and corticosteroid-induced osteoporosis. The drug is reported to reduce bone turnover and decrease resorption, chiefly through its effects on osteoclasts, with no undesirable effect on cortical porosity, thickness, or cancellous bone volume [104]. G-MSCs treated with Risedronate showed notable negative alterations in the morphology of the cells with fewer, rounder cells, alterations in the cytoskeletal organization, and reduced viability with decreased CCK-8 values [105].

10.5. Hypoxia. Hypoxia may be a promising preconditioning agent to promote the regenerative/reparative potential of G-MSCs in cell-based therapies. 2% hypoxic stimulation promoted the immunomodulatory properties of G-MSCs, through enhancing their suppressive effects on peripheral blood mononuclear cells (PBMCs), inhibiting their proliferation and increasing their apoptosis. This effect was attributed to the expression of FasL, which through its binding with its receptor induces cell apoptosis, by G-MSCs in the hypoxic environment [42].

Systemically infused G-MSCs enhanced skin wound repair in vivo and a 24-hour hypoxic preinfusion stimulation significantly supported their reparative capacity. The

delivered G-MSCs inhibited the local inflammation of the injured skin through inflammatory cells' suppression, reducing TNF-α and increasing the anti-inflammatory cytokine IL-10. These effects were reinforced by hypoxia [42]. The results point at the positive potential of possible hypoxic preconditioning of G-MSCs, prior to their therapeutic application. Further studies are needed to validate these effects and develop, in light of the obtained results, enhanced standardized G-MSCs' culturing protocols.

11. Experimental Therapeutic Applications of G-MSCs

11.1. Skin Wound Repair. Considering the characteristically observed scarless gingival intraoral wound healing properties, G-MSCs have become an exciting alternative for tissue engineering approaches, aiming at enhanced wound repair in extraoral tissues, originally branded, in secondary healing intentions, by scar formation [11, 13]. The utility of treating wounds with G-MSCs has recently been demonstrated through their systemic infusion for wound repair in a mouse model [106]. Besides a local enrichment in multipotent and self-renewing G-MSCs at the wound site, one of the mechanisms by which G-MSCs were assumed to improve repair is via their modulation of the local inflammatory response. As discussed above, G-MSCs are proposed to promote polarization of macrophages toward the regenerative (M2) phenotype, causing a rise in the level of anti-inflammatory IL-10 and a concomitant decrease in the expression of M1-cytokines (TNF-α and IL-6), thereby attenuating the local inflammation, promoting angiogenesis, and significantly enhancing wound repair [106]. The previously described immunomodulatory, in addition to the tissue-regenerative effect of G-MSCs, could bring about the observed outstanding wound repair attributes.

11.2. Tendon Regeneration. Tendon injuries are common in sports and in everyday life. The successful repair or regeneration of the injured tendon remains a clinically challenging task, especially in light of the reduced blood supply and cellular activity in the tendon areas of the human body. Earlier studies reported on the positive effect of the application of MSCs in tendon repair and regeneration [107, 108]. G-MSCs encapsulated in an injectable and biodegradable TGF-β3-loaded RGD-coupled alginate hydrogel microspheres scaffold (see scaffolds description above) were tested as an alternative treatment modality for tendon regeneration. Following a subcutaneous encapsulated G-MSCs' transplantation into immunocompromised mice, ectopic de novo tendon regeneration was observed, comparable to that induced by BM-MSCs. The results were evident by a positive immunohistochemical staining of the tissues using antibodies against the specific tendon markers Tenomodulin (Tnmd), Eya1, Eya2, and Scleraxis (Scx), confirming the regenerative capacity of the encapsulated G-MSCs [38]. Further studies are needed to validate the observed tendon repair/regeneration effect.

11.3. Bone Defects Regeneration. Multiple studies outlined the positive potential of G-MSCs in the field of MSCs-based bone reconstruction [18, 22]. eGFP-labelled G-MSCs seeded on Col-I gel implanted into mandibular ($5 \times 2 \times 1$ mm) as well as critical size calvarial defects (5 mm in diameter) in rats showed bone reconstruction potential over 2 months [22]. Transplanted G-MSCs encapsulated in a RGD-coupled alginate microencapsulation system were tested for their regenerative ability in 5 mm diameter critical size calvarial defects in immunocompromised mice. G-MSCs, despite showing reduced osteogenic differentiation capability, were able to repair the critical size defects. These newly formed bony tissues were immune-positive for Runx2 and OC antibodies [39]. G-MSCs preconditioned in an osteogenic differentiation medium showed induction of Runx2, ALP, and osterix expression, with mineralized nodules formation. When transplanted into C57BL/6J mice with mandibular bony defects via the tail vein, G-MSCs homed to the bone defects and promoted bone regeneration [41]. All of these results combined confirm a clear bone regenerative capacity by G-MSCs.

11.4. Periodontal Regeneration. G-MSCs are considered a promising and readily available cell source for periodontal tissue regeneration, including the reestablishment of functional tooth cementum, periodontal ligament, and alveolar bone. In an earlier study, porcine free gingival margin derived stem/progenitor cells isolated via a minimally invasive procedure and magnetically sorted, employing anti-STRO-1 antibodies and delivered on collagen or inorganic bovine bone matrix, showed a remarkable periodontal regenerative capacity in vivo [89]. This result evidently challenged the classical periodontal compartmentalization theory, declaring that the gingiva does not contribute to periodontal regeneration and that it should be excluded via guided tissue regeneration (GTR) barriers [109], showing that its connective tissue harbored multipotent stem/progenitor cells with a significant periodontal regenerative potential.

In a further study, GFP-labelled G-MSCs' cell sheets cultured in the medium supplemented with 100 mg/mL AA were employed for periodontal regeneration in a class III furcation defects dog model. The transplanted G-MSCs significantly enhanced the regeneration of the damaged periodontal tissues, including the alveolar bone, cementum, and periodontal ligament [87].

Recently, periodontal regenerative potential of G-MSCs combined with a short-term releasing IL-1ra hyaluronic acid based hydrogel synthetic extracellular matrix demonstrated a remarkable periodontal regenerative potential in a porcine experimental periodontitis model in vivo, with newly formed bone, cementum, and periodontal ligament fibers [88].

11.5. Peri-Implantitis. Peri-implantitis, one of the most serious medium- and long-term complications following dental implants oral rehabilitation, is characterized by bacterial destructive inflammatory changes in the tissues surrounding and supporting the dental implant [110]. G-MSCs encapsulated in a silver lactate- (SL-) containing RGD-coupled alginate hydrogel scaffold demonstrated antimicrobial properties against *Aggregatibacter actinomycetemcomitans* (Aa) on the surface of titanium disc, mimicking a peri-implantitis

model in vitro, while maintaining the G-MSCs' proliferation and osteogenic differentiation capacity. Silver ions, effectively released from the SL-loaded alginate microspheres for up to two weeks, were responsible for the antibacterial activity and the effect was dose dependent [111]. This in addition to the previously described G-MSCs' anti-inflammatory potential (see the above) could make them attractive agents in peri-implantitis treatment. Further studies are needed to explore this promising therapeutic potential in vivo.

11.6. Antitumor Effect. Tongue squamous cell carcinoma (TSCC) is presently the most prevalent type of oral cancer [112]. It clearly affects the life quality of the affected patients with malfunction of mastication, speech, and deglutition. Despite recent improvements in diagnostic techniques and therapeutic approaches, the number of deaths linked to TSCC increased by over 10% during the past 5 years [113]. G-MSCs therapeutic application could provide a new hope for its management. G-MSCs showed the ability to migrate towards TSCC cell lines (Tca8113 and Cal27) in an in vitro transwell cell-migration-assay, inducing tumor cell necrosis and apoptosis. Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a member of the TNF superfamily, is a type 2 transmembrane death ligand that causes apoptosis of transformed cells, but not in most of the normal cells [114]. TRAIL-transduced G-MSCs were administered to nude mice locally and systemically (mixed injection with tumor cells and tail vein injection). The transduced cells migrated toward TSCC in a large quantity and homed efficiently, reducing or even inhibiting TSCC growth, especially when the ratio of TRAIL-transduced G-MSCs to tumor cells was 1 : 1 [30]. Taking into account the clinical difficulties commonly encountered, as the unexposed tumor sites and the difficulty of topical administration of drugs, the proposed approach could present a future promising solution for local therapeutic delivery of biomolecules and cell.

11.7. Oral Mucositis. One of the major side effects of head and neck anticancer radio- and chemotherapy, affecting patients' life quality, is the resultant oral mucositis, secondary to basal cell layers damage and the subsequent impaired regenerative capacity of the oral epithelium. Anticancer therapy-induced oral mucositis represents a challenging and painful clinical situation showing a persistent oral wound characterized by atrophy, erythema, ulceration, and, eventually, loss of the mucosal barrier functions [115].

Employing an in vivo murine model of chemotherapy-induced oral mucositis, spheroid-derived G-MSCs delivered systemically reserved body weight loss and promoted the regeneration of disrupted epithelial lining of the murine mucositic tongue. 3D spheroid cultures of G-MSCs expressed high levels of reactive oxygen species, hypoxia-inducible factor- (HIF-) 1 and -2a, superoxide dismutase-2 (SOD2), and manganese superoxide dismutase, which improved their resistance to oxidative stress-induced apoptosis. Spheroid cultures derived G-MSCs displayed improved cell plasticity and aptitudes to home to mucositic lesions. The relatively smaller cell sizes and increased expression of CXCR-4 by spheroid cultures derived G-MSCs facilitated their faster trafficking through the lung microvasculature and more efficient distribution into mucositis affected tissues. These effects ameliorated the chemotherapy-induced oral mucositis lesions [34] and hold a promising therapeutic potential, warranting further in-depth research.

11.8. Experimental Colitis. G-MSCs ameliorated dextran sulfate sodium- (DSS-) induced colitis in a mouse model. Systemic infusion of G-MSCs in experimental colitis significantly improved both clinical and histopathological severity of the colonic inflammation, refurbished the injured gastrointestinal mucosal tissues, reversed diarrhea and weight loss, and suppressed the overall disease activity. The therapeutic effect of G-MSCs was suggested to be mediated, in part, by the suppression of inflammatory infiltrates and inflammatory cytokines/mediators, the increased infiltration of regulatory T-cells, and the expression of anti-inflammatory cytokine IL-10 at the colonic sites [25]. The immunomodulatory effect of G-MSCs was further hypothesized to be associated with upregulated expression of the FasL, which plays an important role in MSCs-based immunomodulation (see the above) [36]. Additional studies are needed to further elucidate the exact mechanism underlying the described colitis-ameliorating therapeutic effect.

11.9. Collagen-Induced Arthritis (CIA). G-MSCs may provide a promising therapeutic approach for the treatment of patients suffering from rheumatoid arthritis and other autoimmune diseases. G-MSCs significantly attenuated inflammatory arthritis in a collagen-induced arthritis (CIA) model. The therapeutic effects of G-MSCs depended mainly upon CD39/CD73-induced signals and partially upon the induction and expansion of Tregs (see the above). G-MSCs may suppress CIA directly in a CD39 or CD73 dependent manner. However, G-MSCs may also exert an indirect suppressing effect via promoting Tregs' production through CD39 and CD73 signaling, as was demonstrated by the fact, that G-MSCs pretreatment with CD39 or CD73 inhibitors abolished G-MSC-mediated Tregs' upregulation [116].

11.10. Contact Hypersensitivity. Systemic infusion of G-MSCs prior to sensitization and challenge phase dramatically suppressed hapten-induced murine contact hypersensitivity (CHS), an experimental model for human allergic contact dermatitis (ACD), one of the prevalent skin diseases worldwide. G-MSCs' infusion modulated the function of multiple innate and adaptive immune cells through the COX/PGE_2 pathway, resulting in a decreased infiltration of DCs, CD81 T-cells, Th-17, and MCs, a suppression of a variety of inflammatory cytokines, a reciprocal increased infiltration of Tregs, and an expression of IL-10 at the regional lymph nodes and the allergic contact areas. G-MSCs further blocked de novo synthesis of proinflammatory cytokines by MCs via PGE_2-dependent mechanisms [68] (see the above). All of these effects combined account for the hypersensitivity ameliorating effect of G-MSCs.

12. Conclusion and Outlook

The human gingival connective tissue provides a readily accessible as well as easily obtainable and renewable source of multipotent postnatal stem/progenitor cells for cellular approaches in different tissue repair/engineering/regeneration performances. The striking positive attributes of G-MSCs make them attractive cellular sources in the field of tissue engineering. G-MSCs show remarkable tissue reparative/regenerative potential, noteworthy immunomodulatory properties, and primary experimental therapeutic applications of G-MSCs are very promising, pointing at future biologically based therapeutic techniques, being potentially superior to conventional clinical treatment modalities.

However, numerous biological and technical challenges need to be addressed prior to considering transplantation approaches of G-MSCs a clinical reality in humans. Of prime importance remain the further optimization of techniques for cellular integration and propagation in apt biocompatible scaffolds and the improvement of their properties for clinical handling. Potential ex vivo karyotypic instability with possible gene mutations in prolonged cell-expansion-cultures remains currently hazardous outcome possibilities. Presently, the different inductive/differentiation/growth factors and cellular processes activated during stem/progenitor cells' self-renewal and differentiation are not satisfactorily illuminated. Most of our present understanding and elucidation models stem from in vitro cell culture and in vivo animal models, which do not entirely translate to human clinical situations. Finally, in view of our current knowledge gaps of tissue development processes, deeper understanding of biological processes is required, before reliable biologically based regenerative therapies become a clinical reality.

Competing Interests

The authors declare that they have no competing interests.

References

[1] M.-I. Cho and P. R. Garant, "Development and general structure of the periodontium," *Periodontology 2000*, vol. 24, no. 1, pp. 9–27, 2000.

[2] H. Larjava, C. Wiebe, C. Gallant-Behm, D. A. Hart, J. Heino, and L. Häkkinen, "Exploring scarless healing of oral soft tissues," *Journal of the Canadian Dental Association*, vol. 77, article 18, 2011.

[3] K. M. El-Sayed, S. Paris, C. Graetz et al., "Isolation and characterisation of human gingival margin-derived STRO-1/MACS$^+$ and MACS$^-$ cell populations," *International Journal of Oral Science*, vol. 7, no. 2, pp. 80–88, 2014.

[4] Y. Chai, X. Jiang, Y. Ito et al., "Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis," *Development*, vol. 127, no. 8, pp. 1671–1679, 2000.

[5] Y. Chai and R. E. Maxson Jr., "Recent advances in craniofacial morphogenesis," *Developmental Dynamics*, vol. 235, no. 9, pp. 2353–2375, 2006.

[6] A. R. Ten Cate, C. Mills, and G. Solomon, "The development of the periodontium. A transplantation and autoradiographic study," *Anatomical Record*, vol. 170, no. 3, pp. 365–379, 1971.

[7] C. Morsczeck, W. Götz, J. Schierholz et al., "Isolation of precursor cells (PCs) from human dental follicle of wisdom teeth," *Matrix Biology*, vol. 24, no. 2, pp. 155–165, 2005.

[8] S. Pitaru, C. A. McCulloch, and S. A. Narayanan, "Cellular origins and differentiation control mechanisms during periodontal development and wound healing," *Journal of Periodontal Research*, vol. 29, no. 2, pp. 81–94, 1994.

[9] B.-M. Seo, M. Miura, S. Gronthos et al., "Investigation of multipotent postnatal stem cells from human periodontal ligament," *The Lancet*, vol. 364, no. 9429, pp. 149–155, 2004.

[10] G. D. Sempowski, M. A. Borrello, T. M. Blieden, R. K. Barth, and R. P. Phipps, "Fibroblast heterogeneity in the healing wound," *Wound Repair and Regeneration*, vol. 3, no. 2, pp. 120–131, 1995.

[11] S. L. Schor, I. Ellis, C. R. Irwin et al., "Subpopulations of fetal-like gingival fibroblasts: characterisation and potential significance for wound healing and the progression of periodontal disease," *Oral Diseases*, vol. 2, no. 2, pp. 155–166, 1996.

[12] R. P. Phipps, M. A. Borrello, and T. M. Blieden, "Fibroblast heterogeneity in the periodontium and other tissues," *Journal of Periodontal Research*, vol. 32, no. 1, pp. 159–165, 1997.

[13] L. Häkkinen, V.-J. Uitto, and H. Larjava, "Cell biology of gingival wound healing," *Periodontology 2000*, vol. 24, no. 1, pp. 127–152, 2000.

[14] D. Widera, C. Zander, M. Heidbreder et al., "Adult palatum as a novel source of neural crest-related stem cells," *Stem Cells*, vol. 27, no. 8, pp. 1899–1910, 2009.

[15] T. I. Mitrano, M. S. Grob, F. Carrion et al., "Culture and characterization of mesenchymal stem cells from human gingival tissue," *Journal of Periodontology*, vol. 81, no. 6, pp. 917–925, 2010.

[16] K. Marynka-Kalmani, S. Treves, M. Yafee et al., "The lamina propria of adult human oral mucosa harbors a novel stem cell population," *Stem Cells*, vol. 28, no. 5, pp. 984–995, 2010.

[17] B. P. J. Fournier, F. C. Ferre, L. Couty et al., "Multipotent progenitor cells in gingival connective tissue," *Tissue Engineering Part A*, vol. 16, no. 9, pp. 2891–2899, 2010.

[18] G. B. Tomar, R. K. Srivastava, N. Gupta et al., "Human gingiva-derived mesenchymal stem cells are superior to bone marrow-derived mesenchymal stem cells for cell therapy in regenerative medicine," *Biochemical and Biophysical Research Communications*, vol. 393, no. 3, pp. 377–383, 2010.

[19] K. M. El-Sayed, S. Paris, C. Graetz et al., "Isolation and characterisation of human gingival margin-derived STRO-1/MACS$^+$ and MACS$^-$ cell populations," *International Journal of Oral Science*, vol. 7, no. 2, pp. 80–88, 2015.

[20] L. Tang, N. Li, H. Xie, and Y. Jin, "Characterization of mesenchymal stem cells from human normal and hyperplastic gingiva," *Journal of Cellular Physiology*, vol. 226, no. 3, pp. 832–842, 2011.

[21] K. M. Fawzy El-Sayed, C. Dörfer, F. Fändrich, F. Gieseler, M. H. Moustafa, and H. Ungefroren, "Adult mesenchymal stem cells explored in the dental field," *Advances in Biochemical Engineering/Biotechnology*, vol. 130, pp. 89–103, 2013.

[22] F. Wang, M. Yu, X. Yan et al., "Gingiva-derived mesenchymal stem cell-mediated therapeutic approach for bone tissue regeneration," *Stem Cells and Development*, vol. 20, no. 12, pp. 2093–2102, 2011.

[23] S. H. Ge, K. M. Mrozik, D. Menicanin, S. Gronthos, and P. M. Bartold, "Isolation and characterization of mesenchymal stem cell-like cells from healthy and inflamed gingival tissue: potential use for clinical therapy," *Regenerative Medicine*, vol. 7, no. 6, pp. 819–832, 2012.

[24] S. H. Jin, J. E. Lee, J.-H. Yun, I. Kim, Y. Ko, and J. B. Park, "Isolation and characterization of human mesenchymal stem cells from gingival connective tissue," *Journal of Periodontal Research*, vol. 50, no. 4, pp. 461–467, 2015.

[25] Q. Zhang, S. Shi, Y. Liu et al., "Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis," *The Journal of Immunology*, vol. 183, no. 12, pp. 7787–7798, 2009.

[26] T. El-Bialy, A. Alhadlaq, B. Wong, and C. Kucharski, "Ultrasound effect on neural differentiation of gingival stem/progenitor cells," *Annals of Biomedical Engineering*, vol. 42, no. 7, pp. 1406–1412, 2014.

[27] P. van Pham, N. Y. Tran, N. L.-C. Phan, N. B. Vu, and N. K. Phan, "Vitamin C stimulates human gingival stem cell proliferation and expression of pluripotent markers," *In Vitro Cellular and Developmental Biology—Animal*, vol. 52, no. 2, pp. 218–227, 2016.

[28] Y. Gao, G. Zhao, D. Li, X. Chen, J. Pang, and J. Ke, "Isolation and multiple differentiation potential assessment of human gingival mesenchymal stem cells," *International Journal of Molecular Sciences*, vol. 15, no. 11, pp. 20982–20996, 2014.

[29] Q. Z. Zhang, S. Shi, Y. Liu et al., "Mesenchymal stem cells derived from human gingiva are capable of immunomodulatory functions and ameliorate inflammation-related tissue destruction in experimental colitis," *Journal of Immunology*, vol. 183, no. 12, pp. 7787–7798, 2009.

[30] L. Xia, R. Peng, W. Leng et al., "TRAIL-expressing gingival-derived mesenchymal stem cells inhibit tumorigenesis of tongue squamous cell carcinoma," *Journal of Dental Research*, vol. 94, no. 1, pp. 219–228, 2015.

[31] A. J. Friedenstein, R. K. Chailakhyan, N. V. Latsinik, A. F. Panasyvk, and I. V. Keiliss-Borok, "Stromal cells responsible for transferring the microenvironment of the hemopoietic tissues: cloning in vitro and retransplantation in vivo," *Transplantation*, vol. 17, no. 4, pp. 331–340, 1974.

[32] M. Dominici, K. Le Blanc, I. Mueller et al., "Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement," *Cytotherapy*, vol. 8, no. 4, pp. 315–317, 2006.

[33] Q. Z. Zhang, A. L. Nguyen, W. H. Yu, and A. D. Le, "Human oral mucosa and gingiva: a unique reservoir for mesenchymal stem cells," *Journal of Dental Research*, vol. 91, no. 11, pp. 1011–1018, 2012.

[34] Q. Z. Zhang, A. L. Nguyen, S. Shi et al., "Three-dimensional spheroid culture of human gingiva-derived mesenchymal stem cells enhances mitigation of chemotherapy-induced oral mucositis," *Stem Cells and Development*, vol. 21, no. 6, pp. 937–947, 2012.

[35] H. Yang, L.-N. Gao, Y. An et al., "Comparison of mesenchymal stem cells derived from gingival tissue and periodontal ligament in different incubation conditions," *Biomaterials*, vol. 34, no. 29, pp. 7033–7047, 2013.

[36] X. Xu, C. Chen, K. Akiyama et al., "Gingivae contain neural-crest- and mesoderm-derived mesenchymal stem cells," *Journal of Dental Research*, vol. 92, no. 9, pp. 825–832, 2013.

[37] I. Gay, A. Cavender, D. Peto et al., "Differentiation of human dental stem cells reveals a role for microRNA-218," *Journal of Periodontal Research*, vol. 49, no. 1, pp. 110–120, 2014.

[38] A. Moshaverinia, X. Xu, C. Chen et al., "Application of stem cells derived from the periodontal ligament or gingival tissue sources for tendon tissue regeneration," *Biomaterials*, vol. 35, no. 9, pp. 2642–2650, 2014.

[39] A. Moshaverinia, C. Chen, X. Xu et al., "Bone regeneration potential of stem cells derived from periodontal ligament or gingival tissue sources encapsulated in RGD-modified alginate scaffold," *Tissue Engineering Part A*, vol. 20, no. 3-4, pp. 611–621, 2014.

[40] S.-M. Wu, H.-C. Chiu, Y.-T. Chin et al., "Effects of enamel matrix derivative on the proliferation and osteogenic differentiation of human gingival mesenchymal stem cells," *Stem Cell Research and Therapy*, vol. 5, no. 2, article 52, 2014.

[41] Q.-C. Xu, Z.-G. Wang, Q.-X. Ji et al., "Systemically transplanted human gingiva-derived mesenchymal stem cells contributing to bone tissue regeneration," *International Journal of Clinical and Experimental Pathology*, vol. 7, no. 8, pp. 4922–4929, 2014.

[42] C. M. Jiang, J. Liu, J. Y. Zhao et al., "Effects of hypoxia on the immunomodulatory properties of human gingiva-derived mesenchymal stem cells," *Journal of Dental Research*, vol. 94, no. 1, pp. 69–77, 2015.

[43] X. Yin, Y. Li, J. Li et al., "Generation and periodontal differentiation of human gingival fibroblasts-derived integration-free induced pluripotent stem cells," *Biochemical and Biophysical Research Communications*, vol. 473, no. 3, pp. 726–732, 2016.

[44] S. J. Morrison and J. Kimble, "Asymmetric and symmetric stem-cell divisions in development and cancer," *Nature*, vol. 441, no. 7097, pp. 1068–1074, 2006.

[45] L. Hayflick and P. S. Moorhead, "The serial cultivation of human diploid cell strains," *Experimental Cell Research*, vol. 25, no. 3, pp. 585–621, 1961.

[46] A. R. Sanz, F. S. Carrión, and A. P. Chaparro, "Mesenchymal stem cells from the oral cavity and their potential value in tissue engineering," *Periodontology 2000*, vol. 67, no. 1, pp. 251–267, 2015.

[47] A. Moshaverinia, C. Chen, K. Akiyama et al., "Alginate hydrogel as a promising scaffold for dental-derived stem cells: an in vitro study," *Journal of Materials Science: Materials in Medicine*, vol. 23, no. 12, pp. 3041–3051, 2012.

[48] S. Treves-Manusevitz, L. Hoz, H. Rachima et al., "Stem cells of the lamina propria of human oral mucosa and gingiva develop into mineralized tissues in vivo," *Journal of Clinical Periodontology*, vol. 40, no. 1, pp. 73–81, 2013.

[49] F. C. Ferré, H. Larjava, L.-S. Loison-Robert et al., "formation of cartilage and synovial tissue by human gingival stem cells," *Stem Cells and Development*, vol. 23, no. 23, pp. 2895–2907, 2014.

[50] C. M. Jacque, C. Vinner, M. Kujas, M. Raoul, J. Racadot, and N. A. Baumann, "Determination of glial fibrillary acidic protein (GFAP) in human brain tumors," *Journal of the Neurological Sciences*, vol. 35, no. 1, pp. 147–155, 1978.

[51] U. Roessmann, M. E. Velasco, S. D. Sindely, and P. Gambetti, "Glial fibrillary acidic protein (GFAP) in ependymal cells during development. An immunocytochemical study," *Brain Research*, vol. 200, no. 1, pp. 13–21, 1980.

[52] G. Buniatian, P. Traub, M. Albinus et al., "The immunoreactivity of glial fibrillary acidic protein in mesangial cells and podocytes of the glomeruli of rat kidney in vivo and in culture," *Biology of the Cell*, vol. 90, no. 1, pp. 53–61, 1998.

[53] M. S. Davidoff, R. Middendorff, E. Köfüncü, D. Müller, D. Ježek, and A.-F. Holstein, "Leydig cells of the human testis possess astrocyte and oligodendrocyte marker molecules," *Acta Histochemica*, vol. 104, no. 1, pp. 39–49, 2002.

[54] V. Kasantikul and S. Shuangshoti, "Positivity to glial fibrillary acidic protein in bone, cartilage, and chordoma," *Journal of Surgical Oncology*, vol. 41, no. 1, pp. 22–26, 1989.

[55] M. Atari, C. Gil-Recio, M. Fabregat et al., "Dental pulp of the third molar: a new source of pluripotent-like stem cells," *Journal of Cell Science*, vol. 125, no. 14, pp. 3343–3356, 2012.

[56] J. H. Hanna, K. Saha, and R. Jaenisch, "Pluripotency and cellular reprogramming: facts, hypotheses, unresolved issues," *Cell*, vol. 143, no. 4, pp. 508–525, 2010.

[57] K. Takahashi and S. Yamanaka, "Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors," *Cell*, vol. 126, no. 4, pp. 663–676, 2006.

[58] M. Z. Ratajczak, E. K. Zuba-Surma, B. MacHalinski, J. Ratajczak, and M. Kucia, "Very small embryonic-like (VSEL) stem cells: purification from adult organs, characterization, and biological significance," *Stem Cell Reviews*, vol. 4, no. 2, pp. 89–99, 2008.

[59] E. K. Zuba-Surma, M. Kucia, J. Ratajczak, and M. Z. Ratajczak, "'Small stem' cells in adult tissues: very small embryonic-like stem cells stand up!," *Cytometry Part A*, vol. 75, no. 1, pp. 4–13, 2009.

[60] H. Egusa, K. Okita, H. Kayashima et al., "Gingival fibroblasts as a promising source of induced pluripotent stem cells," *PLoS ONE*, vol. 5, no. 9, Article ID e12743, 2010.

[61] N. Wada, B. Wang, N.-H. Lin, A. L. Laslett, S. Gronthos, and P. M. Bartold, "Induced pluripotent stem cell lines derived from human gingival fibroblasts and periodontal ligament fibroblasts," *Journal of Periodontal Research*, vol. 46, no. 4, pp. 438–447, 2011.

[62] S. Yamanaka, "Induced pluripotent stem cells: past, present, and future," *Cell Stem Cell*, vol. 10, no. 6, pp. 678–684, 2012.

[63] S. M. I. Hussein, K. Nagy, and A. Nagy, "Human induced pluripotent stem cells: the past, present, and future," *Clinical Pharmacology & Therapeutics*, vol. 89, no. 5, pp. 741–745, 2011.

[64] K. Hynes, D. Menicanin, K. Mrozik, S. Gronthos, and P. M. Bartold, "Generation of functional mesenchymal stem cells from different induced pluripotent stem cell lines," *Stem Cells and Development*, vol. 23, no. 10, pp. 1084–1096, 2014.

[65] Y. Umezaki, Y. Hashimoto, N. Nishishita, S. Kawamata, and S. Baba, "Human gingival integration-free iPSCs; a source for MSC-like cells," *International Journal of Molecular Sciences*, vol. 16, no. 6, pp. 13633–13648, 2015.

[66] J. Ji, X. Tong, X. Huang et al., "Sphere-shaped nano-hydroxyapatite/chitosan/gelatin 3D porous scaffolds increase proliferation and osteogenic differentiation of human induced pluripotent stem cells from gingival fibroblasts," *Biomedical Materials*, vol. 10, no. 4, Article ID 045005, 2015.

[67] R. Clark and T. Kupper, "Old meets new: the interaction between innate and adaptive immunity," *Journal of Investigative Dermatology*, vol. 125, no. 4, pp. 629–637, 2005.

[68] W.-R. Su, Q.-Z. Zhang, S.-H. Shi, A. L. Nguyen, and A. D. Le, "Human gingiva-derived mesenchymal stromal cells attenuate contact hypersensitivity via prostaglandin E2-dependent mechanisms," *Stem Cells*, vol. 29, no. 11, pp. 1849–1860, 2011.

[69] T. Kawai and S. Akira, "The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors," *Nature Immunology*, vol. 11, no. 5, pp. 373–384, 2010.

[70] D. R. Getts, E. M. L. Chastain, R. L. Terry, and S. D. Miller, "Virus infection, antiviral immunity, and autoimmunity," *Immunological Reviews*, vol. 255, no. 1, pp. 197–209, 2013.

[71] K. Fawzy-El-Sayed, M. Mekhemar, S. Adam-Klages, D. Kabelitz, and C. Dorfer, "TlR expression profile of human gingival margin-derived stem progenitor cells," *Medicina Oral Patología Oral y Cirugia Bucal*, vol. 21, no. 1, pp. e30–e38, 2016.

[72] O. DelaRosa and E. Lombardo, "Modulation of adult mesenchymal stem cells activity by toll-like receptors: implications on therapeutic potential," *Mediators of Inflammation*, vol. 2010, Article ID 865601, 9 pages, 2010.

[73] S. J. Galli, N. Borregaard, and T. A. Wynn, "Phenotypic and functional plasticity of cells of innate immunity: macrophages, mast cells and neutrophils," *Nature Immunology*, vol. 12, no. 11, pp. 1035–1044, 2011.

[74] K. Kawahara, H. Hohjoh, T. Inazumi, S. Tsuchiya, and Y. Sugimoto, "Prostaglandin E2-induced inflammation: relevance of prostaglandin e receptors," *Biochimica et Biophysica Acta—Molecular and Cell Biology of Lipids*, vol. 1851, no. 4, pp. 414–421, 2015.

[75] M. Rodríguez, E. Domingo, C. Municio et al., "Polarization of the innate immune response by prostaglandin E2: a puzzle of receptors and signals," *Molecular Pharmacology*, vol. 85, no. 1, pp. 187–197, 2014.

[76] W. J. Lee, S. Tateya, A. M. Cheng et al., "M2 macrophage polarization mediates anti-inflammatory effects of endothelial nitric oxide signaling," *Diabetes*, vol. 64, no. 8, pp. 2836–2846, 2015.

[77] B. A. Sayed, A. Christy, M. R. Quirion, and M. A. Brown, "The master switch: the role of mast cells in autoimmunity and tolerance," *Annual Review of Immunology*, vol. 26, pp. 705–739, 2008.

[78] T. Kunikata, H. Yamane, E. Segi et al., "Suppression of allergic inflammation by the prostaglandin E receptor subtype EP3," *Nature Immunology*, vol. 6, no. 5, pp. 524–531, 2005.

[79] S. Sakaguchi, T. Yamaguchi, T. Nomura, and M. Ono, "Regulatory T cells and immune tolerance," *Cell*, vol. 133, no. 5, pp. 775–787, 2008.

[80] S. G. Zheng, J. H. Wang, J. D. Gray, H. Soucier, and D. A. Horwitz, "Natural and induced $CD4^+CD25^+$ cells educate $CD4^+CD25^-$ cells to develop suppressive activity: the role of IL-2, TGF-β, and IL-10," *Journal of Immunology*, vol. 172, no. 9, pp. 5213–5221, 2004.

[81] S. G. Zheng, J. H. Wang, M. N. Koss, F. Quismorio Jr., J. D. Gray, and D. A. Horwitz, "$CD4^+$ and CD8+ regulatory T cells generated ex vivo with IL-2 and TGF-β suppress a stimulatory graft-versus-host disease with a lupus-like syndrome," *The Journal of Immunology*, vol. 172, no. 3, pp. 1531–1539, 2004.

[82] Q. Lan, H. Fan, V. Quesniaux, B. Ryffel, Z. Liu, and S. G. Zheng, "Induced $Foxp3^+$ regulatory T cells: a potential new weapon to treat autoimmune and inflammatory diseases?" *Journal of Molecular Cell Biology*, vol. 4, no. 1, pp. 22–28, 2012.

[83] X. Chen, C. Hu, G. Wang et al., "Nuclear factor-κB modulates osteogenesis of periodontal ligament stem cells through competition with β-catenin signaling in inflammatory microenvironments," *Cell Death and Disease*, vol. 4, no. 2, article e510, 2013.

[84] M. Chen, W. Su, X. Lin et al., "Adoptive transfer of human gingiva-derived mesenchymal stem cells ameliorates collagen-induced arthritis via suppression of Th1 and Th17 cells and enhancement of regulatory T cell differentiation," *Arthritis and Rheumatism*, vol. 65, no. 5, pp. 1181–1193, 2013.

[85] G. Tour, M. Wendel, and I. Tcacencu, "Cell-derived matrix enhances osteogenic properties of hydroxyapatite," *Tissue Engineering Part A*, vol. 17, no. 1-2, pp. 127–137, 2011.

[86] T. Kaully, K. Kaufman-Francis, A. Lesman, and S. Levenberg, "Vascularization—the conduit to viable engineered tissues," *Tissue Engineering—Part B: Reviews*, vol. 15, no. 2, pp. 159–169, 2009.

[87] X. Yu, S. Ge, S. Chen et al., "Human gingiva-derived mesenchymal stromal cells contribute to periodontal regeneration in beagle dogs," *Cells Tissues Organs*, vol. 198, no. 6, pp. 428–437, 2013.

[88] K. M. Fawzy El-Sayed, M. K. Mekhemar, B. E. Beck-Broichsitter et al., "Periodontal regeneration employing gingival margin-derived stem/progenitor cells in conjunction with IL-1ra-hydrogel synthetic extracellular matrix," *Journal of Clinical Periodontology*, vol. 42, no. 5, pp. 448–457, 2015.

[89] K. M. Fawzy El-Sayed, S. Paris, S. T. Becker et al., "Periodontal regeneration employing gingival margin-derived stem/progenitor cells: an animal study," *Journal of Clinical Periodontology*, vol. 39, no. 9, pp. 861–870, 2012.

[90] S.-H. Jin, H. Kweon, J.-B. Park, and C.-H. Kim, "The effects of tetracycline-loaded silk fibroin membrane on proliferation and osteogenic potential of mesenchymal stem cells," *Journal of Surgical Research*, vol. 192, no. 2, pp. E1–E9, 2014.

[91] L. Trombelli and R. Farina, "Clinical outcomes with bioactive agents alone or in combination with grafting or guided tissue regeneration," *Journal of Clinical Periodontology*, vol. 35, no. 8, supplement, pp. 117–135, 2008.

[92] C. Robinson, S. J. Brookes, R. C. Shore, and J. Kirkham, "The developing enamel matrix: nature and function," *European Journal of Oral Sciences*, vol. 106, no. 1, pp. 282–291, 1998.

[93] L. Hammarström, "Periodontal regeneration in a buccal dehiscence model in monkeys after application of enamel matrix proteins," *Journal of Clinical Periodontology*, vol. 24, no. 9, pp. 669–677, 1997.

[94] M. T. Van Der Pauw, T. Van Den Bos, V. Everts, and W. Beertsen, "Enamel matrix-derived protein stimulates attachment of periodontal ligament fibroblasts and enhances alkaline phosphatase activity and transforming growth factor β1 release of periodontal liqament and ginqival fibroblasts," *Journal of Periodontology*, vol. 71, no. 1, pp. 31–43, 2000.

[95] S. Gestrelius, C. Andersson, D. Lidström, L. Hammarström, and M. Somerman, "In vitro studies on periodontal ligament cells and enamel matrix derivative," *Journal of Clinical Periodontology*, vol. 24, no. 9, pp. 685–692, 1997.

[96] S. S. Hakki, J. E. Berry, and M. J. Somerman, "The effect of enamel matrix protein derivative on follicle cells in vitro," *Journal of Periodontology*, vol. 72, no. 5, pp. 679–687, 2001.

[97] K. M. Fawzy El-Sayed, C. Dörfer, H. Ungefroren, N. Kassem, J. Wiltfang, and S. Paris, "Effect of Emdogain enamel matrix derivative and BMP-2 on the gene expression and mineralized nodule formation of alveolar bone proper-derived stem/progenitor cells," *Journal of Cranio-Maxillofacial Surgery*, vol. 42, no. 5, pp. 568–576, 2014.

[98] S.-H. Jeong, J.-E. Lee, S.-H. Jin, Y. Ko, and J.-B. Park, "Effects of Asiasari radix on the morphology and viability of mesenchymal stem cells derived from the gingiva," *Molecular Medicine Reports*, vol. 10, no. 6, pp. 3315–3319, 2014.

[99] S.-H. Jeong, J.-E. Lee, B.-B. Kim, Y. Ko, and J.-B. Park, "Evaluation of the effects of cimicifugae Rhizoma on the morphology and viability of mesenchymal stem cells," *Experimental and Therapeutic Medicine*, vol. 10, no. 2, pp. 629–634, 2015.

[100] S.-H. Jeong, B.-B. Kim, J.-E. Lee, Y. Ko, and J.-B. Park, "Evaluation of the effects of *Angelicae dahuricae* radix on the morphology and viability of mesenchymal stem cells," *Molecular Medicine Reports*, vol. 12, no. 1, pp. 1556–1560, 2015.

[101] J. H. Kim, W.-K. Kim, Y. K. Sung et al., "The molecular mechanism underlying the proliferating and preconditioning effect of vitamin C on adipose-derived stem cells," *Stem Cells and Development*, vol. 23, no. 12, pp. 1364–1376, 2014.

[102] M. A. Esteban and D. Pei, "Vitamin C improves the quality of somatic cell reprogramming," *Nature Genetics*, vol. 44, no. 4, pp. 366–367, 2012.

[103] Y. Gao, Z. Han, Q. Li et al., "Vitamin C induces a pluripotent state in mouse embryonic stem cells by modulating microRNA expression," *FEBS Journal*, vol. 282, no. 4, pp. 685–699, 2015.

[104] C. J. Dunn and K. L. Goa, "Risedronate: a review of its pharmacological properties and clinical use in resorptive bone disease," *Drugs*, vol. 61, no. 5, pp. 685–712, 2001.

[105] B. B. Kim, Y. Ko, and J. B. Park, "Effects of risedronate on the morphology and viability of gingiva-derived mesenchymal stem cells," *Biomedical Reports*, vol. 3, no. 6, pp. 845–848, 2015.

[106] Q.-Z. Zhang, W.-R. Su, S.-H. Shi et al., "Human gingiva-derived mesenchymal stem cells elicit polarization of M2 macrophages and enhance cutaneous wound healing," *Stem Cells*, vol. 28, no. 10, pp. 1856–1868, 2010.

[107] H. A. Awad, D. L. Butler, G. P. Boivin et al., "Autologous mesenchymal stem cell-mediated repair of tendon," *Tissue Engineering*, vol. 5, no. 3, pp. 267–277, 1999.

[108] A. K. S. Chong, J. Chang, and J. C. H. Go, "Mesenchymal stem cells and tendon healing," *Frontiers in Bioscience*, vol. 14, no. 12, pp. 4598–4605, 2009.

[109] J. Gottlow, S. Nyman, T. Karring, and J. Lindhe, "New attachment formation as the result of controlled tissue regeneration," *Journal of Clinical Periodontology*, vol. 11, no. 8, pp. 494–503, 1984.

[110] G. Tabanella, H. Nowzari, and J. Slots, "Clinical and microbiological determinants of ailing dental implants," *Clinical Implant Dentistry and Related Research*, vol. 11, no. 1, pp. 24–36, 2009.

[111] I. M. A. Diniz, C. Chen, S. Ansari et al., "Gingival mesenchymal stem cell (GMSC) delivery system based on RGD-coupled alginate hydrogel with antimicrobial properties: a novel treatment modality for peri-implantitis," *Journal of Prosthodontics*, vol. 25, no. 2, pp. 105–115, 2016.

[112] X. Yu and Z. Li, "MicroRNA expression and its implications for diagnosis and therapy of tongue squamous cell carcinoma," *Journal of Cellular and Molecular Medicine*, vol. 20, no. 1, pp. 10–16, 2016.

[113] J. S. Hwa, O. J. Kwon, J. J. Park et al., "The prognostic value of immunohistochemical markers for oral tongue squamous cell carcinoma," *European Archives of Oto-Rhino-Laryngology*, vol. 272, no. 10, pp. 2953–2959, 2015.

[114] A. Ashkenazi, R. C. Pai, S. Fong et al., "Safety and antitumor activity of recombinant soluble Apo2 ligand," *Journal of Clinical Investigation*, vol. 104, no. 2, pp. 155–162, 1999.

[115] S. Al-Ansari, J. A. Zecha, A. Barasch, J. de Lange, F. R. Rozema, and J. E. Raber-Durlacher, "Oral mucositis induced by anticancer therapies," *Current Oral Health Reports*, vol. 2, no. 4, pp. 202–211, 2015.

[116] M. G. Chen, W. Su, X. Lin et al., "Adoptive transfer of human gingiva-derived mesenchymal stem cells ameliorates collagen-induced arthritis via suppression of Th1 and Th17 cells and enhancement of regulatory T cell differentiation," *Arthritis & Rheumatism*, vol. 65, no. 5, pp. 1181–1193, 2013.

Hindawi Publishing Corporation
Stem Cells International
Volume 2016, Article ID 1290561, 10 pages
http://dx.doi.org/10.1155/2016/1290561

Research Article

Clonal Heterogeneity in the Neuronal and Glial Differentiation of Dental Pulp Stem/Progenitor Cells

Fraser I. Young,[1,2] Vsevolod Telezhkin,[3,4] Sarah J. Youde,[1] Martin S. Langley,[1] Maria Stack,[1] Paul J. Kemp,[3] Rachel J. Waddington,[1] Alastair J. Sloan,[1] and Bing Song[1]

[1]*Oral and Biomedical Sciences, School of Dentistry, Cardiff University, Heath Park, Cardiff CF14 4XY, UK*
[2]*Neuroscience and Mental Health Research Institute, School of Medicine, Cardiff University, Hadyn Ellis Building, Maindy Road, Cardiff CF24 4HQ, UK*
[3]*Cardiff School of Biosciences, Cardiff University, Sir Martin Evans Building, Museum Avenue, Cardiff CF10 3AX, UK*
[4]*Department of Neuroscience, Physiology & Pharmacology, University College London, London WC1E 6BT, UK*

Correspondence should be addressed to Fraser I. Young; youngf1@cardiff.ac.uk and Bing Song; songb3@cardiff.ac.uk

Received 14 January 2016; Revised 24 March 2016; Accepted 8 May 2016

Academic Editor: George Huang

Cellular heterogeneity presents an important challenge to the development of cell-based therapies where there is a fundamental requirement for predictable and reproducible outcomes. Transplanted Dental Pulp Stem/Progenitor Cells (DPSCs) have demonstrated early promise in experimental models of spinal cord injury and stroke, despite limited evidence of neuronal and glial-like differentiation after transplantation. Here, we report, for the first time, on the ability of single cell-derived clonal cultures of murine DPSCs to differentiate *in vitro* into immature neuronal-like and oligodendrocyte-like cells. Importantly, only DPSC clones with high nestin mRNA expression levels were found to successfully differentiate into Map2 and NF-positive neuronal-like cells. Neuronally differentiated DPSCs possessed a membrane capacitance comparable with primary cultured striatal neurons and small inward voltage-activated K^+ but not outward Na^+ currents were recorded suggesting a functionally immature phenotype. Similarly, only high nestin-expressing clones demonstrated the ability to adopt Olig1, Olig2, and MBP-positive immature oligodendrocyte-like phenotype. Together, these results demonstrate that appropriate markers may be used to provide an early indication of the suitability of a cell population for purposes where differentiation into a specific lineage may be beneficial and highlight that further understanding of heterogeneity within mixed cellular populations is required.

1. Introduction

Stem cell heterogeneity poses a significant obstacle to the clinical implementation of cell-based therapies. Mixed cultures may contain a combination of stem cells with a broad range of differentiation potentials and long term proliferative abilities, as well as more lineage-committed progenitor cells. Such variability amongst cells in the same transplantable population can lead to the adoption of adverse phenotypes, potentially limiting improvements in outcome.

Dental Pulp Stem/Progenitor Cells (DPSCs) possess typical mesenchymal progenitor properties *in vitro*, demonstrating the ability to differentiate into odontoblasts/osteoblasts, adipocytes, and chondrocytes [1–3]. Furthermore, endothelial, myogenic, hepatocytic, and melanocytic differentiation capabilities have also been reported, suggesting a diverse range of potential therapeutic applications [4–8]. However, DPSCs represent a highly heterogeneous population of cells with distinct clonal differences in proliferation and mineralisation capabilities [1, 9]. Such variability could potentially hinder progress in the development of DPSC-based treatments. Nevertheless, transplanted mixed populations of DPSCs have demonstrated promise at improving functional outcome in experimental models of spinal cord injury, stroke, and Parkinson's disease [10–15]. These effects are mostly protective and mediated through the release of supportive growth factors. It is, as yet, unclear whether DPSCs can differentiate into, and functionally compensate for, neuronal and glial cell types after transplantation. *In vitro* studies have indicated potential for rodent and human DPSCs

to differentiate into neuronal-like cells [16]. Furthermore, independent studies have described a degree of functionality of such neuronally differentiated DPSCs [17–21]. However, only a small fraction of cells within such cultures develop a functional phenotype [18]. Similarly, outcomes may differ between patient samples subjected to the same differentiation protocol [22]. Understanding the cellular biology behind such heterogeneity poses a substantial challenge to researchers. The identification of an appropriate marker of neural differentiation capabilities, for which to screen DPSC cultures beforehand, could help to minimise such variability between studies resulting in more defined and predictable outcomes.

In this study, single cell-derived clonal populations of murine DPSCs (mDPSCs) were isolated and differences in the expression of early stage neural markers identified. Only clones with high levels of nestin expression were found to differentiate into immature neuronal-like cells, displaying minimal electrical activity. Similarly, a novel differentiation protocol was developed for the derivation of oligodendrocyte-like cells from high nestin-expressing mDPSC clones. Together, these findings suggest that nestin may act as a suitable marker for use in assessing the ability of mDPSCs to differentiate into neuronal-like and glial-like cells *in vitro*.

2. Experimental Procedures

2.1. Isolation of mDPSCs. All procedures were approved by the Cardiff University Biological Standards Office. For each DPSC isolation, the incisor pulpal tissue of 4-5 × 21–28-day-old C57/Bl6 mice, sacrificed by CO_2 asphyxiation in accordance with Schedule 1 of the UK Animals (Scientific Procedures) Act 1986, was pooled. Following collagenase/dispase digestion to a cellular suspension, the preferential adherence to fibronectin selection technique was used to select for progenitor cells by isolating cells of more immature phenotypes based on β1 integrin functionality [23, 24]. After 12 days of primary culture, individual colonies of fibronectin-adherent cells, displaying typical DPSC bipolar fibroblastic-like morphology and numbering greater than 32 cells, were selected for clonal isolation and expansion as described previously using cloning rings [25]. Clonal DPSCs were subsequently expanded in αMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 20% (v/v) heat-inactivated foetal bovine serum (all from Life Technologies) and 100 μM l-ascorbic acid 2-phosphate (Sigma-Aldrich). Cell counts were performed at every passage and used to track population doublings over time in culture:

$$\text{Population doublings} = \frac{\log_{10}(\text{cell count at passage}) - \log_{10}(\text{no. of cells initially seeded})}{\log_{10}(2)}. \quad (1)$$

Cells of between 20 and 40 population doublings were used for all experiments. Four individual mDPSC clones that expanded sufficiently to allow multiple reproducible differentiation experiments were used in this study, each derived from a separate pulpal extraction ($n = 4$).

2.2. Neuronal Differentiation. mDPSC clones were seeded at 10,000 cells/cm^2 on poly-d-lysine/laminin-coated culture surfaces in DMEM/F12 (1 : 1) containing L-glutamine and HEPES buffer, 100 units/mL penicillin, 100 μg/mL streptomycin, and 1 × N2 supplement (all from Life Technologies), 1 × NEAA (Sigma-Aldrich), and 20 ng/mL basic fibroblast growth factor (bFGF) and 20 ng/mL epidermal growth factor (both from Peprotech). After 5 days, cultures were washed with PBS and changed to neurobasal medium supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 2 mM L-glutamine (all from Life Technologies), 1 × NEAA (Sigma-Aldrich), and 10 ng/mL brain-derived neurotrophic factor, 10 ng/mL nerve growth factor, and 10 ng/mL neurotrophin-3 (all from Peprotech). RNA was extracted on days 0, 5, 10, and 15 of differentiation for use in qPCR and cells were fixed on day 15 for immunocytochemistry.

2.3. Oligodendrocyte Differentiation. Clonal mDPSC cultures were seeded at 10,000 cells/cm^2 on poly-d-lysine-coated culture surfaces in DMEM containing 100 units/mL penicillin, 100 μg/mL streptomycin, SATO supplement (16 μg/mL putrescine, 62 ng/mL progesterone, 5 ng/mL sodium selenite, and 100 μg/mL bovine serum albumin (BSA)), 50 μg/mL holo-Transferrin, and 5 μg/mL insulin (all from Sigma-Aldrich) and 10 ng/mL platelet-derived growth factor-aa and 20 ng/mL bFGF (both Peprotech). After 10 days differentiation cells were fixed for immunocytochemistry.

2.4. Isolation of mSTM Neurons. Mouse striatal (mSTM) neuronal tissues were dissected in PBS from P0 mice, digested using Accutase, and plated on poly-l-lysine-coated glass coverslips in Advanced DMEM/F-12 supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, 1.8 mM $CaCl_2$, 0.5 mM/L valproic acid, and 1 x B27-supplement (without vitamin A) (all from Life Technologies).

2.5. Reverse Transcriptase PCR. Total RNA was extracted using an RNeasy Mini Kit with on-column DNase digestion (QIAGEN) according to manufacturer's directions and cDNA synthesised using MMLV reverse transcriptase (Promega). PCR reactions were performed using GoTaq Polymerase (Promega) and product-specific primers (Supplemental Table 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2016/1290561). RNA extracted from primary cultured E14.5 cortical neural stem cells, isolated as described previously [26], was used as a positive control.

2.6. Real Time Quantitative PCR. For qPCR readings, cDNA samples generated from three separate experiments per clone

were used ($n = 3$) and each was measured in triplicate using an ABI Prism 7000 machine (Advanced Biosystems). Target-specific primers (Supplemental Table 2) were added to each cDNA sample together with Precision MasterMix with ROX and SYBRgreen (PrimerDesign). Dissociation curves were recorded to check for specificity of reactions and products were electrophoresed on 1.4% agarose gels in order to confirm product size. Relative changes in expression were calculated using the $2^{-\Delta\Delta CT}$ method [27]. Statistical analyses were performed using Graphpad Prism Software.

2.7. Immunocytochemistry. Cells were fixed with 4% (w/v) paraformaldehyde for 15 min at room temperature and permeabilised in 0.1% (v/v) Triton X-100 for 10 min. Nonspecific antibody binding was blocked by incubating in 2% (w/v) BSA for 30 min. Cells were incubated overnight with the following primary antibodies: nestin (Santa Cruz), musashi (Life Technologies), microtubule-associated protein 2 (Map2 (Millipore)), neurofilament light chain NF-l (Abcam), Olig1 and Olig2 (both from Millipore), myelin basic protein (MBP (Abcam)), and β-actin (Cell Signalling). On the following day, complementary Alexa Fluor 488- and 594-conjugated secondary antibodies (Life Technologies) were applied. Glass coverslips were mounted using mounting media supplemented with DAPI stain (VectorLabs) and preparations imaged under a fluorescent microscope.

2.8. Patch Clamp Electrophysiological Recordings. Transmembrane currents of primary cultured mSTM neurons from days 3 to 21 of culture and mDPSCs neuronally differentiated for 15 days were recorded in conventional whole-cell configuration. The bath solution contained 135 mM NaCl, 5 mM KCl, 1.2 mM $MgCl_2$, 1.25 mM $CaCl_2$, 10 mM d-glucose, and 5 mM HEPES; pH was adjusted to 7.4 using NaOH. The pipette solution contained 117 mM KCl, 10 mM NaCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2 mM Na_2ATP, 1 mM Na_2GTP, 11 mM HEPES, and 11 mM ethylene glycol tetra acetic acid; free $[Ca^{2+}]_i$ was adjusted to 100 nM; pH was adjusted to 7.2 with KOH. All recordings were performed at room temperature ($22 \pm 0.5^{\circ}C$) using an Axopatch 200B amplifier and Digidata 1320 A/D interface (Axon Instruments). Holding voltages were set to −70 mV and transmembrane currents recorded using a voltage step protocol of 80 ms duration in voltage range from −120 to +80 mV. Series resistance and membrane capacitance were compensated ≈90%. Pipette resistances were ≈5–10 MΩ when filled with the pipette solution. All recordings were filtered with an 8-pole Bessel filter at 5 kHz and digitized at 10 kHz. Tetraethylammonium chloride (TEA) was purchased from Sigma.

2.9. Data Analysis. The patch clamp data were analyzed using Clampfit 9.0, Microsoft Office Excel 2003, and Microcal Origin 6.0 software. Transient inward Na^+ currents were presented as peak values whereas outward steady-state K^+ currents were presented as means. Current densities (pA/pF) were plotted against command voltage (mV). Statistical comparisons of the means were performed using independent t-test; differences were considered significant at $p < 0.05$.

3. Results

3.1. Isolation, Expansion, and Characterisation of Clonal mDPSC Cultures. Dental pulp cells were successfully isolated and cultured from murine incisors. One day following isolation, sparsely distributed fibroblastic-like cells were identified growing on fibronectin-coated culture surfaces. A number of these rapidly expanded clonally to form discrete individual colonies. Clones were isolated at day 12 and found to expand over extended periods, some reaching 50+ population doublings, confirming a highly proliferative phenotype (Figure 1(a)). Presence of the neural progenitor markers nestin and musashi was identified within expanding clones using immunocytochemistry (Figure 1(b)). RNA was extracted from each clone between 20 and 40 population doublings and used in RT-PCR to identify further similarities in marker expression between four mDPSC clones and primary cultured murine neural stem cells (mNSCs) (Figure 1(c)). mDPSC clones were found to express a range of transcripts also expressed by NSCs including CD90, SCA1, GLAST, Sox2, Pax6, Myt1l, P75, BLBP, musashi, and NF-l. However, marked clonal differences were observed, with only the general stem cell marker SCA1, the neural crest-associated marker P75, and the radial glial protein BLBP being expressed by all mDPSC clones tested. No expression of CD133 was found in any mDPSC clone. Furthermore, clonal differences were identified in the mRNA expression levels of nestin. Based on a semiquantitative analysis, 8 out of 11 isolated clones appeared to express nestin mRNA transcripts at higher levels than the remaining 3 clones. However, only 4 of these clones continued to expand to allow the extraction of further RNA samples for a more accurate quantification of nestin expression and thus are subsequently described in this study. qPCR identified that clone 1 and clone 2 each expressed significantly higher levels ($p < 0.01$) of nestin transcripts than both clone 3 and clone 4 (Figure 1(d)). Subsequently, clones 1 and 2 were defined as high nestin-expressing clones and clones 3 and 4 as low nestin-expressing clones.

3.2. Neuronal-Like Differentiation. mDPSCs were typically bi/tripolar and fibroblastic-like in morphology prior to differentiation. Following 15 days of differentiation, clones initially identified as having high levels of nestin mRNA expression adopted a more neuronal-like phenotype with multiple neurite-like extensions. Conversely, no significant changes in morphology were observed in low nestin-expressing clones (Figure 2(a)). Immunocytochemical staining of the mature neuronal proteins Map2 and NF-l was identified in high, but not low, nestin-expressing mDPSC clones after 15 days of differentiation (Figure 2(b)). Changes in the mRNA expression of early and mature neuronal markers by high nestin-expressing clones over 15 days of differentiation were analyzed using qPCR (Figure 2(c)). By day 5 of differentiation, mRNA expression of SCA1 was strongly downregulated suggesting a transition from the default mesenchymal phenotype associated with mDPSCs. From day 5 onwards, after transferring to the neutrophin-containing maturation medium, expression of nestin and Map2 was seen to increase, indicating a more neuronal-like phenotype.

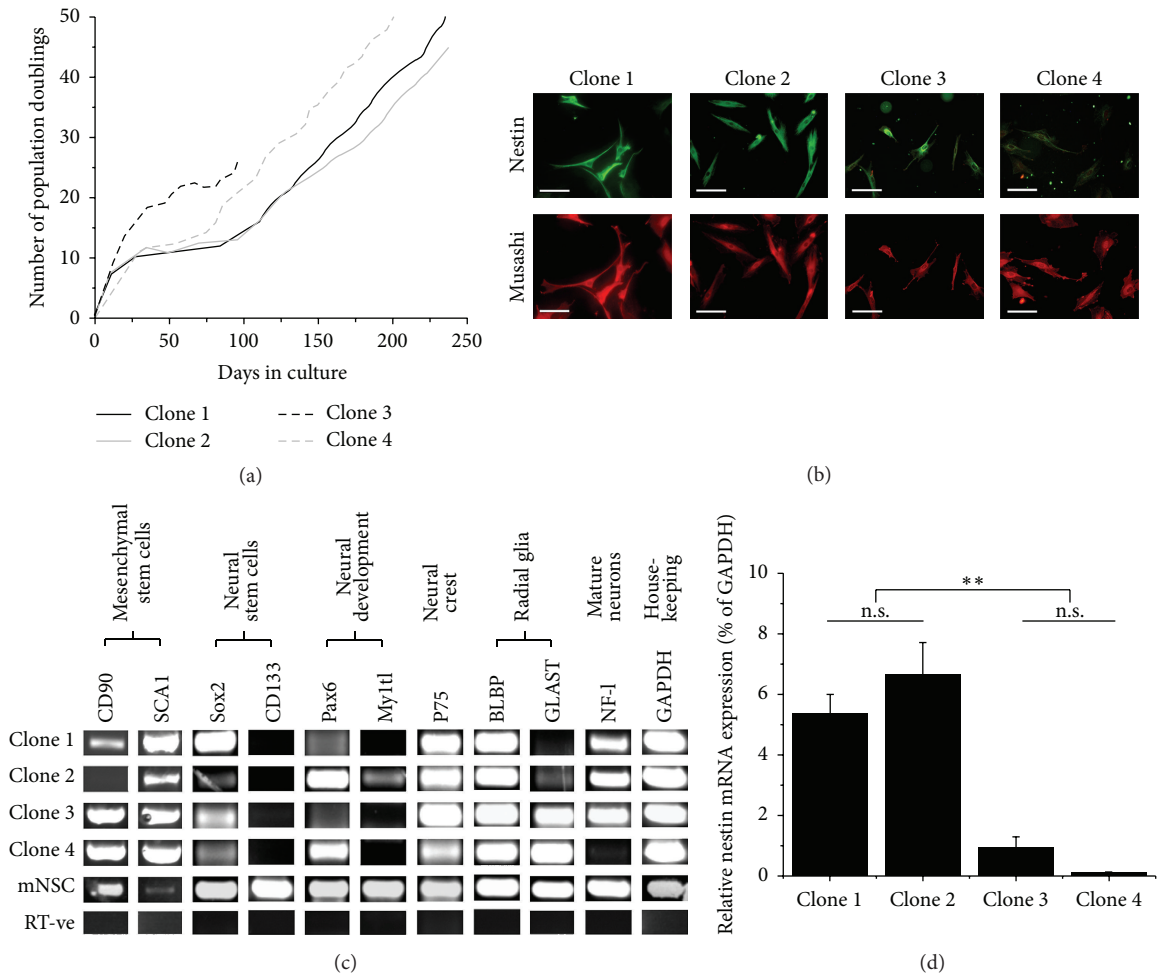


FIGURE 1: *In vitro* expansion and heterogeneity in the expression of developmental markers by clonal mDPSC cultures. (a) Single cell-derived clones, each expanded from a separate pulpal extraction, proliferated steadily for up to 240 days of culture reaching 50+ population doublings ($n = 4$ clones). Traces represent continuous culture growth from day of primary isolation, and cryopreserved cells continued to proliferate beyond the population doublings indicated. (b) Double immunostaining of clonal cultures for neural progenitor markers nestin and musashi. (c) RNA extracted from each clone between 20 and 40 population doublings was used in RT-PCR to identify clonal differences in the expression of RNA transcripts for CD90, stem cell antigen 1 (SCA1), glutamate aspartate transporter (GLAST), Sox2, Pax6, myelin transcription factor 1-like (Myt1l), P75, musashi, neurofilament light chain (NF-l), and CD133. (d) qPCR analysis of nestin mRNA expression by four mDPSC clones. Clones 1 and 2 were each individually found to express significantly higher levels of nestin than both clones 3 and 4. DPSC cultures were subsequently divided into strongly nestin-positive clones (clone 1 and clone 2) and weakly nestin-positive clones (clone 3 and clone 4). Nestin expression was calculated as a relative percentage of GAPDH ± SEM using the $2^{-\Delta\Delta CT}$ method ($n = 3$, RNA samples extracted from three separate passages per clone between 16 and 40 population doublings). One-way ANOVA with Tukey-Kramer posttest: n.s. = not significant, $^{**}p < 0.01$. Scale bars = 100 μm.

mRNA expression levels for NF-l were found to be unchanged during the 15 days of differentiation.

Patch clamp recordings were taken to characterise the electrophysiological properties of high nestin-expressing mDPSC clones prior to, and after, 15 days of differentiation. Primary cultured mSTM neurons provided a positive control for comparison purposes. Only outward K^+ currents were detected in mDPSCs, both before and after differentiation. These currents were effectively inhibited with 1 mM TEA, a nonselective blocker of K^+ channels (Figures 3(a) and 3(b)). At the same time, both voltage-activated K^+ and Na^+ currents were recorded in P0 mSTM neurons (Figure 3(c)). At a voltage of +80 mV, significantly higher K^+ current densities were recorded in P0 mSTM neurons (155.4 ± 10.1 pA/pF) compared to undifferentiated (6.4 ± 1.5 pA/pF) and neuronally differentiated mDPSCs (7.3 ± 1.4 pA/pF): $p < 3.0E - 13$ and $p < 6.0E - 15$, respectively. No significant difference was found between the current densities of undifferentiated and neuronally differentiated mDPSCs; however, membrane capacitance varied dramatically (Figure 3(d)). Differentiated mDPSCs (30.7 ± 4.0) possessed statistically significant lower membrane capacitances than undifferentiated mDPSCs (62.0 ± 10.2): $p < 0.005$. This lower capacitance was directly comparable with p0 mSTM

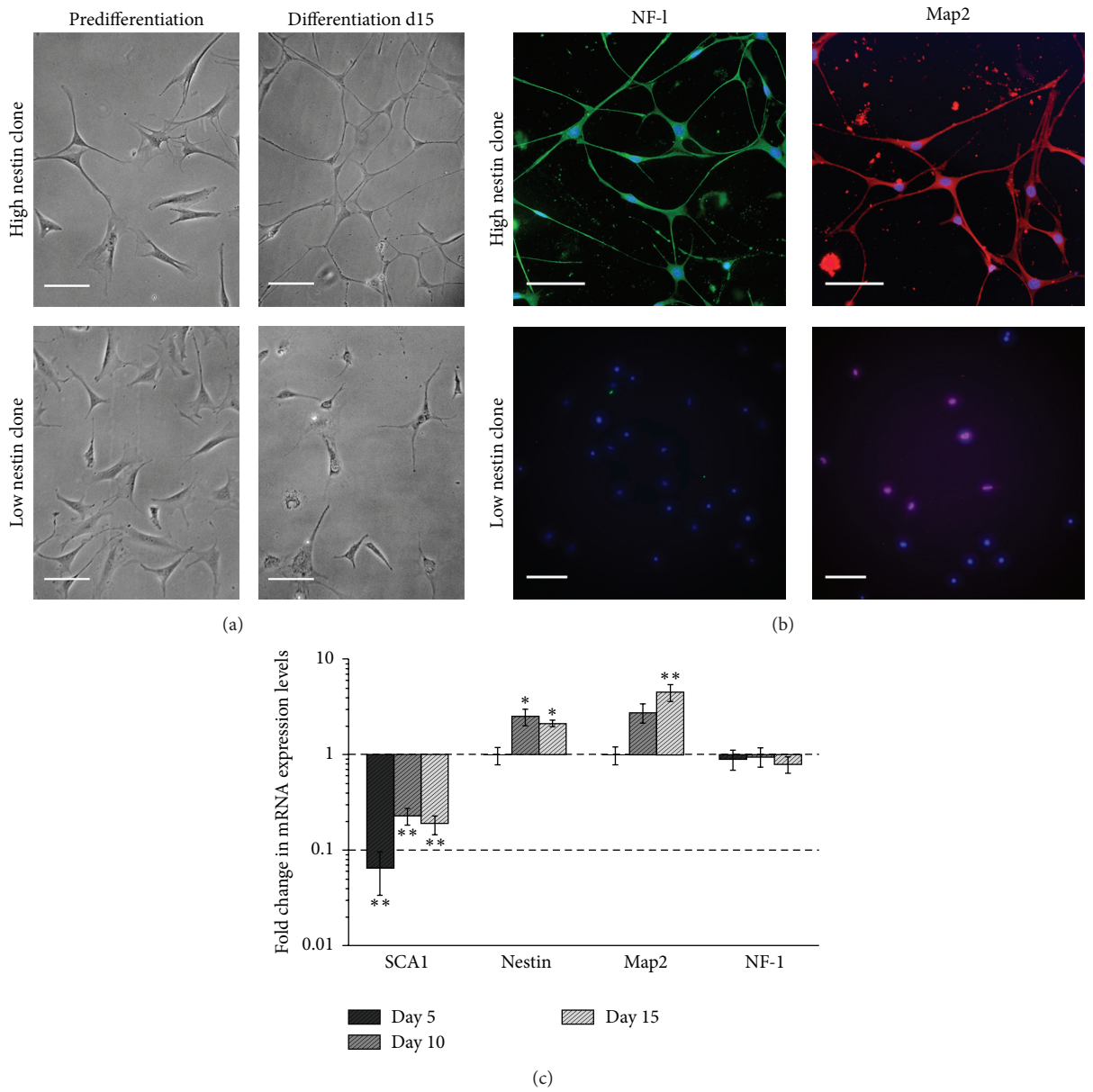


Figure 2: Only high nestin-expressing mDPSC clones possess the ability to differentiate into neuronal-like cells. (a) Representative phase contrast images of high (clone 2) and low (clone 3) nestin-expressing clones prior to and following 15 days of neuronal differentiation demonstrating a more neuronal-like morphology in high nestin-expressing clones with small refractive cell somas extending multiple interconnecting processes. (b) Immunocytochemical staining identified the presence of microtubule-associated protein 2 (Map2) and NF-l in high, but not low, nestin-expressing mDPSC clones following 15 days of neuronal differentiation. (c) Changes in mRNA expression of mesenchymal and neural markers during neuronal differentiation of high nestin-expressing mDPSC clones (clone 2). Expression levels of target genes were normalized against GAPDH and the $2^{-\Delta\Delta CT}$ method for qPCR analysis used to calculate fold change in expression relative to predifferentiation cells on day 0 ± SEM ($n = 3$ independent differentiation experiments). One-way ANOVA with Dunnett multiple comparisons posttest to identify significant increases/decreases in expression compared to day 0 cells: $^{*}p < 0.05$ and $^{**}p < 0.01$. Scale bars = 100 μm.

neurons (20.9 ± 6.6), with no significant difference observed ($p > 0.2$). Together, these results suggest that, despite appropriate morphology, the presence of mature neuronal proteins after 15 days of differentiation, and a comparable cell capacitance to primary cultured neurons, high nestin-expressing mDPSC clones maintain an electrophysiologically immature phenotype.

3.3. Oligodendrocyte-Like Differentiation. Following 10 days of differentiation, clones with initially high levels of nestin were seen to adopt a highly branched oligodendrocyte-like morphology. Although some branching was observed, low nestin-expressing clones largely failed to survive 10 days of differentiation (Figure 4(a)). Immunocytochemical staining of myelin basic protein (MBP), Olig1, and Olig2

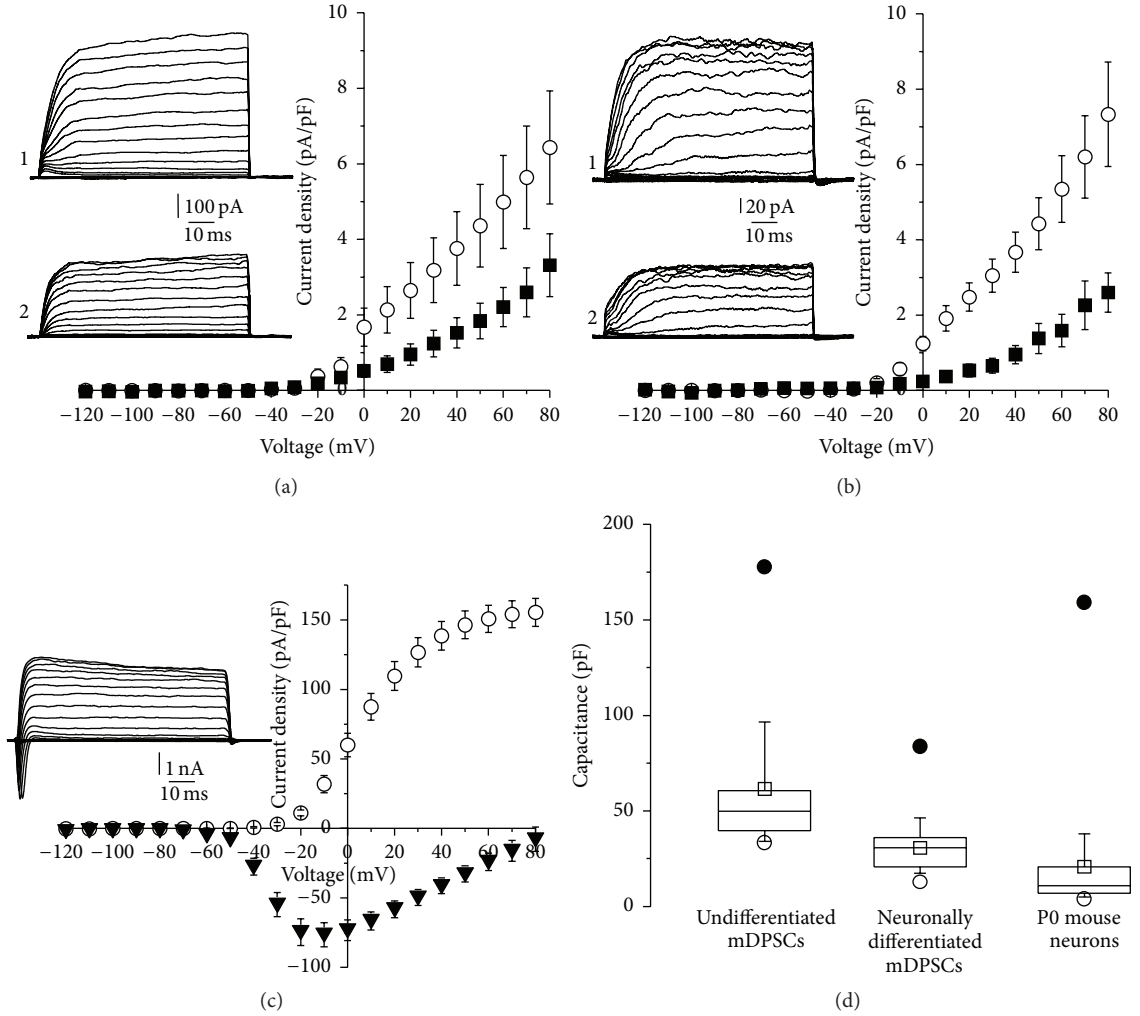


Figure 3: Neuronally differentiated high nestin-expressing mDPSCs show immature electrophysiology properties. Current density-voltage relationships of transmembrane K^+ currents of undifferentiated (a) and neuronally differentiated (b) high nestin-expressing mDPSCs (clone 2) in the absence (○, inlet 1 illustrates exemplar trace of currents, $n = 14$ and 17 cells, resp.) and presence of TEA (1 mM) (■, inlet 2 illustrates exemplar trace of currents, $n = 9$ for each differentiation condition). (c) Current density-voltage relationships of transmembrane K^+ current (○) and Na^+ currents (▼) of mSTM neurons ($n = 23$ cells). Inlet illustrates exemplar trace of currents. The mean ± SEM current densities at +80 mV of undifferentiated (6.4 ± 1.5 pA/pF) and neuronally differentiated mDPSCs (7.3 ± 1.4 pA/pF) showed a significant difference in comparison with primary cultured mSTM neurons (155.4 ± 10.1 pA/pF): $p < 3.0E - 13$ and $p < 6.0E - 15$, respectively. (d) Comparison of capacitances of all three cell types. The mean values ± SEM (□) of undifferentiated mDPSCs (62.0 ± 10.2) and neuronally differentiated high nestin-expressing mDPSCs (30.7 ± 4.0), as well as undifferentiated mDPSCs and P0 mSTM neurons (20.9 ± 6.6), were considered as significantly different: $p < 0.005$ and $p < 0.002$, respectively. ● and ○ are maximal and minimal values, respectively.

was only observed in high nestin-expressing mDPSC clones (Figure 4(b)). The expression of oligodendrocyte-associated proteins, together with appropriate morphology, suggests that this novel protocol may be used to derive an oligodendrocyte-like phenotype from mDPSCs with high levels of nestin expression.

4. Discussion

In this study we have identified heterogeneity in the ability of single cell-derived clonal cultures of mDPSCs to differentiate into neuronal-like and glial-like cells. Those clones possessing the highest levels of mRNA expression for the neuronal progenitor-associated intermediate filament protein, nestin, showed a greater potential for differentiation down both neural lineages. Although some evidence of variability in the neural differentiation potential of heterogeneous DPSC has been previously described [18, 22], our findings suggest that nestin may act as a suitable marker for which to screen DPSC cultures *in vitro* prior to use in neural tissue engineering applications. The problems associated with cellular heterogeneity are increasingly becoming recognised in the stem cell research field and gaining a fuller understanding of the variability within transplantable populations will help maximise the potential of any stem cell-based therapy.

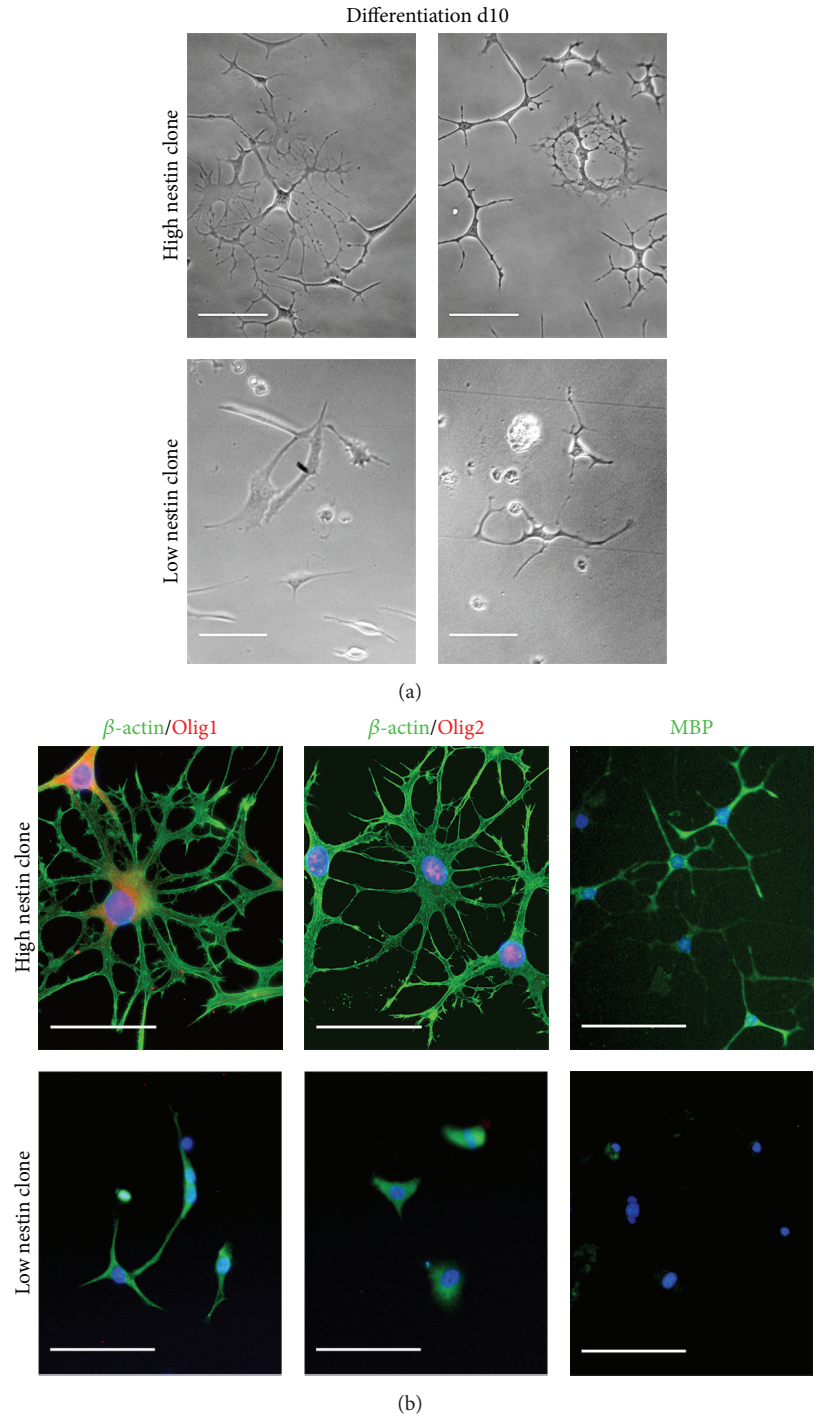


FIGURE 4: Only high nestin-expressing mDPSC clones display the ability to differentiate into oligodendrocyte-like cells. (a) Representative phase contrast images of high (clone 2) and low (clone 3) nestin-expressing clones following 15 days of oligodendrocyte-like differentiation. Clones with higher levels of nestin mRNA were found to adopt a more highly branched oligodendrocyte-like morphology compared to lower nestin-expressing clones. (b) Immunocytochemical staining identified the presence of myelin basic protein (MBP) and the oligodendrocyte transcription factors Olig1 and Olig2 in high, but not low, nestin-expressing mDPSC clones following 10 days of differentiation. β-actin staining was performed to demonstrate highly branched morphology. Scale bars = 100 μm.

Prior to differentiation, the expression of a range of developmental and neural progenitor markers by single cell-derived mDPSC cultures was extensively analyzed and compared with primary mNSCs. Although mDPSCs were found to express a number of markers also associated with NSCs including Sox2, Pax6, GLAST, BLBP, nestin, and NF-l, expression patterns were highly variable between clones, demonstrating the degree of heterogeneity that exists within the mixed populations of DPSCs typically used for neural transplantation studies [10–15]. Importantly, only those clones identified with high levels of nestin mRNA expression displayed the ability to differentiate into a neuronal-like phenotype based on cell morphology and increased expression levels of the more mature neuronal marker Map2. To test the electrophysiological properties of these cells, patch clamp recordings were made. The electrical properties of neuronal-like cells derived from murine DPSCs remain largely uncharacterised in contrast to human DPSCs in which voltage-activated Na^+ and K^+ currents and ATP-activated Ca^{2+} surges have been recorded [17, 18, 20, 21]. In the only previous functional study using rodent DPSCs, mixed populations of mDPSCs differentiated using an established protocol displayed voltage-activated Ca^{2+} but not K^+ or Na^+ currents, directly contradicting recordings taken when the same protocol was applied to human hDPSCs [18, 19]. Single cell-derived cultures of high nestin-expressing mDPSCs differentiated using the protocol described in this report, on the other hand, display TEA-sensitive voltage-gated K^+ currents, demonstrating the presence of functional voltage-activated K^+ channels in neuronally differentiated rodent DPSCs for the first time. Although the amplitude of these currents is reduced when compared to mSTM neurons, similar membrane capacitances were measured for each cell type. This reduction in capacitance is indicative of cells with an ability to store electrical charge directly comparable to primary cultured striatal neurons, confirming a more neuronal-like phenotype after differentiation. Although a fully functional phenotype with the ability to fire action potentials has yet to be derived from either human or rodent DPSCs, there is sufficient evidence here to suggest that high nestin-expressing mDPSCs may be promoted to differentiate, at least partially, along this lineage. However, further steps will be required to obtain a more mature neuronal-like phenotype and future studies might focus on incorporating a supporting cell type in coculture to provide appropriate trophic factor support for the development and maturation of functional properties, for example, astrocytes [28, 29].

Oligodendrocyte-like differentiation of DPSCs has only previously been described *in vivo*, following mixed population transplantation into a rat model of spinal cord injury [12]. Using a novel protocol adapted from those used in the culture and differentiation of oligodendrocyte progenitor cells (OPCs) [30, 31], mDPSC clones with high levels of nestin mRNA expression adopted a highly branched oligodendrocyte-like morphology and stained positive for oligodendrocyte markers Olig1, Olig2, and MBP. Despite the expression of MBP in differentiated mDPSCs, there was no observation of membranous sheets associated with mature myelinating oligodendrocytes *in vitro* [30, 32]. This suggests that, similar to neuronal differentiation, high nestin-expressing mDPSCs are able to differentiate partially to an immature premyelinating phenotype, but further differentiation steps may be required for full functionality. Nevertheless, the development of this protocol represents a significant finding and may provide a useful *in vitro* research tool for further studies into mechanisms through which DPSCs may promote central nervous system repair and regeneration.

Unlike bone marrow, another common source of mesenchymal stem cells, dental pulp is a nonhaematopoetic tissue and clonal DPSC cultures may be more lineage-restricted in nature [9]. Their highly heterogeneous nature is purported to be attributable to multiple populations of progenitor cells residing in different locations of the pulp which may possess different proliferative and differentiation capabilities. Different niches have been identified *in situ* associated with the vasculature, within the pulpal stroma, in the subodontoblast layer and amongst peripheral nerve-associated glial cells [33–38]. During development, the dental pulp and central nervous system both derive from the embryonic ectoderm. Following neurulation, multipotent neural crest cells migrate away from the neural tube into developing craniofacial tissues. At the initiation of tooth morphogenesis, these cells populate the underlying mesenchyme, eventually giving rise to the cellular components of pulpal tissue [39]. Multipotent adult DPSCs that maintain neural crest stem cell characteristics and may represent a source of cells with greater potential for neuronal and glial differentiation given their developmental origin have been isolated from different niches within the pulp [25, 38, 40, 41]. A recent study compared the proliferative and differentiation potentials of human DPSCs based on the expression of the pericyte-associated cell surface antigen CD34 [42]. Only $CD34^+$ hDPSCs were found to express nestin and possess the ability to differentiate down neuronal lineages, similar to the high nestin-expressing mDPSC described here, suggesting that they may be neural crest in origin and derived from a perivascular-associated niche. It may prove beneficial to select for such DPSCs in future neural tissue engineering studies.

Together, the results presented herein suggest that mRNA levels of nestin may be indicative of the potential of mDPSCs for neuronal-like and oligodendrocyte-like differentiation. Nestin expression is associated with stem cells in the developing neural tube as well as specific subtypes of OPCs [43, 44]. As such, its link to mDPSCs with neuronal and oligodendrocyte-like differentiation capabilities fits. However, nestin-positive cells make up only a small fraction of the total cellular component of dental pulp, less than 3.5% reported in isolates from rat incisors [45]. Most published studies utilise such mixed populations of cells and so likely contain a significant proportion of other cell types, perhaps explaining previous inconsistencies in response to neuronal differentiation cues [18, 22]. The use of clonally derived cultures allows investigations to be carried out at the single cell level and the subsequent identification of differences between individual clonal cell lines. Large differences in the proliferation and mineralisation potential

of clonal DPSC cultures have been previously reported in this manner [1, 9]. Similarly, differences in the neuronal-like and oligodendrocyte-like differentiation potential of mDPSC clones are reported here. The use of single cell-derived clones is unlikely to be therapeutically applicable due to scalability issues within short time frames. However, clonal cultures serve as an extremely useful research tool to identify desirable properties of cells within mixed populations. In future studies, the screening of single cell-derived clones on a larger scale to that described in this report will serve to further our understanding of cellular heterogeneity and its implications for the development of stem cell-based therapies.

5. Conclusions

Significant heterogeneity exists between clonal cultures of mDPSCs and clones with comparatively higher levels of nestin expression possess a greater capacity for differentiation into neural lineages. These findings help explain previous reports of only small numbers of transplanted DPSCs adopting neuronal-like and glial-like phenotypes after transplantation, as well as inconsistencies in *in vitro* differentiation studies. In conclusion, high nestin-expressing DPSCs may represent a more desirable cell source for promoting central nervous system repair and regeneration.

Competing Interests

The authors declare that they have no competing interests.

Acknowledgments

This study was funded by European Research Council StG Grant 243261, Wellcome Trust Grant WT082887, and the Royal Society URF Award UF051616 to Bing Song.

References

[1] S. Gronthos, J. Brahim, W. Li et al., "Stem cell properties of human dental pulp stem cells," *Journal of Dental Research*, vol. 81, no. 8, pp. 531–535, 2002.

[2] A. Balic, H. L. Aguila, M. J. Caimano, V. P. Francone, and M. Mina, "Characterization of stem and progenitor cells in the dental pulp of erupted and unerupted murine molars," *Bone*, vol. 46, no. 6, pp. 1639–1651, 2010.

[3] V. Govindasamy, A. N. Abdullah, V. S. Ronald et al., "Inherent differential propensity of dental pulp stem cells derived from human deciduous and permanent teeth," *Journal of Endodontics*, vol. 36, no. 9, pp. 1504–1515, 2010.

[4] K. Iohara, L. Zheng, H. Wake et al., "A novel stem cell source for vasculogenesis in ischemia: subfraction of side population cells from dental pulp," *STEM CELLS*, vol. 26, no. 9, pp. 2408–2418, 2008.

[5] I. Kerkis, C. E. Ambrosio, A. Kerkis et al., "Early transplantation of human immature dental pulp stem cells from baby teeth to golden retriever muscular dystrophy (GRMD) dogs: local or systemic?" *Journal of Translational Medicine*, vol. 6, article 35, 2008.

[6] F. Paino, G. Ricci, A. De Rosa et al., "Ecto-mesenchymal stem cells from dental pulp are committed to differentiate into active melanocytes," *European Cells & Materials*, vol. 20, pp. 295–305, 2010.

[7] R. Nakatsuka, T. Nozaki, Y. Uemura et al., "5-Aza-2′-deoxycytidine treatment induces skeletal myogenic differentiation of mouse dental pulp stem cells," *Archives of Oral Biology*, vol. 55, no. 5, pp. 350–357, 2010.

[8] N. Ishkitiev, K. Yaegaki, T. Imai et al., "High-purity hepatic lineage differentiated from dental pulp stem cells in serum-free medium," *Journal of Endodontics*, vol. 38, no. 4, pp. 475–480, 2012.

[9] J. Harrington, A. J. Sloan, and R. J. Waddington, "Quantification of clonal heterogeneity of mesenchymal progenitor cells in dental pulp and bone marrow," *Connective Tissue Research*, vol. 55, no. 1, pp. 62–67, 2014.

[10] F. M. De Almeida, S. A. Marques, B. D. S. Ramalho et al., "Human dental pulp cells: a new source of cell therapy in a mouse model of compressive spinal cord injury," *Journal of Neurotrauma*, vol. 28, no. 9, pp. 1939–1949, 2011.

[11] M. Sugiyama, K. Iohara, H. Wakita et al., "Dental pulp-derived $CD31^{-}/CD146^{-}$ side population stem/progenitor cells enhance recovery of focal cerebral ischemia in rats," *Tissue Engineering—Part A*, vol. 17, no. 9-10, pp. 1303–1311, 2011.

[12] K. Sakai, A. Yamamoto, K. Matsubara et al., "Human dental pulp-derived stem cells promote locomotor recovery after complete transection of the rat spinal cord by multiple neuro-regenerative mechanisms," *The Journal of Clinical Investigation*, vol. 122, no. 1, pp. 80–90, 2012.

[13] C.-Z. Fang, Y.-J. Yang, Q.-H. Wang, Y. Yao, X.-Y. Zhang, and X.-H. He, "Intraventricular injection of human dental pulp stem cells improves hypoxic-ischemic brain damage in neonatal rats," *PLoS ONE*, vol. 8, no. 6, article e66748, 2013.

[14] A. Yamamoto, K. Sakai, K. Matsubara, F. Kano, and M. Ueda, "Multifaceted neuro-regenerative activities of human dental pulp stem cells for functional recovery after spinal cord injury," *Neuroscience Research*, vol. 78, no. 1, pp. 16–20, 2014.

[15] H. Fujii, K. Matsubara, K. Sakai et al., "Dopaminergic differentiation of stem cells from human deciduous teeth and their therapeutic benefits for Parkinsonian rats," *Brain Research*, vol. 1613, pp. 59–72, 2015.

[16] F. Young, A. Sloan, and B. Song, "Dental pulp stem cells and their potential roles in central nervous system regeneration and repair," *Journal of Neuroscience Research*, vol. 91, no. 11, pp. 1383–1393, 2013.

[17] A. Arthur, G. Rychkov, S. Shi, S. A. Koblar, and S. Gronthose, "Adult human dental pulp stem cells differentiate toward functionally active neurons under appropriate environmental cues," *Stem Cells*, vol. 26, no. 7, pp. 1787–1795, 2008.

[18] M. Király, B. Porcsalmy, Á. Pataki et al., "Simultaneous PKC and cAMP activation induces differentiation of human dental pulp stem cells into functionally active neurons," *Neurochemistry International*, vol. 55, no. 5, pp. 323–332, 2009.

[19] K. M. Ellis, D. C. O'Carroll, M. D. Lewis, G. Y. Rychkov, and S. A. Koblar, "Neurogenic potential of dental pulp stem cells isolated from murine incisors," *Stem Cell Research and Therapy*, vol. 5, article 30, 2014.

[20] M. Kanafi, D. Majumdar, R. Bhonde, P. Gupta, and I. Datta, "Midbrain cues dictate differentiation of human dental pulp stem cells towards functional dopaminergic neurons," *Journal of Cellular Physiology*, vol. 229, no. 10, pp. 1369–1377, 2014.

[21] P. Gervois, T. Struys, P. Hilkens et al., "Neurogenic maturation of human dental pulp stem cells following neurosphere generation induces morphological and electrophysiological characteristics of functional neurons," *Stem Cells and Development*, vol. 24, no. 3, pp. 296–311, 2015.

[22] R. Aanismaa, J. Hautala, A. Vuorinen, S. Miettinen, and S. Narkilahti, "Human dental pulp stem cells differentiate into neural precursors but not into mature functional neurons," *Stem Cell Discovery*, vol. 2, no. 3, pp. 85–91, 2012.

[23] P. H. Jones and F. M. Watt, "Separation of human epidermal stem cells from transit amplifying cells on the basis of differences in integrin function and expression," *Cell*, vol. 73, no. 4, pp. 713–724, 1993.

[24] G. P. Dowthwaite, J. C. Bishop, S. N. Redman et al., "The surface of articular cartilage contains a progenitor cell populations," *Journal of Cell Science*, vol. 117, no. 6, pp. 889–897, 2004.

[25] R. J. Waddington, S. J. Youde, C. P. Lee, and A. J. Sloan, "Isolation of distinct progenitor stem cell populations from dental pulp," *Cells Tissues Organs*, vol. 189, no. 1–4, pp. 268–274, 2009.

[26] X. Meng, W. Li, F. Young et al., "Electric field-controlled directed migration of neural progenitor cells in 2D and 3D environments," *Journal of Visualized Experiments*, no. 60, 2012.

[27] K. J. Livak and T. D. Schmittgen, "Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C_T}$ method," *Methods*, vol. 25, no. 4, pp. 402–408, 2001.

[28] X. Tang, L. Zhou, A. M. Wagner et al., "Astroglial cells regulate the developmental timeline of human neurons differentiated from induced pluripotent stem cells," *Stem Cell Research*, vol. 11, no. 2, pp. 743–757, 2013.

[29] D. Prè, M. W. Nestor, A. A. Sproul et al., "A time course analysis of the electrophysiological properties of neurons differentiated from human induced Pluripotent Stem Cells (iPSCs)," *PLoS ONE*, vol. 9, no. 7, Article ID e103418, 2014.

[30] A. Jagielska, A. L. Norman, G. Whyte, K. J. V. Vliet, J. Guck, and R. J. M. Franklin, "Mechanical environment modulates biological properties of oligodendrocyte progenitor cells," *Stem Cells and Development*, vol. 21, no. 16, pp. 2905–2914, 2012.

[31] B. Zhu, C. Zhao, F. I. Young, R. J. M. Franklin, and B. Song, "Isolation and long-term expansion of functional, myelinating oligodendrocyte progenitor cells from neonatal rat brain," *Current Protocols in Stem Cell Biology*, 2014.

[32] C. A. Dyer and J.-M. Matthieu, "Antibodies to myelin/oligodendrocyte-specific protein and myelin/oligodendrocyte glycoprotein signal distinct changes in the organization of cultured oligodendroglial membrane sheets," *Journal of Neurochemistry*, vol. 62, no. 2, pp. 777–787, 1994.

[33] S. Shi and S. Gronthos, "Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp," *Journal of Bone and Mineral Research*, vol. 18, no. 4, pp. 696–704, 2003.

[34] H. Løvschall, M. Tummers, I. Thesleff, E.-M. Füchtbauer, and K. Poulsen, "Activation of the Notch signaling pathway in response to pulp capping of rat molars," *European Journal of Oral Sciences*, vol. 113, no. 4, pp. 312–317, 2005.

[35] O. Téclès, P. Laurent, S. Zygouritsas et al., "Activation of human dental pulp progenitor/stem cells in response to odontoblast injury," *Archives of Oral Biology*, vol. 50, no. 2, pp. 103–108, 2005.

[36] J. Feng, A. Mantesso, C. De Bari, A. Nishiyama, and P. T. Sharp, "Dual origin of mesenchymal stem cells contributing to organ growth and repair," *Proceedings of the National Academy of Sciences of the United States of America*, vol. 108, no. 16, pp. 6503–6508, 2011.

[37] N. F. Lizier, A. Kerkis, C. M. Gomes et al., "Scaling-up of dental pulp stem cells isolated from multiple niches," *PLoS ONE*, vol. 7, no. 6, Article ID e39885, 2012.

[38] N. Kaukua, M. K. Shahidi, C. Konstantinidou et al., "Glial origin of mesenchymal stem cells in a tooth model system," *Nature*, vol. 513, no. 7519, pp. 551–554, 2014.

[39] Y. Chai, X. Jiang, Y. Ito et al., "Fate of the mammalian cranial neural crest during tooth and mandibular morphogenesis," *Development*, vol. 127, no. 8, pp. 1671–1679, 2000.

[40] K. Janebodin, O. V. Horst, N. Ieronimakis et al., "Isolation and characterization of neural crest-derived stem cells from dental pulp of neonatal mice," *PLoS ONE*, vol. 6, no. 11, Article ID e27526, 2011.

[41] S. Abe, K. Hamada, M. Miura, and S. Yamaguchi, "Neural crest stem cell property of apical pulp cells derived from human developing tooth," *Cell Biology International*, vol. 36, no. 10, pp. 927–936, 2012.

[42] A. Pisciotta, G. Carnevale, S. Meloni et al., "Human Dental pulp stem cells (hDPSCs): isolation, enrichment and comparative differentiation of two sub-populations Integrative control of development," *BMC Developmental Biology*, vol. 15, no. 1, article 14, 2015.

[43] U. Lendahl, L. B. Zimmerman, and R. D. G. McKay, "CNS stem cells express a new class of intermediate filament protein," *Cell*, vol. 60, no. 4, pp. 585–595, 1990.

[44] G. Almazán, J. M. Vela, E. Molina-Holgado, and C. Guaza, "Re-evaluation of nestin as a marker of oligodendrocyte lineage cells," *Microscopy Research and Technique*, vol. 52, no. 6, pp. 753–765, 2001.

[45] M. Takeyasu, T. Nozaki, and M. Daito, "Differentiation of dental pulp stem cells into a neural lineage," *Pediatric Dental Journal*, vol. 16, no. 2, pp. 154–162, 2006.