Stem Cells International

Stem Cell Approaches to Dental Tissue Engineering and Regenerative Dentistry

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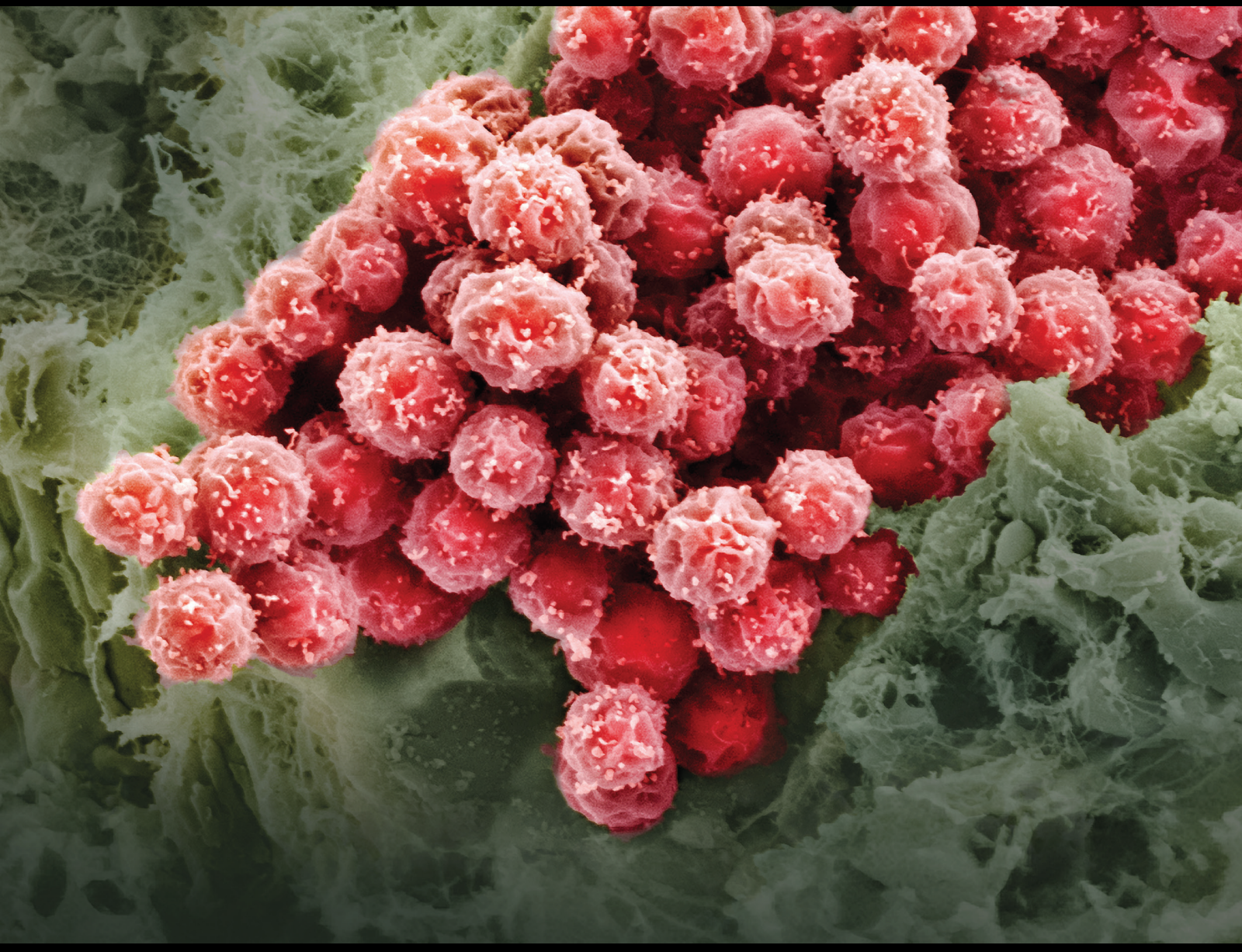

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Review Article

Induced Pluripotent Stem Cells in Dental and Nondental Tissue Regeneration: A Review of an Unexploited Potential

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Guest Editor: Alireza Moshaverinia

Cell-based therapies currently represent the state of art for tissue regenerative treatment approaches for various diseases and disorders. Induced pluripotent stem cells (iPSCs), reprogrammed from adult somatic cells, using vectors carrying definite transcription factors, have manifested a breakthrough in regenerative medicine, relying on their pluripotent nature and ease of generation in large amounts from various dental and nondental tissues. In addition to their potential applications in regenerative medicine and dentistry, iPSCs can also be used in disease modeling and drug testing for personalized medicine. The current review discusses various techniques for the production of iPSC-derived osteogenic and odontogenic progenitors, the therapeutic applications of iPSCs, and their regenerative potential in vivo and in vitro. Through the present review, we aim to explore the potential applications of iPSCs in dental and nondental tissue regeneration and to highlight different protocols used for the generation of different tissues and cell lines from iPSCs.

1. Introduction

Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of the blastocyst. They can give rise to tissues derived from the three germ layers and are regarded as a renewable potent cell source for the regeneration of all body tissues [1–4]. However, ES usage in regenerative medicine faces a lot of obstacles as their isolation requires destruction of human embryos which raises justified ethical objections. ES can also elicit an immune response upon transplantation in patients [5]. In 2006, Takahashi et al. [6] demonstrated that mature differentiated cells can be reprogrammed and dedifferentiated into embryonic-like cells, with ES-like properties. Mature murine fibroblast cell lines were reversed into pluripotency via retroviral transduction of 4 transcription factors, POU domain class 5 transcription factor 1 (Oct3/4), the sex-determining region Y-box2 (Sox2), Kruppel-like factor 4 (Klf4), and myelocytomatosis oncogene (c-Myc), giving rise to induced pluripotent stem cells (iPSCs). Those four transcription factors (also referred to as OSKM factors) were postulated to be responsible for the maintenance of ES inherent pluripotency. Over the subsequent years, iPSCs were generated from a variety of adult tissues [7–9] and were similar to ES in morphology, proliferative rates, surface antigens, expressed genes, and in vivo teratoma formation [6].

2. iPSC Source and Generation (Reprogramming) Methods

iPSCs were successfully generated from different dental and nondental tissues (Figure 1) including fibroblasts, keratinocytes, melanocyte blood cells, bone marrow cells, adipose cells, tissue-resident progenitor cells, and gingival and

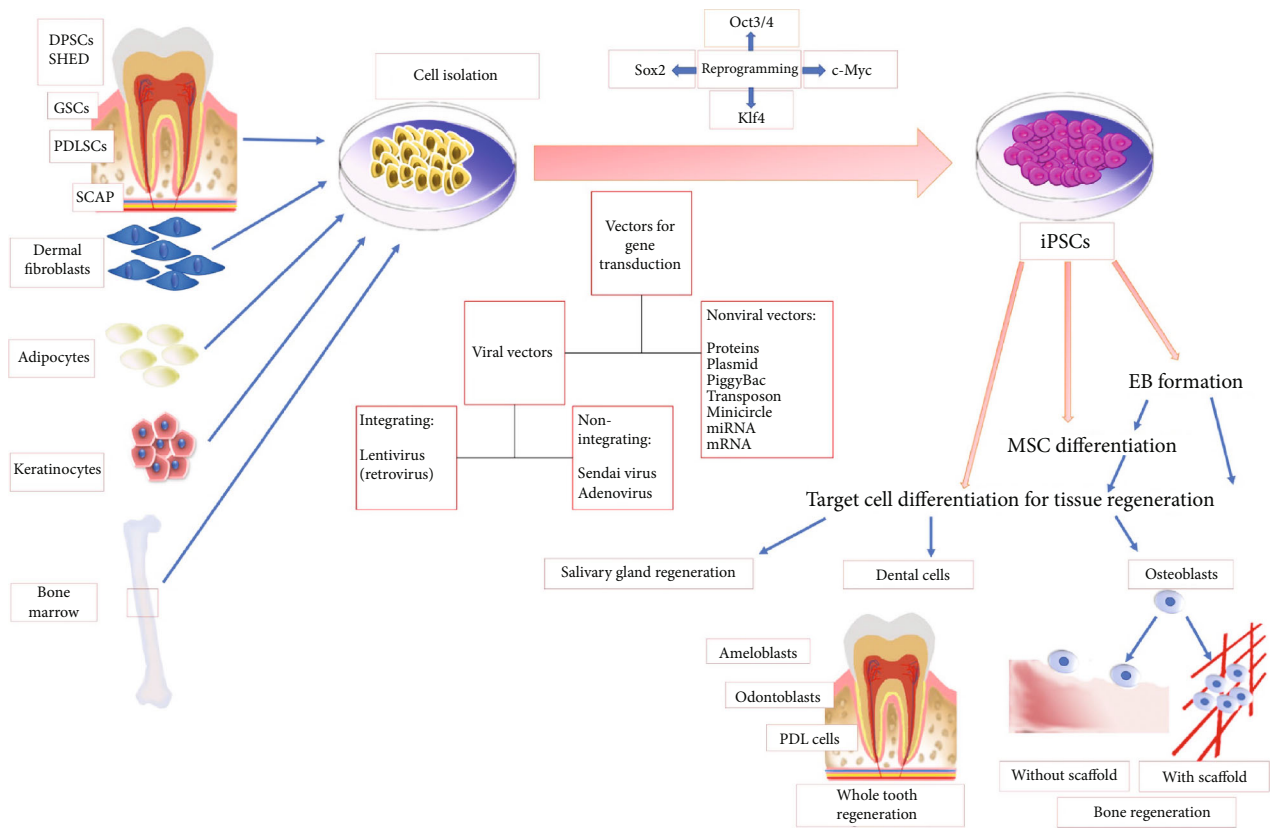

Figure 1: Diagram summarizing iPSC source, methods of gene transduction, and iPSC differentiation. Dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHED), gingival stem cells (GSCs), stem cells from apical dental papilla (SCAP), embryoid bodies (EB), mesenchymal stem cells (MSCs), and induced pluripotent stem cells (iPSCs).

periodontal ligament fibroblasts [10–13] via transduction of Oct3/4, Sox2, and Klf4 [14, 15]. iPSCs were also successfully generated from dental pulp stem cells (DPSCs) [16–18], stem cells from human exfoliated deciduous teeth (SHED) [18, 19], and stem cells from apical dental papilla [18]. Gingival fibroblast-derived iPSCs were considered to be advantageous over dermal fibroblasts (DF) as they could be easily acquired during routine dental treatment and were effectively reprogrammed into iPSCs [14].

As mentioned above, generation of iPSCs depends on the transduction of specific transcription factors into the somatic cell genome via vectors for its reprogramming [20]. Vectors used during the generation of iPSCs can be divided into integrative viral vectors, integrative free vectors, and nonviral vectors [21]. Originally, lentivirus (a retrovirus), an integrating viral vector, was used for iPSC generation with high reprogramming efficacy [6]. Despite offering a high transduction ability, integrating viral vectors insert their whole genome into recipient cells and may introduce oncogenes or genetic mutations into the host cells [22] (Figure 1).

Nonintegrating viruses, such as Sendai virus and adenovirus, were subsequently introduced in an attempt to overcome these drawbacks [23]. Tashiro et al. [24] compared four types of promoters (RSV, CMV, cytomegalovirus enhancer/b-actin (CA), and elongation factor-1a (EF-1a)) using adenovirus vectors for iPSC induction. An adenovirus vector containing EF-1a and CA promoter efficiently transduced transgenes into mouse iPSCs, without a decrease in pluripotency or viability. An optimized adenovirus vector that was developed by the authors enhanced adipocyte and osteoblast differentiation, confirmed by significant gene expressions of peroxisome proliferator-activated receptor c and runt-related transcription factor 2 (RUNX2), respectively, by iPSCs.

To avoid an increased risk of tumor generation and chromosomal instability, nonviral vectors were subsequently introduced for the somatic reprogramming process, including proteins, plasmid, piggyBac transposon, minicircle vector, miRNA, and mRNA [25–30]. Gene-editing technologies like CRISPR/Cas9, zinc finger nucleases, and transcription activator-like effector nucleases (TALENs) were additionally employed for genome editing of iPSCs to introduce certain traits for disease modeling and cancer research or to alter their gene expression for possible application in the field of regenerative medicine [31].

3. Assessment of Pluripotency

Following iPSC generation, cells have to be assessed via pluripotency assays, including morphological and histological analysis, and certain gene expressions, proving their ability to differentiate into tissues derived from the three germ layers

and teratoma formation [32]. Teratoma assays involve injection of iPSCs into immunocompromised experimental animals and subsequent formed tissue analysis to assure teratoma formation [33]. Alternatively, in vitro embryoid body (EB) generation can be used to ascertain pluripotency; EB is a mass of cells derived from all three germ layers [32], generated from iPSCs upon culturing in proper media [32, 34, 35]. EB generation encompasses the homogeneous method as the liquid suspension method and the heterogeneous method as the hanging drop culture. While the heterogeneous method is considered the easiest way to generate EB, the resulting cell masses are largely heterogeneous in size [36], which are irreproducible [37] and negatively affect subsequent iPSC differentiation towards a specific cell line [38]. The homogeneous method, on the other hand, creates cell masses of more homogeneous, uniform sizes that subsequently enhance cell viability and facilitate their subsequent differentiation into specific cell lines [33, 39]. To avoid tumor formation, prior to implantation, iPSCs are either differentiated into mesenchymal stem cells (MSCs) or targeted tissue cell types with or without EB formation (Figure 1).

4. iPSCs in Dental and Nondental Tissue Regeneration (Table 1)

4.1. iPSCs and Bone Regeneration. Although autogenous bone graft remains to be the gold standard for reconstruction of bone defects [40], it carries the risk of bone resorption and donor site infection and the graft may not always be available in sufficient amounts [41]. iPSC technologies may provide a suitable alternative to autogenous grafting, whereby patients' somatic cells are induced into bone-forming cells that are loaded on an appropriate scaffold in combination with proper bioactive molecules for bone tissue engineering [42]. To induce osteogenic differentiation of iPSCs, a variety of agents were proposed in isolation or combination, including osteogenic media, ascorbic acid, b-glycerophosphate, dexamethasone, bone morphogenetic proteins (BMPs), and vitamin D_3 [43–46]. Osteogenic differentiation is followed by proper characterization of generated bone cells through their expression of osteogenesis-related genes (RUNX2, osteopontin (OPN), osterix (OSX), osteocalcin (OCN), and collagen type I (COL1A1)) [47–50] in addition to the evaluation of in vitro mineralization and alkaline phosphatase (ALP) activity [51, 52].

Osteogenic potential of human iPSCs was demonstrated on polymeric nanofibrous polyethersulfone (PES) scaffold with upregulated expressions of osteogenic genes and alkaline phosphatase activity in vitro [48, 53]. The expression of key osteoblast-related genes in undifferentiated iPSCs was nearly 30 times higher than in undifferentiated ES cells. On the contrary, the expression of the same genes in ES- and iPSC-derived osteoblasts was not significantly different except for OPN and COL1A1, which were significantly higher in iPSC-derived osteoblasts [51]. Evidence revealed that ES cells and iPSCs generated from transgenic mice expressing rat 2.3 kb type I collagen promoter-driven green fluorescent protein (Col2.3GFP) successfully differentiated into osteoblast lineage cells that expressed Col2.3GFP in vitro [54]. Gene expression profiles proved that ES- and iPSC-derived osteoblasts resemble osteoblasts present in the calvaria [54].

The osteoinductive properties of iPSC-derived bone cells and their capability in treating bone defects were further assessed in vivo by their implantation into a severe combined immunodeficiency (SCID) mouse model. Bone formation was confirmed four weeks following implantation by soft X-ray images [43], X-ray microcomputed tomography (μCT) [55], cone beam computed tomography imaging [49], and histological tissue specimens [43, 47–52]. In a cleidocranial dysostosis model, the mutation in RUNX2 gene was repaired in iPSCs derived from mucosal tissues of affected patients. The reverted cells revealed marked upregulation of osteoblast differentiation markers after being cultured in OM for nine days. Loading the differentiated osteoblasts originating from iPSCs with a corrected mutation on a peptide nanofiber scaffold and implanting them into SCID rats' calvarial bone defects revealed reossification four weeks after transplantation with a significant increase in bone volume and bone mineral content [52]. Similarly, osteogenic cells differentiating from EB derived from iPSCs showed positive results in bone regeneration and healing following implantation in the rats' critical-sized calvarial defect [53, 56, 57] and long bone segmental defect rat model [57] after being loaded on polymeric nanofibrous PES scaffold [53], fibrin glue scaffold [57], hydroxyapatite (HA)/b-tricalcium phosphate scaffold [57], or self-assembling peptide nanofiber hydrogel scaffold [56]. Moreover, iPSCs differentiated into functional osteoblasts and demonstrated a bone regenerative effect comparable to human bone marrow-(BM-) MSCs in vivo [57].

4.1.1. Osteogenic Potential of iPSCs-MSCs Obtained through EB Formation. This method entails the differentiation of MSCs from EB-derived iPSCs. It was suggested to possess notable advantages over direct differentiation of iPSCs into osteoblasts, with the resulting osteogenic cells demonstrating a significant upregulation of osteoblast-related genes including ALP, RUNX2, COL1A1, and OCN [58, 59]. Several factors were demonstrated to influence the osteogenic potential of iPSC-derived MSCs including the incorporation of retinoic acid, transforming growth factor-beta (TGF-β) [60, 61], or metformin into the culture media [62] as well as coseeding with other cell types [63–65]. The suspension time of EB and genetic modification of iPSCs-MSCs also proved to affect their osteogenic capability [66–68]. Culturing EB generated from dermal fibroblast iPSCs in media supplemented with TGF-β induced MSC differentiation. Two populations of MSCs were recognized, early MSCs that migrated from EB during days 2–5 and late MSCs that crawled from EB during days 5–8. The two iPSC-derived MSC populations and BM-MSCs were transduced with BMP-6 plasmid. Resulting cells were either suspended in fibrin gel and injected into thigh muscles of SCID rats or loaded on collagen scaffolds and implanted in a nonunion radial fracture SCID rat model. No or limited bone formation was acquired upon ectopic injection of BMP-6-late MSCs,

Table 1: Studies investigating the regenerative potential of iPSCs.

Authors, year	Cell source	Study model	Scaffold	Outcome
Bone				
Tashiro et al., 2009 [24]	20D17, 38C2, and stm99-1 from mouse EF	In vitro In vivo	—	CA promoter potently transduced iPSCs with enhanced osteogenic differentiation.
Kao et al., 2010 [112]	Murine germ line-competent from rat EF	In vitro In vivo	—	Resveratrol had antiapoptotic effect and enhanced osteogenic differentiation of iPSCs.
Li et al., 2010 [60]	Mouse tail-tip fibroblasts	In vitro In vivo	—	Retinoic acid and TGF-β enhanced osteogenic differentiation of iPSCs.
Bilousova et al., 2011 [81]	Mouse dermal fibroblasts	In vitro In vivo	3D gelatin scaffold	3D gelatin scaffold enhanced functional osteoblastic differentiation of iPSCs.
Ye et al., 2011 [104]	Mouse tail-tip fibroblasts	In vitro In vivo	Silk scaffolds	SATB2 facilitated iPSC differentiation towards osteoblast lineage cells with enhanced bone formation and mineralization.
Hayashi et al., 2012 [43]	iPS-MEF-Ng-20D-17	In vitro In vivo	PES scaffolds	2 Gray irradiation prior to transplantation inhibited teratoma formation.
Levi et al., 2012 [100]	Human adipose-derived stromal cells	In vivo	HA-coated, BMP-2–releasing PLA scaffold	HA-coated, BMP-2–releasing PLA scaffold promoted osteogenesis.
Li and Niyibizi, 2012 [61]	Murine tail-tip fibroblasts	In vitro In vivo	HA/TCP scaffolds	TGF-β promoted iPSC-derived EBs towards osteogenic lineage.
Villa-Diaz et al., 2012 [165]	Human fibroblasts	In vitro In vivo	Poly [2-(methacryloyloxy) ethyl dimethyl-(3-sulfopropyl) ammonium hydroxide] scaffold	hiPSCs cultured in a xeno-free system can differentiate into MSCs and form bone in vivo.
Ardeshirylajimi et al., 2013 [53]	Human iPSC line	In vitro In vivo	PES scaffolds	Plasma-treated PES scaffolds promoted osteogenic differentiation of iPSCs.
Ardeshirylajimi et al., 2013 [48]	Human iPSC line	In vitro	PES scaffolds	PES scaffold enhanced differentiation of iPSCs into osteoblast-like cells.
de Peppo et al., 2013 [86]	11C and 1013A iPSC (dermal fibroblast), BC1-iPSC (bone marrow)	In vitro In vivo	Decellularized bone scaffold	Different reprogramming methods can influence osteogenic potential of iPSCs.
Jin et al., 2013 [106]	Cat SC101AiPSC	In vitro In vivo	Macrochanneled PCL scaffolds	iPSCs exhibited in vitro transcription and translation of osteogenesis-related molecules and in vivo bone induction.
Liu et al., 2013 [66]	Human B1 cell line	In vitro	CPC immobilized with RGD (Arg-Gly-Asp)	iPSCs transduced with BMP-2 showed enhanced osteogenic differentiation.
Nasu et al., 2013 [91]	Human BMSCs and DFs	In vitro	—	No difference was noticed in chondrogenic and osteogenic differentiation of iPSCs from different origins.

Table 1: Continued.

Authors, year	Cell source	Study model	Scaffold	Outcome
Thein Han et al., 2013 [72]	Human BC1 cell line	In vitro	Biofunctionalized CPC	Biofunctionalized CPC enhanced osteogenic differentiation and mineralization.
Zou et al., 2013 [90]	Human fibroblast	In vitro In vivo	PCL or PHT	Increased ALP activity and calcium deposition on PHT scaffold in vitro as well as ectopic bone formation in vivo in comparison to PCL
Ardeshirylajim et al., 2014 [97]	Human fibroblast iPSC lines	In vitro	—	iPSCs showed a higher capacity for osteogenic differentiation compared to AT-MSCs.
Dogaki et al., 2014 [93]	Mouse embryonic fibroblast	In vitro	—	iPSCs revealed higher osteogenic differentiation capability in comparison to BM-MSCs.
Hong et al., 2014 [87]	Rhesus macaques' BMSCs, skin fibroblasts, and CD34+ cells	In vitro In vivo	HA/TCP	iPSCs demonstrated robust bone formation.
Hynes et al., 2014 [88]	Gingival fibroblasts, periodontal ligament cells, and human lung	In vitro In vivo	HA/TCP	iPSCs derived from PDL showed a superior capability to form mature bone.
Lee et al., 2014 [58]	Human fibroblasts	In vitro	—	MSC CM enhanced osteogenic differentiation of iPSCs.
Liu et al., 2014 [67]	Human BC1 cell line	In vitro	CPC immobilized with RGD	NELL1 gene overexpression enhanced osteogenesis.
Kang et al., 2014 [89]	Human fibroblast	In vitro	PCL or PCL-nHA	Increased expression of osteogenic genes in both OC scaffolds was highly expressed in PCL-nHA in comparison to PCL scaffolds.
Kang et al., 2014 [107]	IMR90p18-iPS	In vitro	Mineralized gelatin methacrylate-based matrices	Osteogenic differentiation of hiPSCs was achieved through biomaterial-based cues alone.
Kanke et al., 2014 [114]	Human neonatal dermal fibroblasts Mouse fibroblasts	In vitro	—	An effective strategy for differentiation of mESCs, miPSCs, and hiPSCs into osteoblasts was deviced.
Ko et al., 2014 [57]	Human iPSC line (SC802A-1)	In vivo In vitro	HA/b-tricalcium phosphate scaffold Fibrin glue scaffold	iPSCs differentiated into functional osteoblasts and demonstrated bone regenerative effect comparable to human BM-MSCs in vivo. Osteoinduced hiPSCs showed relatively lower and delayed expressions of the osteogenic marker in vitro.
Ochiai-Shino et al., 2014 [109]	Human iPSCs (line 201B7) from adult fibroblasts	In vitro	—	TNAP-positive cell hiPSC-derived EBs expressed high levels of osteogenic genes.
Phillips et al., 2014 [99]	Human SFs (NIHi2 and NIHi7)	In vitro In vivo	HA/TCP	BM-MSCs cultured on HA/TCP promoted bone formation.
Tang et al., 2014 [77]	BC1 cell line	In vitro	CPC	CPC scaffold promoted osteoblastic differentiation.

Table 1: Continued.

Authors, year	Cell source	Study model	Scaffold	Outcome
Wu et al., 2014 [115]	Tail-tip mouse fibroblasts	In vitro In vivo	CCHS	Alox5 affects the osteogenic and adipogenic abilities of iPSCs.
Ardeshirylajimi and Soleimani, 2015 [110]	Human iPSC line	In vitro	—	Combination of OM and ELF-EMF promoted bone differentiation.
Ishiy et al., 2015 [94]	SHED and human dermal fibroblast	In vitro	—	Osteogenic potential of SHED-iPSCs and iPSCs-fibroblasts-iPSCs is higher than osteoinduced SHED.
Ji et al., 2015 [121]	Human gingival fibroblasts	In vitro In vivo	nHA/CG scaffolds	Sphere-nHA/CG increased hiPSC osteogenic differentiation and bone formation.
Kang et al., 2015 [95]	Human dermal fibroblast	In vitro	—	iPSCs showed osteogenic efficacy comparable to BM-MSCs.
Lepage et al., 2016 [96]	Equine fibroblast	In vitro	—	iPSCs showed early mineralization indicating early osteogenesis.
Wang et al., 2015 [73]	BC1 cell line	In vitro In vivo	RGD-coated macroporous CPC	Enhanced osteogenic differentiation of iPSCs
Wang et al., 2015 [105]	Umbilical cord mesenchymal cells	In vitro	Synthetic peptide-decorated 2D microenvironment via pDA chemistry and CMC	Peptide-decorated niche promoted osteogenic differentiation of human iPSCs.
Hayashi et al., 2016 [56]	Human iPSCs (line 201B7)	In vivo In vitro	Peptide nanofiber hydrogel scaffold	Increased bone regeneration using iPSCs delivered in the nanofiber scaffold.
Jeon et al., 2016 [80]	Dermal fibroblasts	In vitro In vivo	PLGA/PLLA	3D biomaterials promoted osteogenic differentiation of iPSCs.
Ji et al., 2016 [49]	Human gingival fibroblasts	In vitro In vivo	HCG	Osteogenic differentiation of hiPSCs was improved by HCG scaffold.
Kang et al., 2016 [102]	IMR90p18-iPS cell line	In vitro In vivo	Macroporous synthetic matrices	Adenosine induced hiPSC differentiation into functional osteoblasts.
Sheyn et al., 2016 [68]	Dermal fibroblasts	In vitro In vivo	—	Genetic modification of iPSCs-MSCs and the suspension time of EB can effectively influence bone regeneration.
Sladkova et al., 2016 [76]	1013A cell line obtained from dermal fibroblasts	In vitro	Macroporous CPC using PEG particle	Enhanced osteogenic differentiation
Wang et al., 2016 [78]	Human BC1 cell line	In vitro	Injectable CPC with hydrogel fibers	Injectable CPC with hydrogel fibers promoted osteogenesis.
Wang et al., 2016 [79]	BC1 cell line and clone 1 from human foreskin fibroblast	In vitro	Injectable CPC with hydrogel fibers	Injectable CPC with cell-encapsulating hydrogel fibers was associated with enhanced bone regeneration.

Table 1: Continued.

Authors, year	Cell source	Study model	Scaffold	Outcome
Wang et al., 2016 [69]	Human BC1 cell line	In vitro In vivo	CPC alginate microbeads	Osteoinduction or transduction with BMP-2 promoted osteogenic differentiation.
Xie et al., 2016 [74]	Mouse MiPS-01 cell line	In vitro In vivo	Biomimetic nanofiber HA/Col/CTS	Biomimetic nanofiber HA/Col/CTS was associated with upregulation of osteogenic genes.
Zhang et al., 2016 [85]	Human foreskin fibroblasts	In vitro In vivo	Porous β-TCF	Dimethyloxaloylglycine promoted iPSC angiogenesis.
Chijimatsu et al., 2017 [92]	Mouse neural crest cells	In vitro In vivo	—	iPSCs failed to repair rat osteochondral knee defects although chondrogenic and osteogenic capacity in vitro was comparable to human BM-MSCs.
Deng et al., 2017 [101]	hNF-C1 line obtained from dermal fibroblasts	In vitro	Peptide-conjugated nanofiber scaffold	Nanofiber scaffolds facilitated osteodifferentiation of hiPSCs.
Liu et al., 2017 [64]	Human BC1 cell line	In vitro In vivo	CPC	HUVECs promoted mineralization of iPSCs.
Ma et al., 2017 [51]	E14 mouse embryonic fibroblasts	In vitro	—	ES and iPSCs were similar in their osteogenic differentiation potential.
Zhang et al., 2017 [65]	Human BC1 cell line	In vitro In vivo	CPC	HUVECs and pericytes promoted mineralization of iPSCs.
Chen et al., 2018 [63]	Human BC1 cell line	In vitro In vivo	CPC	HUVECs promoted mineralization of iPSCs.
Oudina et al., 2018 [122]	Human adult myoblasts	In vitro In vivo	Coral scaffold	Undifferentiated hiPSC implantation promoted the formation of bone-like structures of murine origin.
Saito et al., 2018 [52]	Oral mucosa of 2 CCD patients	In vitro In vivo	Peptide nanofiber scaffold	Repairing RUNX2 mutation in iPSCs-CCD promoted osteogenesis.
Wang et al., 2018 [62]	Human BC1 cell line	In vitro	CPC	Metformin promoted osteogenic differentiation of iPSCs.
Wu et al., 2018 [70]	Human foreskin fibroblasts	In vitro In vivo	Injectable alginate microbeads	3G7 promoted antibody-mediated osseous regeneration.
Abazari et al., 2019 [120]	Human iPSC line	In vitro	PVDF/Col/PRP scaffolds	PRP-incorporated PVDF/col promoted iPSC osteogenesis.
Abazari et al., 2019 [47]	Human iPSC line	In vitro	PCL-PVDF (bFGF)	Incorporating bFGF in PCL-PVDF scaffolds promoted osteogenesis.
Al-Wahabi et al., 2019 [75]	Mouse MEF-NG-20D-17 cell line	In vitro	Polystyrene substrate	Different scaffold topography enhanced osteogenic differentiation.
Hosseini et al., 2019 [117]	Human iPSC line from HEK293T cells	In vitro	PHBV nanofiber scaffold	Nanofiber-based PHBV increased osteogenic differentiation.

Table 1: Continued.

Authors, year	Cell source	Study model	Scaffold	Outcome
Hosseini et al., 2019 [118]	Human iPSC line from HEK293T cells	In vitro	PCL-PLLA (poly-P) electrospun scaffolds	Poly-P in PCL-PLLA enhanced osteogenesis.
Kawai et al., 2019 [103]	414C2 and 409B2: human fibroblasts 1231A3: human PBMC 317-12: human fibroblast OI#1: skin fibroblasts OI#2: skin fibroblasts	In vitro In vivo	—	Retinoic acid induced the osteogenic differentiation iPSCs and bone formation.
Mao et al., 2019 [98]	Adipose-derived stem cells	In vivo	nHP gelatin cryogel scaffolds	ASC-iPSCs showed osteogenic differentiation.
Mirzaei et al., 2019 [116]	Human iPSC line from HEK293T cells	In vitro	2D and 3D PVDF	3D scaffold enhanced differentiation of bone-forming cells.
Ramaraju and Kohn, 2019 [71]	Human fibroblasts	In vitro In vivo	Mineralized scaffolds coated with DPI-VTK	Enhanced osteogenesis and angiogenesis
Saburi et al., 2019 [119]	Human iPSC line from HEK293T cells	In vitro	GO-PVDF	GO significantly improved osteoconductivity of the PVDF.
Sladkova et al., 2019 [108]	Mesenchymal progenitors derived from pluripotent stem cell line 1013A (1013A-MPs)	In vitro	Decellularized cow and human bone scaffolds	Both scaffolds equally supported cell viability, tissue growth, and formation of mineralized bone matrix.
Tahmasebi et al., 2019 [50]	Human iPS cell line	In vitro	PCL nanofibers with miRNA-22 and miRNA-126	miRNAs incorporated in PCL scaffold promoted osteogenesis.
Xu et al., 2019 [55]	Human fibroblasts	In vitro In vivo	HA derived from PLCL with peptide H1 in a core silk fibroin	Increased proliferation and osteogenic differentiation of iPSCs as well as fast bone formation in vivo
Zhong et al., 2019 [59]	Murine iPSCs derived from MiPS-01	In vitro	—	Osteoblast conditioned medium enhanced osteogenic differentiation.
Zhu et al., 2019 [54]	Human embryonic kidney line 293T	In vitro In vivo	—	Gene profiles of ESC and iPSC-derived osteoblasts are similar.
Salivary glands				
Alaa El-Din et al., 2019 [123]	Human skin fibroblasts	In vitro In vivo	—	iPSCs treated salivary gland carcinomas.
Ono et al., 2015 [124]	Stomach cells	In vitro In vivo	—	iPSCs accelerated salivary gland development and regeneration.
Periodontal tissues				
Duan et al., 2011 [127]	iPSCs (foreskin)-1-DL-1 from human foreskin fibroblasts	In vitro In vivo	Silk scaffold	EMD combined with iPSCs enhanced periodontal tissue regeneration.
Hynes et al., 2013 [132]	Human foreskin	In vitro In vivo	Fibrinogen and thrombin	iPSCs-MSCs enhanced periodontal tissue regeneration.

Table 1: Continued.

Authors, year	Cell source	Study model	Scaffold	Outcome
Yang et al., 2014 [134]	Rat embryonic fibroblasts	In vitro In vivo	—	iPSCs transduced with TSG-6 were associated with decreased inflammation and alveolar bone loss.
Yin et al., 2016 [128]	Human gingival fibroblasts	In vitro In vivo	—	EMD and GDF-5 induced periodontal differentiation of iPSCs.
Li et al., 2017 [131]	Human gingival fibroblasts	In vitro	—	Increasing culturing time had no effect on periodontal differentiation potential of iPSCs.
Yin et al., 2017 [129]	Peripheral blood mononuclear cells	In vitro In vivo	Hyaluronic acid hydrogels	rhGDF-5 promoted periodontal differentiation of iPSCs-MSCs.
Chien et al., 2018 [130]	Rat fibroblasts	In vitro In vivo	G/C/GP hydrogel phosphate	BMP-6-iPSCs on hydrogel scaffold promoted periodontal tissue regeneration.
Hamano et al., 2018 [126]	Skin fibroblasts	In vitro	—	iPSC-NCLC-PDL cells showed upregulated expression of periodontal tissue-related genes.
Hynes et al., 2018 [133]	Tail-tip fibroblasts from NOD/Lt mice	In vitro In vivo	—	iPSCs decreased inflammation and periodontal tissue destruction.
Li et al., 2018 [125]	Human gingival fibroblasts and human neonatal skin fibroblast	In vitro In vivo	Hydrogel	Gingival iPSCs demonstrated better expression of periodontal cells' markers.
Enamel				
Arakaki et al., 2012 [135]	Mouse embryonic fibroblast	In vitro	—	iPSCs cocultured with dental epithelial cells differentiated into ameloblasts.
Yoshida et al., 2015 [136]	Mouse embryonic fibroblast	In vitro	—	iPSCs differentiated into ameloblast-like cells cultured with epithelial cell rests of Malassez cell conditioned medium and gelatin-coated dishes.
Abdullah et al., 2019 [137]	Mouse embryonic fibroblast	In vitro	—	Neurotrophin-4 in addition to iPSCs promoted its differentiation into dental epithelial-like cells.
Dentin pulp complex				
Otsu et al., 2012 [140]	Mouse embryonic fibroblast	In vitro	—	iPSCs differentiated into NCLC could further differentiate into iPSC-derived dental mesenchymal cells including odontoblasts.
Ozeki et al., 2013 [138]	Mouse embryonic fibroblast	In vitro	Collagen type I scaffold combined with BMP-4	iPSCs differentiated into functional odontoblast-like cells.
Ozeki et al., 2015 [139]	Mouse iPSC line (iPS-MEF-Ng-20D-17)	In vitro		Treatment with inorganic polyphosphate induced MMP-3 that physiologically accelerated both the proliferation and differentiation of odontoblast-like cells derived from iPSCs.
Seki et al., 2015 [141]	Mouse iPSCs	In vitro	—	Gene transfection of Pax9 and BMP-4 into iPSC-derived NCLCs promoted their differentiation into odontoblast-like cells.

Table 1: Continued.

Authors, year	Cell source	Study model	Scaffold	Outcome
Xie et al., 2018 [142]	Dental pulp stem cells	In vitro In vivo	Dentin discs with PLA scaffolds	iPSCs cultured on dentin discs with PLA scaffolds formed pulp-like tissue with the presence of tubular dentin.
Whole tooth regeneration				
Wen et al., 2012 [145]	Mouse embryonic fibroblast	In vitro In vivo	Collagen hemisphere	iPSCs combined with epithelial and mesenchymal cells formed bone and dental pulp-like structures.
Cai et al., 2013 [143]	Human urine cells	In vitro In vivo	—	iPSCs cocultured with mouse dental mesenchyme formed tooth-like structure.
Liu et al., 2016 [144]	Mouse iPSC line (C5 cell line)	In vitro In vivo	Fibrin gel	iPSCs cultured in ameloblast serum-free conditioned medium supplemented with BMP-4 differentiated into ameloblast- and odontoblast-like cells.
Liu et al., 2020 [146]	Mouse iPSC line (C5 cell line)	In vitro	—	Ameloblasts serum-free CM increased the gene and protein expression of enamelin, ameloblastin, and CK-14, as well as phosphorylated Smad1/5, p38 MAPK, and ERK1/2 MAPK in miPSCs as compared with miPSCs cultured in epithelial cell medium for 14 days.

while opposite results were obtained upon injecting BMP-6-early MSCs. It was concluded that iPSCs-MSCs obtained at early EB suspension time possessed a more pronounced stem cell phenotype and were capable of ectopic bone formation, whereas those cells obtained later acquired a more differentiated phenotype of osteoblasts and were capable of significant bone formation in vivo [68].

Similarly, genetic modification of human iPSCs-MSCs by either BMP-2 or NELL1 overexpression, followed by seeding of the modified cells on calcium phosphate cement (CPC) scaffold immobilized with RGD (Arg-Gly-Asp), showed significantly high expression of RUNX2, OCN, and COL1A1 [66, 67]. Additionally, human iPSCs-MSCs that were either osteoinduced or transduced with BMP-2 demonstrated high expression levels of osteoblast-related genes [69]. Incorporating retinoic acid combined with TGF-β1 or TGF-β1 into murine iPSC-derived EB culture media enhanced mineralization and osteogenic differentiation [60, 61]. Additionally, human iPSCs-MSCs cultured in the presence of metformin and seeded on CPC scaffolds showed upregulated expression of osteoblast-related genes and proteins as well as increased mineralization. Induction of adenosine monophosphate-(AMP-) activated protein kinase phosphorylation concomitant with increased RUNX2 expression was also evident [62]. Moreover, coseeding of human iPSCs-MSCs with human umbilical vein endothelial cells (HUVECs) on CPC scaffolds [63, 64] or coseeding with pericytes [65] enhanced osteogenesis and vascularization in vitro and in vivo with an upregulation expression of osteogenic (ALP, OCN, and COL1A1) and angiogenic genes (vascular endothelial growth factor (VEGF) and vascular endothelial cadherin).

Antibody-mediated osseous regeneration was recently described to impact in vivo bone regeneration. Human iPSCs-MSCs were combined with 3G7, an anti-BMP-2 antibody, that were hypothesized to facilitate the engagement of BMP-2 to their receptors on iPSCs-MSCs. 3G7 and iPSCs-MSCs were subsequently loaded on biocompatible, biodegradable alginate microbeads that were injected subcutaneously in rats. In vivo enhanced bone formation, mineralization, and vascularization associated with in vitro enhanced osteogenic differentiation were mediated through activation of the BMP-2/Smad1/RUNX2 pathway [70].

Biofunctionalization of the scaffold was further suggested to promote human iPSCs-MSCs osteogenic differentiation and vascularization, where human iPSCs-MSCs seeded on CPC scaffolds, treated with biofunctional agents and bioactive peptides [71–73] as well as murine iPSCs-MSCs seeded on biomimetic nanofibers of hydroxyapatite/collagen/chitosan (HA/COL/CTS), showed upregulation of RUNX2, OSX, ALP, and COL1A1 gene expression levels [74]. Furthermore, outgrowing cells from mouse iPSCs cultured on different polystyrene substrate topographies displayed upregulation of COL1A1 and RUNX2 [75]. Human iPSCs-MSCs seeded on microporous CPC scaffolds using polyethylene glycol (PEG) particles showed upregulation of RUNX2, COL1A1, ALP, OPN, and platelet-derived growth factor receptor-beta (PDGF-R-β) [76]. Similarly, human iPSCs-MSCs seeded on CPC [62, 77–79] or poly lactic-co-glycolic acid/poly L-lactic acid (PLGA/PLLA) scaffold combined with macrophages [80] or fast degradable alginate microbeads [69] showed high expression of osteoblast-related genes. Moreover, murine iPSC-derived MSCs seeded onto three-dimensional gelatin scaffold revealed upregulation of several osteoblast-related genes in vitro and in vivo, following subcutaneous implantation in rats [81]. Demonstrating the key role of osteoprotegerin/receptor activator of nuclear factor κ B ligand (OPG/RANKL) in orchestrating osteoblastic and osteoclastic action in bone remodeling, human iPSCs-MSCs were cocultured with iPSCs-macrophages committed to osteoblastogenesis and osteoclastogenesis, respectively, on HA-based PLGA/PLLA 3D scaffolds. Enhanced expression of bone-related genes upon monoculturing human iPSCs-MSCs on HA-5 PLGA/PLLA was demonstrated as compared to HA-0 PLGA/PLLA. Coculturing induced upregulated expression of late osteogenic markers (OPN and OCN) and downregulated expression of early osteogenic markers (COL1A1, ALP, and RUNX2). Similar results were attained in vivo through implantation of HA-PLGA/PLLA scaffold loaded with human iPSCs-MSCs and iPSCs-macrophages subcutaneously in rodents [80].

4.1.2. Osteogenic Potential of iPSCs-MSCs Obtained without EB Formation. Another method proposed to obtain iPSCs-MSCs relies on the dissociation of iPSC colonies, without prior formation of EB, into a single cell suspension. The resulting cells are characterized as MSCs, either through flow cytometry or through cell passaging protocols, followed by osteogenic differentiation [82–84]. Dimethyloxaloylglycine (DMOG) promoted iPSCs-MSCs derived from human foreskin fibroblast angiogenesis in critical-sized calvarial rat defects [85]. DMOG enhanced the expression of angiogenic factors (hypoxia-inducible factor 1-α (HIF-1α) and VEGF) through PI3K/Akt intracellular pathway activation, with improved bone formation.

The osteogenic potential of iPSCs-MSCs in combination with different scaffolds was investigated in several studies [55, 86–90]. The subcutaneous implantation of osteoinduced episomal-iPSCs (generated using an episomal vector) derived from BM stromal cells and retro-iPSCs (generated using a retroviral vector) derived from DF cultured on decellularized bone scaffold in SCID mice for 12 weeks revealed high mineral content in the episomal-iPSCs as compared to retro-iPSCs [86]. On the other hand, retro-iPSCs displayed the formation of a uniform bone-like matrix with embedded cells, while episomal-iPSCs exhibited areas of dystrophic calcification [86]. The osteogenic potential of human fibroblast-derived iPSCs was evaluated in vitro and in vivo on synthetic polymer polycaprolactone (PCL) scaffold or PCL scaffold functionalized with natural polymer hyaluronan and ceramic tricalcium phosphate ceramic poly (3-hexylthiophene (TCP-PHT)) [90]. The osteoinduced iPSCs revealed a significant increase in ALP activity and calcium deposition on PHT scaffold in vitro as well as ectopic bone formation in vivo in comparison to PCL. Moreover, human fibroblast-derived iPSCs on PCL nanofibers alone or combined with nano-HA showed an increased expression of osteogenic genes (RUNX2, ALP, COL1A1, and OCN) in both scaffolds, even though they were

expressed at a different time intervals, OCN was highly expressed in PCL-nano-HA in comparison to PCL scaffolds [89]. Similarly, the incorporation of short hydrophilic peptide H1 derived from connective tissue growth factor in a core silk fibroin (SF) combined with HA derived from poly (L-lactic acid-co-ε-caprolactone) (PLCL) resulted in increased proliferation and osteogenic differentiation of iPSCs-MSCs derived from human fibroblasts [55].

The interaction between HA/TCP ceramic particles and iPSCs-MSCs was subsequently investigated in vivo [87, 88]. Rhesus macaques' iPSC-derived mesodermal stromal-like cells mixed with HA/TCP demonstrated robust bone formation when implanted subcutaneously for eight weeks [87]. Furthermore, the osteogenic potential of iPSCs-MSCs from gingival fibroblasts, periodontal ligament cells, and human lung combined with HA/TCP was compared following implantation in SCID mice subcutaneously [88]. Although the three types of iPSCs-MSCs were able to form mineralized tissue, iPSCs-MSCs derived from periodontal ligament cells showed superior capability to form mature bone and connective tissue, which led to a controversial assumption that even after induction, iPSCs may retain epigenetic memory of their origin [91]. The combination of HA derived from PLCL with osteoinductive peptide H1 in a core SF and iPSCs-MSCs derived from human fibroblasts resulted in faster bone formation in vivo as compared to SF/PLCL following eight weeks of implantation in calvarial mouse defects [55].

Yet, although most of the aforementioned studies highlighted the osteogenic potential of iPSCs-MSCs in bone regeneration, Chijimatsu et al. reported that MSCs derived from iPSCs-neural crest cells failed to repair rat osteochondral knee defects in vivo despite their demonstrated chondrogenic and osteogenic capacity comparable to human BM-MSCs in vitro [92].

4.1.3. Osteogenic Differentiation Capability of iPSCs Compared to Other Types of Cells. The osteogenic differentiation ability of iPSCs-MSCs in comparison to MSCs was examined in a variety of studies [86, 93–95]. A study on iPSCs showed a delayed expression of osteogenic markers such as COL1A1 and bone sialoprotein (BSP) as well as weaker osteoblastic differentiation and mineral deposition, compared to human BM-MSCs in vitro [57]. Human fibroblast-derived iPSCs reprogrammed by mRNA (mRNA-iPSCs) or polycistronic lentiviral vector (lenti-iPSCs) were compared to BM-MSCs [95]. Both methods of transduction produced cells that were similar in their morphology and surface antigen to BM-MSCs. lenti-iPSCs revealed faster and more homogeneous calcium staining than mRNA-iPSCs. Although the expression of RUNX2, ALP, and OCN was stronger in BM-MSCs as compared to iPSCs-MSCs, the opposite was demonstrated for COL1A1 expression. Both iPSCs-MSCs showed osteogenic efficacy comparable to BM-MSCs. Similarly, osteoinduced mouse iPSCs-MSCs revealed the same surface antigen profile and higher osteogenic differentiation as BM-MSCs [93]. ALP, OSX, RUNX2, and OCN were intensely upregulated in osteoinduced iPSCs-MSCs aside from the formation of a mineralized matrix at day 14 of osteogenic induction. retro-iPSCs and episomal-iPSCs exhibited higher ALP gene expression than human ES cells [86]. Moreover, the osteogenic potential of iPSCs-MSCs derived from either human deciduous teeth or human DF was higher than that of osteoinduced SHED [94]. iPSCs-MSCs derived from equine fibroblast iPSCs were compared to MSCs derived from newborn foals' umbilical cord blood (CB-MSCs) [96]. Von Kossa and alizarin red staining of iPSCs-MSCs showed early mineralization indicating early osteogenesis which was consistent with the results obtained from CB-MSCs.

Similarly, Ardeshirylajimi et al. [97] compared the biological behavior and osteogenic differentiation potential of human iPSCs and adipose tissue (AT-MSCs). iPSCs confirmed high osteogenic differentiation potential and superior ALP activity and mineralization level. Notably, AT-MSCs expressed greater levels of RUNX2, while iPSCs expressed higher levels of OCN and osteonectin during differentiation which may be a result of their increased proliferation rate compared to AT-MSCs [97]. In vivo comparison of osteogenic potentials between adipose-derived stem cells (ASCs) and ASC-iPSCs loaded on nano-HA gelatin cryogel scaffolds revealed a superior osteogenic differentiation with enhanced osteogenic marker expression of COL1A1 and RUNX2 in the ASC-iPSCs group, proposing ASC-iPSCs as an alternative cell source in bone tissue engineering with a good differentiation ability [98].

On the other hand, the osteogenic potential of iPSCs derived from human skin fibroblasts was compared to iPSCs derived from BM-MSCs cultured on HA/TCP implanted subcutaneously in nude mice [99]. No differences in bone formation were revealed between iPSCs from different origins. In addition, the bone regeneration ability of adipose-derived stromal cells- (AS-) iPSCs was compared to human ES cells cultured on HA-coated PLGA scaffold with or without releasing BMP-2 in calvarial mouse defects [100]. Greater bone regeneration as well as upregulation of osteogenic markers was found in both AS-iPSCs and ES cells loaded on HA-PLGA releasing BMP-2 as compared to nonreleasing BMP-2 [100].

4.1.4. Factors to Improve the Osteogenic Potential of iPSCs (Figure 2). Exploring the therapeutic potential of iPSCs-MSCs in dental and nondental tissue regeneration entails the optimization of the factors that would enhance their osteogenic potential for future clinical applications. Genes, isozymes, laser application, suspension time of EBs, transduction method, natural antioxidant and anticancer products, and constituents of the scaffold material are factors that could enhance or affect the osteogenic potential of iPSCs. In order to attain iPSC osteogenic commitment, various inductive factors were applied including chemical inducers, biomolecules [101–103], growth factors [100], gene modification [104], two-dimensional culture environment [105], and modified three-dimensional scaffolds [100, 101, 106–108]. Tissue-nonspecific alkaline phosphatase (TNAP) was demonstrated to influence the osteogenic differentiation potential of iPSCs, where TNAP-positive cells isolated from human EBs derived from iPSCs and cultured in osteogenic media expressed high levels of OSX, RUNX2, COL1A1,

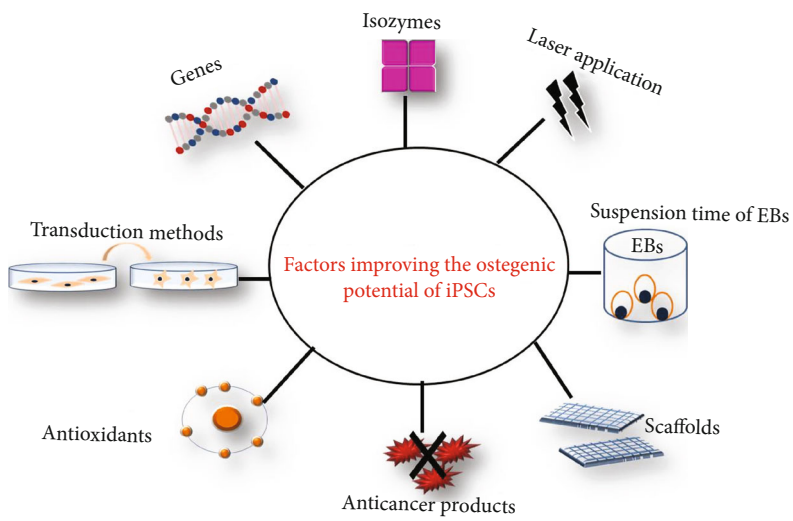

Figure 2: Diagram summarizing factors which may affect osteogenic potential of iPSCs.

BSP, and OCN as well as generated mineralized nodules and revealed a significant expression of osteocyte marker genes, including sclerostin, neuropeptide Y, and reelin [109]. Similarly, extremely low-frequency electromagnetic field (ELF-EMF) (50 Hz and 1.5 mT) also significantly improved the osteogenic potential of iPSCs [110]. Resveratrol a natural polyphenol found largely in red grapes, nuts, pomegranates, and red wine [111] was also found to facilitate osteogenic differentiation of iPSCs, with increased osteogenic gene expression and mineralization content [112]. Growth factors such as recombinant human- (rh-) BMP-2 have been shown to positively modulate osteogenic transformation of iPSCs. Adding rh-BMP-2 to the osteogenic media improved the osteogenic potential of iPSCs derived from human AS through significant upregulation of osteogenic markers RUNX2 and OCN [100]. In vitro results showed that 3 wt/vol% nano-HA in chitosan/gelatin (CG) and miRNAs increased the expression of osteogenic-related genes [49, 50], formed bone-like tissue in vivo [49], and upregulated the OCN and OPN protein expression on day 21 after culturing [50].

Even though growth factors can endorse the osteogenic differentiation of iPSCs, their effects are limited due to their short half-lives and uncontrolled degradation. In contrast, gene modification of iPSC-derived cells can attain a long-term effect via retaining a relatively stable local concentration of these factors [113]. Certain genes such as nuclear matrix protein SATB2 have been transduced into iPSCs to promote osteodifferentiation [104]. An efficacious strategy for differentiating human iPSCs into osteoblasts involves using four small molecules including CHIR99021 (CHIR), cyclopamine (Cyc), smoothened agonist (SAG), and helioxanthin-derivative 4-(4-methoxyphenyl) pyrido [4′,3′:4,5] thieno [2,3-b] pyridine-2-carboxamide (TH) under chemically well-defined conditions [114]. Ex vivo gene therapy of SATB2-modified iPSCs increased the levels of calcium nodule formation, ALP activity, and osteogenic genes in vitro. Subsequent implantation of the transduced cells on silk scaffold encouraged bone regeneration in critical-sized calvarial defects [104]. On the contrary, iPSCs derived from tail-tip fibroblasts of Alox5 knockout mouse demonstrated significant downregulation of early and late osteogenic gene levels with significant upregulation of adipogenic markers. Still, loading Alox5-KO-iPSCs on collagen/chitosan/hydroxyapatite scaffolds induced significantly less new bone formation in rat cranial critical-sized defects as compared to wild-iPSCs [115].

Interestingly, iPSC origin demonstrated no effect on iPSC osteogenic potential. The osteogenic differentiation properties of human iPSCs derived from BM-MSCs and DFs demonstrated no marked differences in gene expression profiles as well as in the methylation profile. Moreover, the chondrogenic and osteogenic differentiation properties of iPSCs from different cells' origin showed no significant differences, although a higher tendency was reported in DF-derived iPSCs [91]. Yet, different reprogramming methods could affect the osteogenic differentiation of iPSCs [86]. iPSCs derived from DF reprogrammed by retroviral vectors (retro-iPSCs) or Sendai virus (Sendai-iPSCs) cultured on decellularized bone scaffold in perfusion bioreactors demonstrated a new bone-like matrix with the highest cell density in Sendai-iPSCs, while retro-iPSCs showed poor osteogenic differentiation [86].

Human iPSCs derived from human embryonic kidney-EB were utilized to compare the osteoinductive properties of 3D nanofibrous scaffold of polyvinylidene fluoride (PVDF) with 2D scaffold [116] as well as to assess electrospun poly (3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV) nanofiber scaffold [117]. iPSCs revealed significantly high ALP activity, calcium content, and osteogenic-related genes after seeding on 3D PVDF [116] and PHBV scaffolds [117]. Moreover, OCN and OPN protein expressions were elevated on day 21 after cell seeding [116, 117]. Utilizing different ratios from nano-HA [49] or different miRNAs (miR-22 and miR-126) [50] in chitosan/gelatin (CG) scaffold or electrospun PCL nanofiber

scaffold, respectively, was also reported to affect the osteogenic differentiation of human iPSCs. Furthermore, incorporating basic fibroblast growth factor (bFGF) in PCL-PVDF scaffold [47] or polyphosphate (poly-P) in PCL/PLLA electrospun scaffolds [118] or graphene oxide (GO) in PVDF nanofibers [119] or platelet-rich plasma in PVDF/collagen nanofibrous scaffolds [120] significantly increased the survival rate of iPSCs and upregulated ALP activity, mineralization content, and expression of preosteoblast- and osteoblast-related genes in iPSCs loaded on PCL-PVDF (bFGF), PCL-PLLA (poly-P), PVDF-GO, or PVDF/Col/PRP scaffolds [47, 118–120]. A xeno-free nanofiber scaffold conjugated with vitronectin peptide upheld pluripotency and proliferation of seeded human iPSCs. Interestingly, this osteogenic culture system promoted direct osteodifferentiation of human iPSCs, as confirmed by the cellular morphology, ALP assay, and RT-PCR analysis combined with immunofluorescence results [101]. A recent report confirmed the osteogenic differentiation of human iPSCs into osteoblast-like cells with enhanced calcified nodule formation under the influence of retinoic acid in vitro and membranous bone tissue formation in vivo without scaffolds [103]. Under osteogenic conditions, human iPSCs cultured on PCL scaffolds confirmed osteogenesis by OPN detection using quantitative PCR and by western blotting. Further subcutaneous implantation in mice revealed marked calcium deposition and positive OCN immunostaining, with no signs of teratoma formation, following the osteogenic induction of human iPSCs [106]. The osteogenic potential of human iPSC-derived mesodermal progenitor cells (hiPSC-MP) on decellularized tissue matrices as scaffolding materials and human bone scaffolds in osteogenic medium under dynamic conditions was compared in perfusion bioreactors. Both scaffolds equally promoted cell viability and mineralized tissue formation [108]. Peptide-decorated 2D culture microenvironment developed through polydopamine (pDA) chemistry with subsequent carboxymethyl chitosan successfully promoted osteogenic differentiation of human iPSCs in vitro [105]. These results were supported by enhanced ALP activity, gene expression, and corresponding protein expression as well as the amount of calcium deposition [105]. Human iPSCs isolated from clinically discarded human gingival tissues were seeded on both sphere-shaped or rod-shaped nano-HA/CG scaffolds. Notably, the sphere-shaped nano-HA in HA/CG scaffolds greatly improved the osteogenic differentiation of human iPSCs as compared to rod-shaped. Consequently, human iPSCs and sphere-shaped nano-HA/CG composites generated a significant amount of bone in vivo [121].

Adenosine-induced differentiation of human iPSCs (Ad-iPSCs) loaded on poly (ethylene glycol) diacrylate-co-acryloyl 6-aminocaproic acid (PEGDA-co-A6ACA) macroporous hydrogel into functioning osteoblast, in growth medium lacking any other osteoinductive factors, revealed progressive dense bone tissue formation. Furthermore, Ad-iPSCs implanted in critical-sized cranial bone defects in mice showed uniform hard tissue formation all over the cranial defect that was integrated with the adjacent bone without teratoma formation [102]. Moreover, ex vivo two-dimensional and three-dimensional cultures and mineralized gelatin methacrylate- (GelMA-) based matrices containing CaP mineral endorse the osteogenic differentiation of human iPSCs in osteoinductive factors free growth medium via the dissociation of Ca^{2+} and PO_4^{3-} ions in a permissive environment through various signaling pathways [107]. Similarly, ectopically implanted human iPSCs seeded on coral scaffolds in mice demonstrated the expression of bone-like structures through the release of osteoinductive factors including BMPs [122]. Paradoxically, the rapid disappearance of human iPSCs due to early cell death was associated with an increase in the osteogenic genes. To settle these conflicting trends, the authors investigated the paracrine effect of bioactive CM from human iPSCs. Interestingly, human iPSC CM promoted the osteogenic differentiation of human MSC osteogenic differentiation as well as upregulated the expression of BMP-2, BMP-4, and BMP-6 genes and enhanced extracellular matrix mineralization [122].

4.2. iPSCs and Salivary Gland Regeneration. iPSC therapeutic and regenerative potentials were exploited in the treatment of salivary glands' diseases. In an in vivo study, iPSCs were utilized for treating salivary gland carcinoma induced in mice. Although iPSCs improved salivary gland function detected by a significant increase in the gene expression of α-amylase, the glands retained some malignant architecture including minor acinar, ductal, and vascular degenerative changes [123].

In an attempt to uncover the paracrine role of iPSCs in salivary gland regeneration, embryonic submandibular gland (SG) cells and mouse green fluorescent protein iPSCs (iSG) were cocultured. More developed epithelial structures were evident upon coculturing than in monoculture of embryonic SG cells. Upon morphological analysis of the regenerated tissues, iSG had a greater number of small acinar-like structures than that in SG cells. Additionally, analysis of differentiation markers among groups showed lower Sox2, c-Myc, and Nanog gene expression and higher Klf4 and Aqp5 gene expression in iSG with a remarkable regenerative capacity [124].

4.3. iPSCs and Periodontal Tissue Regeneration. iPSC differentiation into periodontal regenerative cells is affected by a variety of factors including cell source [125], culturing media [126], coculturing with inducing factors such as enamel matrix derivative (EMD) [127, 128], recombinant growth/differentiation factor-5 (GDF-5) [128, 129] or BMP-6 [130], the number of cellular passages [131], and type of scaffold used [130]. EBs generated from human gingival fibroblast and human neonatal skin fibroblast-derived iPSCs were induced into periodontal progenitor cells, which were then implanted on hydrogel scaffold subcutaneously in SCID rats. Owing to the cells' inherent epigenetic memory, iPSCs derived from gingival fibroblasts showed a higher expression of periodontal cell markers in vitro, including BSP, cementum protein 1 and periostin, and a formation of mineralized structure in vivo, with no teratoma formation observed with either cell types [125]. Neural crest cells derived from human skin fibroblast iPSCs cultured in combination with PDL cells' extracellular matrix showed a higher proliferation rate and a stronger expression of periodontal cell markers, including COL1A1, fibrillin-1, OPG, and periostin, as compared to

cells cultured with either fibronectin, laminin, or dermal fibroblast extracellular matrix [126].

Culturing EB derived from human foreskin iPSCs in combination with EMD gel promoted the expression of RUNX2, an early osteogenic marker, but inhibited the expression of OCN, a late osteogenic marker, and mineralization in vitro. To assess the effect of iPSCs and EMD on osteogenic differentiation and periodontal regeneration in vivo, EBs derived from mouse iPSCs were seeded on apatite-coated silk fibroin scaffolds with EMD before implantation in the periodontal fenestration defect rat model. Following iPSCs-EMD in vivo transplantation, OCN, RUNX2, and OSX expression was higher than those in the control group which was attributed to the ability of EMD to recruit a large number of osteogenic cells. Moreover, iPSCs-EMD were able to induce the formation of new bone almost filling the periodontal defect, promoted the formation of new cementum covering the surface of the root, and stimulated the formation of periodontal fibers perpendicular to the root surface proving that iPSCs-EMD can further be an efficient tool in periodontal regeneration [127].

The periodontal differentiation potential of iPSCs derived from human gingival fibroblasts and treated with growth differentiation factor- (GDF-) 5 was investigated at different passages [5, 10, 15, 20]. All iPSCs-GDF-5-treated passages revealed a high proliferative ability and attained fibroblast-like cell morphology, significant production of calcified nodules, and upregulated expression of bone-related gene (OCN and BSP), periodontal ligament-related gene (periostin and vimentin), and cementum-related genes (cementum attachment protein and cementum protein 1) as compared to their untreated controls [131]. Yet, the periodontal differentiation capability of iPSC-derived MSCs, obtained either from human gingival tissues or from peripheral blood mononuclear cells, was significantly increased after their treatment with recombinant human GDF-5 (rhGDF-5) [128, 129]. This was confirmed by the marked expression of periodontal tissue-related genes (OCN, periostin, and cementum attachment protein). On the contrary, BM-MSCs treated with rhGDF-5 demonstrated an insignificant expression of periostin and CAP, despite the high expression of OCN. Similar results were attained upon loading PKH67-labeled iPSCs-MSCs-rhGDF-5 on hyaluronic acid and subsequent implantation into the dorsal surface of 6-8-week-old male athymic nude mice. Moreover, after 4 weeks of culture with rhGDF-5, both BMSCs and iPSCs-MSCs showed noticeable mineralization with nodule formation [129]. Chitosan/gelatin/glycerol phosphate hydrogel 3D scaffold seeded with osteogenic-induced rat fibroblast-derived iPSCs and BMP-6 applied to periodontal defect created on the root surface of the maxillary first molar in rats significantly downregulated inflammatory cytokines interleukin 8 (IL-8), tumor necrosis factor alpha (TNF-α), and IL-1β and promoted bone and periodontal tissue regeneration [130]. Additionally, human foreskin iPSC-derived MSCs, clotted with fibrinogen and thrombin implanted in periodontal fenestration defect in SCID rats, also revealed a significant increase in newly formed mineralized tissue area percentage [132].

Mesenchymal stromal cells derived from tail-tip fibroblast iPSCs (iPSCs-MCs) revealed immunomodulatory capabilities of the periodontal inflammatory destruction, which may offer a potential therapeutic modality for periodontal disease. In this context, a bacterial-induced periodontitis mouse model was established through local application of *Porphyromonas gingivalis* into the oral cavity and its systemic administration, while an acute inflammation model was created via subcutaneous implantation of heat-killed *Porphyromonas gingivalis*-impregnated sponge in rats. Rats were treated by systemic injection of iPSCs-MCs into the tail vein seven days following periodontitis establishment or by local iPSCs-MCs administration into the implantation site. iPSCs-MCs showed a significant reduction in inflammation and alveolar bone loss in the periodontitis rats' model. Moreover, local or systemic iPSC treatment in the acute inflammation model showed a reduced expression of the proinflammatory cytokine CXCL1, while local iPSCs-MCs administration resulted in a significant reduction in the inflammatory score [133]. Similarly, periodontitis was induced around the maxillary first molar bilaterally in female rats by ligature and subsequent infection with *Porphyromonas gingivalis*. The rats were treated intravenously and topically with rat iPSCs-MSCs reprogrammed from rat embryonic fibroblasts and transduced with tumor necrosis factor alpha-stimulated gene-6 (TSG-6) (iPSCs-MSCs/TSG-6). A significant downregulated level of alveolar bone loss, a few number of TRAP-positive osteoclasts, and serum interleukin 1β (IL-1β) and tumor necrosis factor alpha (TNF-α) were demonstrated as compared to untreated rats [134].

4.4. iPSCs and Enamel Regeneration. Ameloblasts are crucial cell populations required for enamel formation. The ability of mouse iPSCs (miPSCs) to differentiate into ameloblast was investigated [135], where miPSCs cocultured with dental epithelial cells differentiated into ameloblasts, exhibiting epithelial cell-like morphology in addition to expressing ameloblastic markers (ameloblastin and enamelin) and epithelial markers (p63 and cytokeratin- (CK-)14), suggesting an epithelial-mesenchymal interaction role in tooth development. Similarly, miPSCs differentiated into ameloblast-like cells under feeder-free conditions, using cultured epithelial rests of Malassez (ERM) cell CM and gelatin-coated dishes [136]. The differentiated ameloblast-like cells demonstrated an increase in expression of CK-14, amelogenin, and ameloblastin in comparison to miPSCs cocultured with ERM cells. The levels of amelogenin expression in ameloblast-like cells were significantly higher than those in miPSCs cocultured with ERM cells throughout the experiment, while ameloblastin increased significantly on day 14. Moreover, the addition of neurotrophin-4 to miPSCs under serum-free culture conditions during EB formation leads to their differentiation into dental epithelial-like cells with the upregulation of epithelial and ameloblastic markers [137]. These studies highlighted the potential differentiation ability of iPSCs into ameloblasts confirming that iPSCs could be a new cell source for enamel regeneration.

4.5. iPSCs and Dentin Pulp Complex Regeneration. The generation of odontoblast cells from iPSCs could open new opportunities for treating dentinal and/or pulpal damage. Epithelial-mesenchymal interactions are required for differentiating iPSCs into odontoblasts. Herein, the study described the "hanging drop" technique for differentiating miPSCs into odontoblast-like cells exploiting such an interaction. iPSCs were differentiated into EBs and then cultured on a collagen scaffold (CS) in combination with BMP-4 (CS/BMP-4). The generated cells intensely expressed mature odontoblast markers, dentin sialoprotein (DSP), and dentin matrix protein-1 (DMP-1) and presented physiological as well as functional features of odontoblasts [138]. Moreover, in an in vitro model, matrix metalloproteinase- (MMP-) 3 small interfering RNA was transfected into odontoblast-like cells derived from iPSCs. Strikingly, treatment with inorganic polyphosphate induced MMP-3 that physiologically accelerated both proliferation and differentiation of odontoblast-like cells, thereby hypothesized to provide some protection to the cells against the detrimental effects of inflammation and pulp capping materials. Additionally, DSPP and DMP-1 mRNA expressions were upregulated [139].

Under modified culture protocols, miPSCs were differentiated into neural crest-like cells (NCLCs) that could further differentiate into iPSC-derived dental mesenchymal cells (DMC) including odontoblast progenitor cells. Results showed that iPSC-derived NCLC expressed NC markers as demonstrated by immunocytochemistry, flow cytometry, and RT-PCR. Furthermore, NCLC expressed MSC markers, in addition to Pax9 and DSP, proving their capacity to differentiate into dental mesenchyme, when cultured with dental epithelium [140]. Interestingly, gene transfection of Pax9 and BMP-4 into iPSC-derived NCLCs promoted their differentiation into odontoblast-like cells, thus prompting signaling modulation of DMP-1 and DSPP expression, associated with odontoblastic differentiation of miPSCs [141]. In another study, dental pulp stem cells (DPSCs) were reprogrammed into iPSCs; then, the cells were seeded on dentin discs with PLLA scaffolds and implanted subcutaneously in mice. Amazingly, iPSCs generated a pulp-like tissue having tubular dentin, while in vitro, iPSCs maintained the odontogenic and mineralization potential after long-term expansion opposite to DPSCs [142].

4.6. iPSCs and Whole Tooth Regeneration. In addition to ameloblastic and odontoblastic differentiation potential of iPSCs, the capability of iPSCs in whole tooth regeneration was investigated [143–145]. miPSCs which clearly express odontogenic and osteogenic genes following their induction were implanted combined with epithelial and mesenchymal cells in a tooth germ model and transplanted into subrenal mouse capsule [145]. After four weeks of implantation, the formation of bone, dental pulp-like, and irregular tooth-like structures was demonstrated. Additionally, OPN was expressed in the apical region of the tooth-like structure. Notably, implantation of miPSCs alone failed to form dental or bone-like structures in contrast to its combined implantation with epithelial and mesenchymal cells.

Human iPSCs, derived from urine cells, were differentiated into epithelial sheets and cocultured with mouse dental mesenchyme, demonstrating an ability to form tooth-like structures such as enamel organ, enamel space, dentin, and dental pulp with physical and chemical properties similar to human teeth [143]. Further, through specific human antigen expression, it was revealed that iPSC epithelial sheets differentiated into ameloblast, while dental mesenchymal cells gave rise to the rest of the formed dental tissues. Interestingly, mouse dental mesenchymal cells alone formed bone-like tissue rather than tooth-like structure. Furthermore, miPSCs cultured in ameloblast serum-free CM supplemented with BMP-4 displayed the ability to form ameloblast- and odontoblast-like cells [144]. In addition, ameloblast serum-free CM increased the gene and protein expression of enamelin, ameloblastin, and CK-14, as well as phosphorylated Smad1/5, p38 MAPK, and ERK1/2 MAPK in miPSCs as compared with miPSCs cultured in epithelial cell medium for 14 days. Smad1/5 signaling transduction regulates the ameloblastic differentiation of miPSCs induced by ameloblast serum-free CM as the inhibition of Smad1/5 phosphorylation significantly reversed the increased the previously mentioned expression profile [146]. These results raise the possibility of iPSCs' use in whole tooth engineering opening a new gateway for biological tooth replacement.

5. Challenges Facing iPSCs' Human Clinical Applications

One of the major drawbacks that could hinder iPSCs' clinical application is their reported chromosomal instability and the underlying risk of tumor formation, which constitutes a substantial health hazard [12, 147]. Undifferentiated iPSCs' pluripotency and their ability to differentiate into tissues derived from the three germ layers are an incentive to teratoma formation, which is used as an assay to test their pluripotency [33, 148]. Moreover, iPSCs express several oncogenic genes [149]. Owing to iPSCs' unique properties, the generated tumor properties and origin are highly unpredictable and vary with the transplanted cell number as well as the utilized cell line [150]. In addition to their innate tendency for teratoma formation, the method of gene transduction can also increase the risk of tumorigenesis particularly due to the use of viruses that integrate their genome into the reprogrammed cells, as previously discussed. Currently, several attempts are carried out to overcome this through the use of nonviral vectors [25, 26] but are hindered by their lower transfection efficacy, especially following iPSC passaging.

Luckily, utilizing terminally differentiated iPSCs prior to implantation in addition to using nonviral vectors can help reduce risk of tumor formation [151]. Moreover, iPSCs can be reprogrammed via Oct3/4, Sox2, and Klf4, while omitting c-Myc which is a potent oncogene [14, 15, 152]. However, even following iPSC terminal differentiation, some cells may escape differentiation. Residual undifferentiated or partially differentiated iPSCs in the cellular transplants may cause teratoma formation upon implantation in the recipient tissues [153, 154]. Furthermore, iPSCs could retain epigenetic memory, which may affect their subsequent

differentiation and direct them into lineages related to their parent cells [155, 156].

Another limitation associated with most current stem/-progenitor cell isolation and expansion protocols lies in the utilization of xenogeneic-derived products in iPSC protocols. iPSCs are usually cultured on xenogeneic feeder cells that maintain the cells in an undifferentiated state without affecting their pluripotency [157], as well as fetal bovine serum that represents an important culture medium constituent [151, 158]. Using xenogeneic products in clinical trials could elicit an immunogenic reaction, carry a risk of disease transmission [151, 158], and affect reproducibility, as the exact composition of bovine serum varies greatly [159]. An additional problem creating an obstacle for the clinical application of iPSCs is the reduced generation efficacy [23], where iPSC generation efficiency using fibroblasts is extremely low. Even though generation efficiency is 4 to 10 times greater using dental pulp stem cells than fibroblasts, it is still relatively low for application in regenerative medicine [151].

6. Short- and Long-Term Perspectives of iPSC-Mediated Tissue Regeneration

Despite that iPSCs have shown promising results in regenerative medicine, a number of issues are yet to be resolved to allow their translation into clinical application while minimizing their potential side effects. Coculturing iPSCs with cells and growth factors could provide a promising solution to overcome tissue engineering challenges through mimicking in vivo conditions to optimize tissue regeneration results. Upon coculturing iPSCs-MSCs with iPSCs-macrophages committed to osteoblastogenesis and osteoclastogenesis, an OPG/RANKL milieu could be provided [80]. Similarly, coculturing iPSCs with dental epithelial and mesenchymal cells can reproduce epithelial-mesenchymal interaction signals orchestrating the process of tooth development. So far, securing an epithelial-mesenchymal interaction represents a great obstacle in whole tooth regeneration [135, 143]. Epithelial-mesenchymal interaction signals thereby remain to be the key towards inducing the differentiation of iPSCs into ameloblasts and other dental cells, which is the first step in whole tooth regeneration. Moreover, defining the best combination of iPSCs, signaling molecules such as growth factors, and scaffold biomaterials and determining the ideal architectural design of the scaffold 2D or 3D, sphere- or rod-shaped, remain crucial for various applications of iPSCs in dental and paradental tissue regeneration.

Transduction of repaired, edited, and/or modified genes in iPSCs could be a beneficial tool for treating various disorders. In this context, repairing RUNX2 gene mutation in iPSCs derived from cleidocranial dysostosis patients [52] as well as transducing nuclear matrix protein SATB2 [104] and Alox5 gene into iPSCs promoted osteodifferentiation [115]. Besides, Pax9 and BMP-4 gene transfection into iPSC-derived NCLCs promoted odontoblast-like cell differentiation [141] and attained a long-term effect of these factors rather than the short-term effect acquired following their local application [113].

iPSCs' extracellular vesicles, containing protein, mRNA, and miRNA, can further be used in regenerative medicine, seizing the paracrine effect of iPSCs while avoiding the possible risk of tumorigenesis associated with iPSC-based therapy [160]. The paracrine role of iPSCs in salivary gland regeneration has been proven upon coculturing embryonic submandibular gland cells and mouse iPSCs [124]. Besides, human iPSC CM promoted the osteogenic differentiation of human MSCs [122]. Usage of iPSC-derived secretome for tissue regeneration merits further research such as determining the active genes and growth factors expressed in CM from iPSCs.

Defining the optimum and the most accessible cell source to attain iPSCs should be investigated in the future to maximize their differentiation potential as well as their generation efficacy. iPSCs proved to retain their epigenetic memory, which may affect their subsequent differentiation [155, 156]. For example, iPSCs derived from gingival fibroblasts showed a higher expression of periodontal cell markers in vitro [125]. This could be beneficial in using particular cell sources for specific tissue regeneration, but it hinders the wide range of cells that could be derived from iPSCs. Despite that gingival fibroblasts and urine cells could be considered an easy source for attaining iPSCs, the generation efficiency of iPSCs using fibroblasts is extremely low [151].

A better control of the differentiation potential of iPSCs could be achieved by defining the suspension time of EB, since iPSCs-MSCs obtained at early EB suspension time possessed a more stem cell phenotype while those cells obtained later acquired a more differentiated phenotype [68], and by controlling and optimizing the reprogramming method where the highest cell density was attained in Sendai-iPSCs, while retro-iPSCs showed poor osteogenic differentiation [86].

Finally, next-generation sequencing could be alternatively used to assess the pluripotency potential, following iPSC generation instead of the complicated current techniques including teratoma formation and in vitro embryoid body (EB) generation [32].

7. Conclusion

iPSCs represent an autologous cell source, derived from the patient's own tissue, with no risk of immune reaction [161–163]. They have higher proliferative rates than adult stem cells and can be acquired via noninvasive methods [161], all properties that are highly desirable in regenerative medicine. Despite challenges associated with iPSCs' clinical use, their potential impact on medical applications still warrants further research. Carrying the application of iPSCs for tissue regeneration into humans entails strict abiding to the conduct of good manufacturing practice (GMP), as well as properly selecting cell source, culturing media, and vectors for gene transduction and excluding any xenogeneic-derived products from iPSC generation protocols. Recently, iPSCs have been successfully generated using a protocol compliant with GMP from hematopoietic stem cells from peripheral blood [164]. Furthermore, iPSCs were successfully maintained undifferentiated in xenogeneic-free culture medium

and were subsequently differentiated into MSCs and osteogenic cells. Positive results were also attained following implantation in rats' calvarial defects [165] which paves the way for carrying iPSCs into clinical trials. Initial reports documented that the risk of teratoma formation associated with iPSC transplantation could be inhibited by pretreatment with resveratrol [112] or by irradiation of 2 Gray (Gy) prior to transplantation [43]. Finally, iPSCs' extracellular vesicles and secretomes, containing protein, mRNA, and miRNA, can alternatively be used, exploiting the paracrine effect of iPSCs while avoiding the risk of tumorigenesis associated with iPSC-based therapy [160].

It can thus be concluded that even though iPSCs hold a tremendously unexplored potential in the field of regenerative medicine, bone and dental tissue engineering, therapeutic application in bone disorders, gene therapy, and personalized medicine, a number of obstacles must be alleviated to attain their clinical applications. iPSCs still warrant further research focusing on achieving a safe, efficient reprogramming and attaining significant expansion while evading postimplantation tumor risks. Unleashing the full capabilities of iPSCs holds a promise of offering remedies to several genetic disorders in addition to their potential application in bone and dental tissue regeneration.

Abbreviations

ALP: Alkaline phosphatase
ASC-iPSCs: Adipose-derived induced pluripotent stem cells
AT-MSCs: Human adipose tissue
bFGF: Basic fibroblast growth factor
BM-MSCs: Bone marrow mesenchymal stem cells
BMP: Bone morphogenetic protein
BMSC: Bone marrow stromal cells
CA: Cytomegalovirus enhancer/b-actin
CCD: Cleidocranial dysostosis
CCHS: Collagen/chitosan/hydroxyapatite scaffolds
CM: Conditioned media
CMC: Carboxymethyl chitosan
Col2.3GFP: 2.3 kb type I collagen promoter-driven green fluorescent protein
CPC: Calcium phosphate cement
DFs: Dermal fibroblasts
DPI-VTK: Dpiyalswsgma-Vtkhlnqisqsy
EBs: Embryoid bodies
EF: Embryonic fibroblasts
ELF-EMF: Extremely low-frequency electromagnetic field
EMD: Enamel matrix derivatives
ES: Embryonic stem
G/C/GP: Chitosan/gelatin/glycerol phosphate
GDF-5: Growth/differentiation factor-5
GO: Graphene oxide
HA/Col/CTS: Hydroxyapatite/collagen/chitosan
HA/TCP: Hydroxyapatite/tricalcium phosphate
HA: Hydroxyapatite
HCG: Nanohydroxyapatite/chitosan/gelatin
HEK: Human embryonic kidney
hiPSCs: Human induced pluripotent stem cells
HUVECs: Human umbilical vein endothelial cells
iPSCs: Induced pluripotent stem cells
iPS-NC-PDL cells: iPSCs induced into neural crest- (NC-) like cells
iPS-NC cells: p75 neurotrophic receptor-positive cells were cultured on extracellular matrix (ECM) produced by human PDL
MSCs: Mesenchymal stem cells
NCLCs: Neural crest-like cells
nHA/CG scaffolds: Nanohydroxyapatite/chitosan/gelatin scaffolds
nHA: Nanohydroxyapatite
OM: Osteogenic media
PCL: Polycaprolactone
PCL-PLLA: Polycaprolactone-poly-L-lactic acid
PCL-PVDF: Polycaprolactone-polyvinylidene fluoride
pDA: Polydopamine
PBMC: Peripheral blood mononuclear cell
PDL: Periodontal ligaments
PEG: Polyethylene glycol
PES: Polyethersulfone
PHT: Polymer hyaluronan and ceramic tricalcium phosphate ceramic particles
PLA: Poly-L-lactic acid
PLCL: Poly (L-lactic acid-co-ε-caprolactone)
PLGA/PLLA: Poly lactic-co-glycolic acid/poly L-lactic acid
Poly-P: Polyphosphate
PVDF: Polyvinylidene fluoride
PVDF/Col/PRP: Polyvinylidene fluoride/collagen/platelet-rich plasma
RGD: Arg-Gly-Asp
RUNX2: Runt-related transcription factor 2
SHED: Human exfoliated deciduous teeth
TGF-β: Transforming growth factor-beta
TNAP: Tissue-nonspecific alkaline phosphatase
TSG-6: Tumor necrosis factor alpha-stimulated gene-6.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Mesenchymal Stem Cells from Human Exfoliated Deciduous Teeth and the Orbicularis Oris Muscle: How Do They Behave When Exposed to a Proinflammatory Stimulus?

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Mesenchymal stem cells (MSCs) have been studied as a promising type of stem cell for use in cell therapies because of their ability to regulate the immune response. Although they are classically isolated from the bone marrow, many studies have sought to isolate MSCs from noninvasive sources. The objective of this study was to evaluate how MSCs isolated from the dental pulp of human exfoliated deciduous teeth (SHED) and fragments of the orbicularis oris muscle (OOMDSCs) behave when treated with an inflammatory IFN-γ stimulus, specifically regarding their proliferative, osteogenic, and immunomodulatory potentials. The results demonstrated that the proliferation of SHED and OOMDSCs was inhibited by the addition of IFN-γ to their culture medium and that treatment with IFN-γ at higher concentrations resulted in a greater inhibition of the proliferation of these cells than treatment with IFN-γ at lower concentrations. SHED and OOMDSCs maintained their osteogenic differentiation potential after stimulation with IFN-γ. Additionally, SHED and OOMDSCs have been shown to have low immunogenicity because they lack expression of HLA-DR and costimulatory molecules such as CD40, CD80, and CD86 before and after IFN-γ treatment. Last, SHED and OOMDSCs expressed the immunoregulatory molecule HLA-G, and the expression of this antigen increased after IFN-γ treatment. In particular, an increase in intracellular HLA-G expression was observed. The results obtained suggest that SHED and OOMDSCs lack immunogenicity and have immunomodulatory properties that are enhanced when they undergo inflammatory stimulation with IFN-γ, which opens new perspectives for the therapeutic use of these cells.

1. Introduction

Mesenchymal stem cells (MSCs) are multipotent cells that have the ability to differentiate into mesodermal cell lineages, including chondroblasts, osteoblasts, and adipocytes [1, 2]. In addition, three basic characteristics must be present to classify a culture of cells isolated from neonatal or adult tissues as a culture of MSCs [3]. First, MSCs are able to adhere to the plastic of the cell culture flasks. Furthermore, at least 95% of an isolated and cultured cell population must express mesenchymal antigens (such as CD29, CD44, CD73, CD90, and CD105) and should not express hematopoietic or immune cell markers (such as CD14, CD19, and CD34) or endothelial cell markers (such as CD31). Finally, MSCs should be able to differentiate into osteoblasts, chondrocytes, and adipocytes in vitro under specific culture conditions [3].

Although they are more commonly isolated from the bone marrow and adipose tissue of donors, recent studies have demonstrated that MSCs can also be obtained from several other tissue types [4, 5] and that the cells from distinct tissues present considerable differences in their proliferative abilities and differentiation potentials [6]. For example, it has been suggested that MSCs obtained from neonatal tissues are more proliferative, have a higher differentiation potential, and can be maintained in culture for longer periods (before reaching cell senescence) than MSCs isolated from the bone

marrow [6]. In addition, a study conducted by Barlow et al. [7] demonstrated that MSCs isolated from the human placenta have a high proliferative capacity and are able to integrate into tissues to be regenerated. Additionally, a study conducted by Kern at et al. [8] showed that MSCs isolated from adipose tissue also have a proliferative capacity superior to that of MSCs isolated from the bone marrow. Furthermore, this study demonstrated that MSCs isolated from adipose tissue have a high adipogenic potential and are able to be cultured for long periods without undergoing senescence or a loss of phenotypic characteristics [8]. Another type of MSC that is currently being investigated due to its potential in tissue engineering applications is stem cells from human exfoliated deciduous teeth (SHED). This type of stem cell has shown great capacities to differentiate into neural precursor cells, odontoblasts, and osteoblasts and has a high proliferative capacity [9]. In addition, Bueno et al. [10] demonstrated that MSCs isolated from the orbicularis oris muscle (OOMDSCs) obtained during cheiloplasty surgery in cleft lip and palate patients are capable of differentiation into chondrogenic, adipogenic, osteogenic, and skeletal muscle cells and present phenotypic and behavioral characteristics similar to those of MSCs isolated from other tissues. Due to their potential to differentiate into multiple cell types, MSCs could be used for the treatment of several diseases, especially for the repair of tissue lesions.

Many of the therapeutic properties of MSCs have also been attributed to the paracrine and endocrine effects of their secreted factors. Notably, MSCs have been shown to be capable of supporting the maturation and proliferation of hematopoietic cells, migrating to an area of tissue injury, recruiting tissue-specific progenitor cells [11], and regulating the immune response through the secretion of immunomodulatory cytokines and microvesicles containing a variety of bioactive molecules such as enzymes, coding and noncoding RNAs, and heat shock proteins [12]. In addition to the minimum criteria for the characterization of MSCs defined by the International Society for Cell Therapy [3], it has been proposed that the immunological properties of MSCs should also be used as one of the characterization criteria for MSCs [13]. The positive results in preclinical trials and demonstration of the immunomodulatory effects of MSCs in in vitro and animal studies have led to a rapid increase in the number of clinical trials in which the therapeutic potential of these cells has been evaluated for the treatment of a variety of diseases [14]. As a result, a large number of distinct cell preparations of MSCs have been tested in nearly 350 clinical trials conducted by academic institutions and corporations in which the safety and efficacy of autologous and allogeneic MSCs have been evaluated. Examples of diseases in which the therapeutic potential of MSCs has been evaluated in clinical and preclinical trials and has presented promising results include acute myocardial infarction [15], graft-versus-host disease [16], systemic lupus erythematosus [17], rheumatoid arthritis [17], Crohn's disease [18], multiple sclerosis [19], amyotrophic lateral sclerosis [20], and type I diabetes mellitus [21].

Although IFN-γ is a proinflammatory cytokine, studies have shown that IFN-γ also influences the osteogenic potential of MSCs. Croes et al. [22] demonstrated that activated $CD4^+$ T lymphocytes cocultured with human MSCs promote the differentiation of the MSCs into osteoblasts, and after blocking secreted IFN-γ with antibodies, osteogenic differentiation of the MSCs was inhibited. In addition, a study conducted by Duque et al. [23] demonstrated that human MSCs secrete IFN-γ that acts by stimulating the osteogenic differentiation potential of the MSCs through the expression of osteogenic transcription factors, such as Runx2. Furthermore, a study conducted by Vidal et al. [24] demonstrated that MSCs isolated from mice with knocked-out IFN-γ receptors (IFN-γ R $^-/^-$) express Runx2 at lower levels than MSCs isolated from wild-type mice and, therefore, have a more limited potential for osteogenic differentiation. In a study conducted by Liu et al. [25], it was demonstrated that MSCs isolated from the bone marrow had their potential for osteogenic differentiation inhibited when treated with 200 ng/mL IFN-γ compared with no stimulation with IFN-γ. However, it was also demonstrated that treatment of MSCs with IFN-γ at a concentration of 50 ng/mL had no inhibitory effect on the osteogenic differentiation potential of the MSCs [25]. This difference was attributed to the increased expression of SMAD6 (a gene that inhibits osteogenic differentiation) and decreased expression of Runx2, osteocalcin, and alkaline phosphatase in the MSCs treated with the highest IFN-γ concentration, whereas the expression of these genes remained unchanged in the MSCs treated with IFN-γ at a 50 ng/mL concentration [25]. Additionally, a study conducted by Sonoda et al. [26] demonstrated that dental pulp stem cells isolated from teeth with irreversible pulpitis and treated with IFN-γ at a 100 ng/mL concentration were able to give rise to a significant number of nodules containing calcium deposits (positive for Alizarin Red staining) after 4 weeks of culture in osteogenic differentiation medium. However, this same study demonstrated that dental pulp stem cells isolated from teeth with irreversible pulpitis that were not previously treated with IFN-γ gave rise to a much smaller number of nodules containing calcium deposits after 4 weeks of culture in osteogenic differentiation medium.

Regarding their immunomodulatory potential, MSCs, when exposed to a proinflammatory stimulus, will secrete molecules that act by inhibiting the maturation of antigen-presenting cells such as monocytes, dendritic cells (DCs), and macrophages. These molecules also promote the polarization of macrophages into M2 macrophages and inhibit the polarization of macrophages into M1 macrophages. MSCs are also able to inhibit the activation and proliferation of natural killer (NK) cells, $CD8^+$ T lymphocytes (inhibiting their cytotoxic effects and cytokine production), and B lymphocytes (inhibiting the production of antibodies by these cells) to promote the activation of regulatory T lymphocytes and inhibit the activation of DCs [16].

It is of utmost importance that MSCs isolated from different tissues, especially those isolated from less invasive sources, are characterized and classified. Additionally, little is known about the effects of proinflammatory stimulation with IFN-γ on the biological properties of MSCs. Since our group works with bone tissue engineering applications for the reconstruction of the alveolar bone in cleft lip and palate

patients, this study investigated the effects of proinflammatory stimulation with IFN-γ on the biological properties of SHED and OOMDSCs. These sources of MSCs are considered noninvasive for cleft lip and palate patients since small fragments of the orbicularis oris muscle are regularly discarded during cheiloplasty surgery [10], and all children have deciduous teeth in exfoliation when they are between six and twelve years old.

The main objective of this work was to study how both OOMDSCs and SHED behave when treated with an inflammatory IFN-γ stimulus, specifically regarding their proliferative, osteogenic, and immunomodulatory potentials. Specifically, this study was aimed at determining whether the proliferative capacities of SHED and OOMDSCs are influenced by treatment with IFN-γ at different concentrations to evaluate the effect of these treatments on the osteogenic potential of OOMDSCs and SHED by assessing the expression of specific costimulatory, immunogenic, and immunomodulatory molecules in populations of both OOMDSCs and SHED exposed to a proinflammatory IFN-γ stimulus or left unexposed.

2. Material and Methods

2.1. Isolation and Culture of SHED and OOMDSCs. For the establishment of primary lines of MSCs, pulps of deciduous teeth and fragments of the orbicularis oris muscle were obtained from the care service for patients with cleft lip and palate at Hospital Municipal Infantil Menino Jesus. SHED and OOMDSCs isolated from five distinct donors each were isolated and used for the experiments described. For the isolation of SHED, tooth pulps were collected by surgical extraction and immediately added to a sterile collector containing 2 mL of Dulbecco's modified Eagle medium/Nutrient Mixture F-12 (DMEM-F12; Gibco Invitrogen, Grand Island, NY) supplemented with 100 IU/mL penicillin and streptomycin (Penicillin-Streptomycin; Gibco Invitrogen, Grand Island, NY). The pulps of deciduous teeth were then washed with 1X phosphate-buffered saline (PBS^-, pH: 7.4; Gibco Invitrogen, Grand Island, NY) twice and digested with 1 mg/mL trypsin solution (TrypLE; Gibco Invitrogen, Grand Island, NY) diluted in $1XPBS^-$ (pH 7.4; Gibco Invitrogen, Grand Island, NY) for 30 minutes at 37°C. After tissue digestion, the samples were centrifuged at 300 x g for 5 minutes. Subsequently, the pulps were cut into two or more fragments, depending on size, and cultured in a 12-well plate, preferably with one fragment per well. Twenty days after this procedure, MSCs were expelled from the dental pulp fragments, thereby establishing a primary culture of SHED. In addition, orbicularis oris muscle fragments were collected during cheiloplasty surgery and immediately added to a sterile collector containing 2 mL of DMEM-F12 (Gibco Invitrogen, Grand Island, NY) supplemented with 100 IU/mL of penicillin and streptomycin (Penicillin-Streptomycin; Gibco Invitrogen, Grand Island, NY). The orbicularis oris muscle fragments were then washed twice with $1XPBS^-$ (pH 7.4; Gibco Invitrogen, Grand Island, NY) and digested in a solution containing 1 mg/mL trypsin (TrypLE-Gibco Invitrogen, Grand Island, NY) diluted in $1XPBS^-$ for 30 minutes at 37°C. After enzymatic digestion, the samples were centrifuged at 300 x g for 5 minutes. The muscle fragments were then cut into three or four parts, depending on size, and cultured in 12-well plates, preferably with one fragment per well. Twenty days after this procedure, MSCs were expelled from the orbicularis oris muscle fragments, and a culture of OOMDSCs was established.

The isolated SHED and OOMDSCs were cultured at 37°C/5% CO_2 in humidified incubators in T25 or T75 flasks in 5 (for T25 flasks) or 10 (for T75 flasks) mL of DMEM-F12 (Gibco Invitrogen, Grand Island, NY) supplemented with 15% fetal bovine serum (FBS; HyClone-GE Heathcare Life Sciences, South Logan, Utah). The culture medium was replaced every 2 or 3 days. The cultured MSCs were transferred to new flasks when approximately 90% confluent to prevent cellular senescence. To this end, the basal medium was aspirated, and the cell culture flasks were washed twice with 5 mL of $1XPBS^-$ to remove all the residual culture medium. Thereafter, the MSCs that adhered to the cell culture flasks were removed from the flasks by incubating the MSCs at 37°C/5% CO_2 for 3 minutes in 1 (for T25 flasks) or 1.5 (for T75 flasks) mL of TrypLE Express Enzyme (1X) (Gibco Invitrogen, Grand Island, NY). After 3 minutes, 3 mL of basal medium was added to the cell culture flasks to neutralize the action of TrypLE Express Enzyme. The cell culture flasks were then washed several times with the basal medium to remove all residual MSCs from the flasks, and the MSC-containing solution was transferred to 15 mL polypropylene tubes (BD Falcon, Heidelberg, Germany). These tubes were subsequently centrifuged at 300 x g for 5 minutes, and the supernatant was aspirated to remove all TrypLE Express Enzyme present in the supernatant. Then, the precipitate containing the MSCs was resuspended in 1 mL of basal medium, and the cells were transferred to new cell culture flasks containing 5 (for T25 flasks) or 10 (for T75 flasks) mL of basal culture medium at a density of 5,000 cells/cm^2 and kept in culture at 37°C/5% CO_2 in humidified incubators. For long-term storage, MSCs were maintained in liquid nitrogen. When necessary, MSCs were thawed and expanded to conduct experiments. All experiments described here were performed according to national and international standards of research ethics and were approved by the Research Ethics Committee of Hospital Sirio Libanes.

2.2. Cell Surface Marker Analysis. To confirm their identities as MSCs, both SHED and OOMDSCs were characterized by flow cytometry for the expression of typical surface markers of MSCs (CD29, CD44, CD90, CD73, CD105, and CD106) and endothelial (CD31) and hematopoietic (C34) cells. All cells were incubated with antibodies (conjugated with fluorochromes) that had the ability to bind specifically to intracellular and cell surface proteins to compare and characterize the cells according to the expression of specific antigens. A total of 1×10^6 cells obtained from cell cultures and diluted in 100 μL of $1XPBS^-$ were transferred to flow cytometry tubes and incubated with the following monoclonal antibodies for 15 minutes at room temperature in the dark for staining: anti-CD29-PE, anti-CD44-PE, anti-CD73-FITC, anti-CD90-FITC, anti-CD105-PE, anti-CD166-PE, anti-CD34-FITC, and anti-CD31-FITC (BD Bioscience,

Becton Dickinson Franklin Lakes, NJ). The samples were then washed with 1XPBS$^-$, resuspended in 500 μL of 1XPBS$^-$, run on a FACSCalibur (BD, Becton Dickinson, Franklin Lakes, NJ) flow cytometer, and subsequently analyzed using FlowJo software (TreeStar Inc.). A sample of unstained cells was prepared for each experiment to eliminate the influence of any nonspecific staining and innate autofluorescence of the cells. As a negative control for the reactions, an isotype control was used for each antibody.

2.3. Osteogenic Differentiation Assay. In addition to being characterized by the expression of cell surface markers, MSCs were also characterized by their potentials for osteogenic, adipogenic and chondrogenic differentiation. For the osteogenic differentiation assays, a total of 5×10^3 MSCs were seeded into 12-well cell culture plates (Corning® Costar®). The MSCs were then allowed to adhere to the surface of the culture plates for 24 hours in basal medium at 37°C/5% CO_2 in humidified incubators prior to the initiation of the osteogenic differentiation protocol. Osteogenesis was then induced by replacing the basal medium with culture medium containing growth factors specific for the induction of osteogenic differentiation in MSCs (StemPro Osteogenesis Differentiation Kit; Gibco Invitrogen, Grand Island, NY). The osteogenic medium was changed every 3-4 days for 21 days, and osteogenic differentiation was observed during this period by assessing the morphological alteration of spindle-shaped cells into star-shaped cubic cells. The MSCs used as negative controls for the osteogenic differentiation process were cultured in the basal medium for the same 21 days. To evaluate the osteogenic differentiation process, the culture of MSCs was stained with Alizarin Red S after 21 days under the differentiation conditions. The staining of the culture of MSCs with Alizarin Red S indicated the presence of a mineralized matrix in the culture and suggested the presence of calcium hydroxyapatite, indicating that a successful osteogenic differentiation process occurred.

2.4. Adipogenic Differentiation Assay. For the characterization of MSCs regarding their adipogenic differentiation potential, a total of 5×10^3 MSCs were seeded into 12-well cell culture plates (Corning® Costar®). The MSCs were then allowed to adhere to the surface of the culture plates for 24 hours in basal medium at 37°C/5% CO_2 in humidified incubators prior to the initiation of the adipogenic differentiation protocol. Adipogenesis was then induced by replacing the basal medium with culture medium containing specific growth factors to induce adipogenic differentiation in the MSCs (StemPro Adipogenesis Differentiation Kit; Gibco Invitrogen, Grand Island, NY). The adipogenic medium was changed every 3-4 days for 18 days, and adipogenic differentiation was observed by evaluating the presence of vacuoles in the MSCs. The MSCs used as negative controls for the adipogenic differentiation process were cultured in the basal medium for the same 18 days. To evaluate the adipogenic differentiation process, the MSC culture was stained with Oil Red O (Sigma-Aldrich, St. Louis, MO) after 18 days of culture under the differentiation conditions. Oil Red O staining indicated the presence of lipid-rich vacuoles within the MSCs.

2.5. Chondrogenic Differentiation Assay. MSCs were also characterized for their chondrogenic differentiation potential. To assess this potential, a total of 5×10^3 MSCs were seeded in 12-well cell culture plates (Corning® Costar®). The MSCs were then allowed to adhere to the surface of the culture plates for 24 hours in basal medium at 37°C/5% CO_2 in humidified incubators prior to the initiation of the chondrogenic differentiation protocol. Chondrogenesis was induced by replacing the basal medium with culture medium containing growth factors specific for the induction of chondrogenic differentiation in the MSCs (StemPro® Chondrogenesis Differentiation Kit; Gibco Invitrogen, Grand Island, NY) once the MSC culture had reached at least 80% confluency. The chondrogenic medium was changed every 3-4 days for 21 days. The MSCs used as a negative control for the process of chondrogenic differentiation were cultured in the basal medium for the same 21 days. To evaluate the process of chondrogenic differentiation, the culture of MSCs was stained with Alcian Blue (Sigma-Aldrich, St. Louis, MO) after 21 days of culture under the differentiation conditions. The blue color observed by Alcian Blue staining indicated the presence of proteoglycans (extracellular matrix) secreted by chondrocytes and showed that successful chondrogenic differentiation occurred.

2.6. Analysis of the Effect of Proinflammatory Stimulation with IFN-γ on the Proliferation and Viability of SHED and OOMDSCs. To evaluate the effect of a proinflammatory IFN-γ stimulus on the cell proliferation of SHED and OOMDSCs, both cell types were seeded in 12-well plates and maintained in culture medium with or without IFN-γ at different concentrations. Initially, a total of 5×10^3 cells were plated in each well of the 12-well plates in culture medium with or without IFN-γ at 10, 25, 50, 100, or 500 ng/mL for 3, 5, or 7 days. After the preestablished time for each culture was reached, analysis of cell proliferation and viability during this period was performed by determining the percentage of viable cells and the total number of cells present in each well. For this purpose, both SHED and OOMDSCs were removed from each well of the 12-well plates used, washed, and resuspended in 300 μL of culture medium. Then, 10 μL of MSC-containing solution was diluted in 10 μL of Trypan Blue (Sigma-Aldrich) so that the dead cells could be identified. Finally, the counting process and determination of viability were performed using a Cell Countess (Sigma-Aldrich).

2.7. Analysis of the Effect of the Proinflammatory IFN-γ Stimulus on the Osteogenic Potential of SHED and OOMDSCs. To study the proinflammatory effect of the IFN-γ stimulus on the osteogenic differentiation potential of SHED and OOMDSCs, both cell types were seeded in 96-well plates. A total of 3×10^3 MSCs were seeded in each well and cultured in culture medium with or without IFN-γ at different concentrations (10, 25, 50, 100, or 500 ng/mL) for 21 days. After 21 days of differentiation, the wells were

stained with Alizarin Red as previously described. Subsequently, 100 μL of 20% methanol solution and 10% acetic acid diluted in 1XPBS$^-$ were added to each well so that the mineralized matrix previously stained with Alizarin Red could be dissolved. Finally, the samples were incubated for 15 minutes at room temperature exposed to light, and the osteogenic differentiation process was quantified by determining the optical density (OD) of the solution in each well with a spectrophotometer at a 480 nm wavelength.

2.8. Analysis of the Effect of the Proinflammatory IFN-γ Stimulus on the Expression of Cell Surface and Intracellular Markers. To study, by flow cytometry, the expression of costimulatory, immunogenic, and immunomodulatory molecules on the cell surface of both OOMDSCs and SHED exposed to a proinflammatory stimulus, both cell types were maintained in T75 flasks in culture medium supplemented with 25 ng/mL IFN-γ (PeproTech) for 48 hours. In addition, untreated cells were maintained in culture medium and used as a control for the experiment. After 48 hours, the stem cells maintained in the medium supplemented with IFN-γ were extracted from their flasks, processed into a single-cell suspension, and prepared for flow cytometric analysis. This technique was used to evaluate the immunological profile of both the OOMDSCs and the SHED maintained in the medium supplemented with 25 ng/mL IFN-γ or no IFN-γ. The immunological profiles of both the OOMDSCs and the SHED were assessed by analyzing the expression of CD40, CD80, CD86, human leukocyte antigen- (HLA-) DR, HLA-A, HLA-B, HLA-C, and HLA-G in cells maintained in the medium supplemented with IFN-γ or without IFN-γ. For the detection of nuclear or cytoplasmic proteins, the cells were fixed and permeabilized to allow the antibodies to cross the cell membrane. A sample of unlabeled cells was prepared for each experimental group to eliminate the influence of nonspecific staining and innate autofluorescence on the results. As a negative control for the reactions, an isotype control was used for each type of immunoglobulin used. The analysis of the expression of the different markers studied (CD40, CD80, CD86, HLA-DR, HLA-A, HLA-B, HLA-C, and HLA-G) in both the SHED and the OOMDSCs treated with IFN-γ or left unstimulated was performed by determining the median fluorescence intensity (MFI) of each marker.

2.9. Statistical Analysis. Descriptive analyses for quantitative data were performed and are presented as the average accompanied by the corresponding standard deviation (±sd). The assumptions of a normal distribution and homogeneity of the variances were evaluated with the Shapiro-Wilk test and the Levene test, respectively. To analyze two distinct factors, two-way ANOVA was used. For one factor analysis, one-way ANOVA was used. When it was necessary to perform multiple comparisons of means, the Bonferroni post hoc test was used. For comparisons of means between two independent groups, an unpaired Student t-test was used. All analyses were performed with the software SigmaPlot for Windows version 11.0 with a significance level of $\alpha = 0.05$.

3. Results

3.1. SHED and OOMDSCs Express a Typical MSC Immunophenotype and Are Capable of Adipogenic, Osteogenic, and Chondrogenic Differentiation. After being maintained under specific conditions of differentiation, populations of both SHED and OOMDSCs were differentiated into adipocytes, chondrocytes, or osteocytes. After 21 days of culture in osteogenic differentiation medium, both the OOMDSCs and the SHED were capable of osteogenic differentiation, as evidenced by the presence of a mineralized matrix detected by Alizarin Red S staining. Similarly, both the OOMDSCs and the SHED, when cultured in chondrogenic differentiation medium, were capable of chondrogenic differentiation after 21 days, as demonstrated by positivity with Alcian Blue staining. Finally, both the SHED and the OOMDSCs were able to differentiate into adipocytes after 18 days of culture in adipogenic differentiation medium, as demonstrated by the presence of lipid vacuoles stained with Oil Red O (Figure 1(a)). In addition, flow cytometric analysis revealed that the population of both SHED (Figure 1(b)) and OOMDSCs (Figure 1(c)) was positive for the mesenchymal stem markers CD29, CD44, CD90, CD105, CD73, and CD166 and negative for endothelial (CD31) and hematopoietic cell markers (CD34) (Table 1).

3.2. The Osteogenic Differentiation Potential of both SHED and OOMDSCs Was Maintained after Treatment with IFN-γ. Alizarin Red S staining after 21 days of osteogenic differentiation in osteogenic differentiation medium with or without IFN-γ at different concentrations (10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, and 500 ng/mL) demonstrated that both SHED (Figure 2(a)) and OOMDSCs (Figure 2(b)) maintained their osteogenic differentiation potential when stimulated with IFN-γ at all concentrations compared with no stimulation. In particular, SHED had their osteogenic differentiation potential significantly stimulated by the addition of 500 ng/mL IFN-γ to the osteogenic differentiation medium compared with the addition of 10 ng/mL IFN-γ (Figure 2(a)).

3.3. The Proliferation of both SHED and OOMDSCs Was Inhibited after Treatment with IFN-γ. The results obtained in this study demonstrated that, regarding their proliferative capacity and cell viability, populations of both SHED and OOMDSCs behaved in a similar manner when treated with a proinflammatory IFN-γ stimulus. After the third day of culture, compared with no treatment, the addition of distinct concentrations of IFN-γ to the culture medium resulted in the inhibition of cell proliferation in both SHED (Figure 3(a)) and OOMDSCs (Figure 3(c)). The addition of IFN-γ to the culture medium significantly inhibited the proliferation of SHED and OOMDSCs during all days of culture, and this inhibition was more evident in the final days of culture (days 5 and 7) than in the initial days. Furthermore, the addition of IFN-γ at higher concentrations (100 ng/mL and 500 ng/mL) more strongly inhibited the proliferation of both SHED and OOMDSCs than the addition of IFN-γ at lower concentrations (10 ng/mL, 25 ng/mL, and 50 ng/mL). In addition, a significant decrease in the viability of both SHED

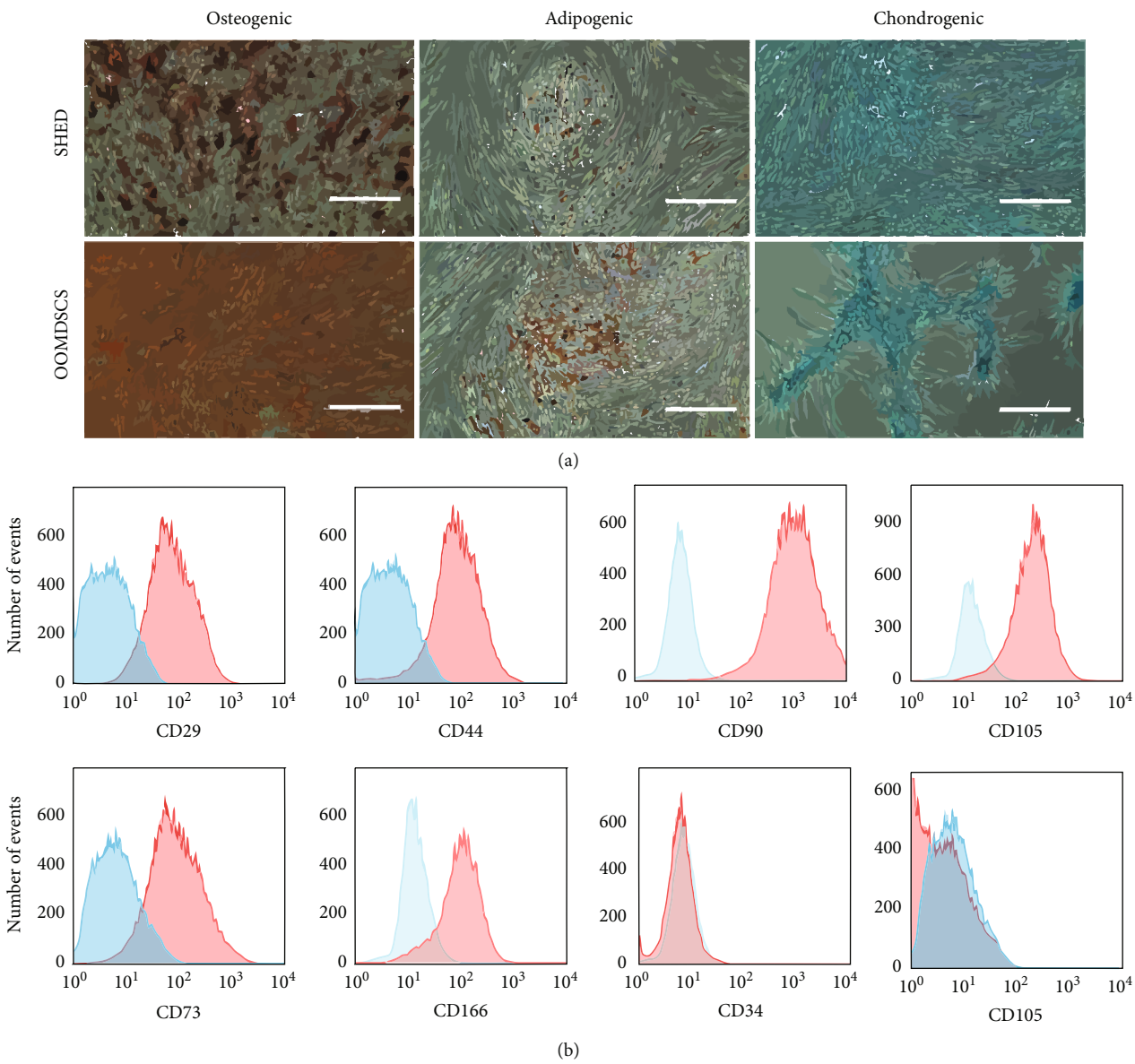


Figure 1: Continued.

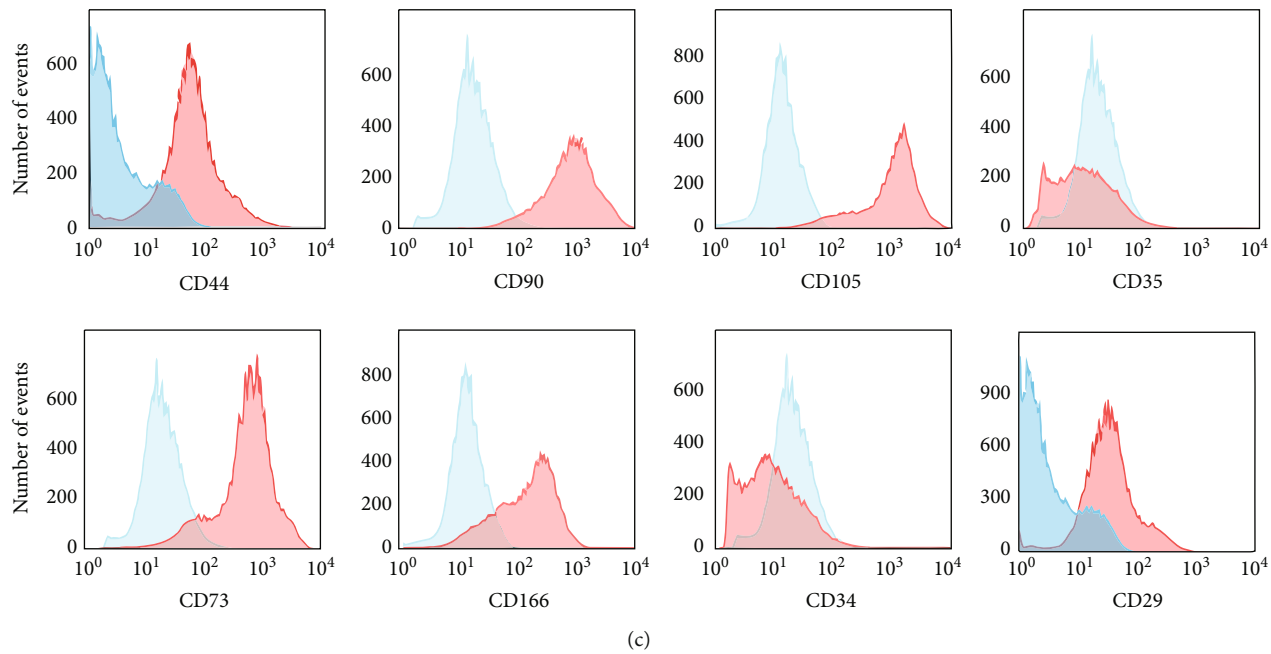


Figure 1: (a) Representative figure of the osteogenic, adipogenic, and chondrogenic differentiation potentials of SHED and OOMDSCs. Both SHED and OOMDSCs were capable of osteogenic, adipogenic, and chondrogenic differentiation after culturing in differentiation medium. Osteogenesis is shown by the presence of a mineralized matrix containing calcium hydroxyapatite (red staining when stained with Alizarin Red S), adipogenesis is shown by the formation of lipid vesicles (red staining when stained with Oil Red O), and chondrogenesis is shown by the production of proteoglycans (blue staining when stained with Alcian Blue). Scale bars represent 400 μm. (b, c) Representative figure of the immunophenotypic profiles of SHED (b) and OOMDSCs (c). Both SHED and OOMDSCs expressed the mesenchymal stem cell markers CD29, CD44, CD90, CD105, CD73, and CD166 but did not express endothelial (CD31) or hematopoietic (CD34) markers on the cell surface. Histograms demonstrate the binding of conjugated antibodies (in red) and isotype controls (in blue) to surface antigens.

Table 1: Average expression of mesenchymal, hematopoietic, and endothelial stem cell markers.

Cell surface marker	Average expression of cell surface markers in SHED (% of positive cells in relation to total)	Average expression of cell surface markers in OOMDSCs (% of positive cells in relation to total)
CD29	91, 3% ± 2, 3%	83, 6% ± 3, 1%
CD44	93, 2% ± 1, 4%	86, 7% ± 2, 7%
CD90	98, 1% ± 0, 6%	94, 8% ± 1, 1%
CD73	90, 2% ± 1, 7%	87, 4% ± 3, 5%
CD105	96, 8% ± 0, 8%	96, 8% ± 0, 5%
CD166	88, 9% ± 4, 1%	71, 8% ± 5, 5%
CD34	0, 42% ± 0, 23%	2, 2% ± 0, 71%
CD31	0, 46% ± 0, 31%	2, 6% ± 0, 84%

(Figure 3(b)) and OOMDSCs (Figure 3(d)) was mainly observed in the initial days of culture and was more evident when the stem cells were treated with the higher concentrations of IFN-γ. After seven days of culture, the viability of most of the IFN-γ-treated populations of both SHED and OOMDSCs returned to levels similar to those of the untreated controls. Due to the fact that IFN-γ was only added to the culture medium in the beginning of the experiment, it can be hypothesized that the increase in cell viability observed is a result of IFN-γ losing its biological function or being depleted from the culture medium after the third day of culture.

3.4. SHED and OOMDSCs Expressed Molecules with Immunomodulatory Properties but Did Not Express Costimulatory Molecules or HLA Class II Molecules after Treatment with IFN-γ. After 48 hours of proinflammatory stimulation with 25 ng/mL IFN-γ, flow cytometric analysis demonstrated that both SHED and OOMDSCs did not express HLA-DR (SHED = 10.95 ± 3.38 MFI, OOMDSCs

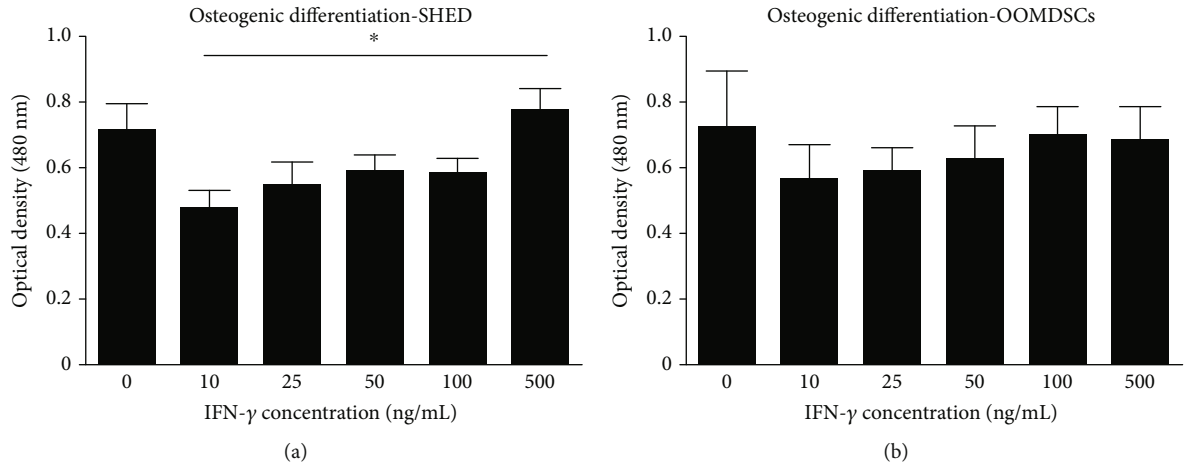


Figure 2: Effects of proinflammatory stimulation with IFN-γ on the osteogenic differentiation potential of SHED and OOMDSCs. (a, b) The addition of IFN-γ at a concentration of 10 ng/mL, 25 ng/mL, 50 ng/mL, 100 ng/mL, or 500 ng/mL maintained the osteogenic differentiation potential of both SHED (a) and OOMDSCs (b) at levels similar to the level observed with control treatment. A statistically significant difference was observed only between SHED maintained in osteogenic differentiation medium supplemented with IFN-γ at a concentration of 500 ng/mL (0.77 ± 0.06 sd) and SHED cultured in osteogenic differentiation medium supplemented with IFN-γ at a concentration of 10 ng/mL (0.47 ± 0.06 sd). $^*p < 0.05$, determined by one-way ANOVA followed by the Bonferroni post hoc test. Five experimental replicates were performed for each group for both SHED and OOMDSCs.

= 7.78 ± 0.5 MFI) or the costimulatory molecules CD40 (SHED = 8.26 ± 1.78 MFI, OOMDSCs = 2.42 ± 0.64 MFI), CD80 (SHED = 7.03 ± 1.17 MFI, OOMDSCs = 4.74 ± 0.52 MFI), and CD86 (SHED = 7.82 ± 2.27 MFI, OOMDSCs = 7.85 ± 1.32 MFI) on their cell surface, and these expression levels were similar to the expression levels of HLA-DR (SHED = 7.53 ± 1.47 MFI, OOMDSCs = 5.54 ± 1.34 MFI), CD40 (SHED = 9.1 ± 2.79 MFI, OOMDSCs = 2.97 ± 0.780 MFI), CD80 (SHED = 7.18 ± 2.28 MFI, OOMDSCs = 5.87 ± 0.19 MFI), and CD86 (SHED = 7.41 ± 2.77 MFI, OOMDSCs = 6.64 ± 0.58 MFI) observed in untreated controls (Figures 4(a) and 4(b)). However, the expression of HLA-A, HLA-B, and HLA-C was detected in the untreated populations of both SHED (172.36 ± 58.94 MFI) and OOMDSCs (159.96 ± 39.16 MFI), and after treatment with IFN-γ, the HLA-A,HLA-B, and HLA-C expression on the cell surface of both SHED (858.8 ± 413.63 MFI) and OOMDSCs (788.8 ± 191.7 MFI) increased (Figure 4(c)). Additionally, the expression of the immunomodulatory molecule HLA-G was detected in the unstimulated populations of both SHED (20.34 ± 2.46 MFI) and OOMDSCs (25.89 ± 4.74 MFI). After treatment with 25 ng/mL IFN-γ, HLA-G expression increased significantly in both SHED (41.08 ± 4.40 MF) and OOMDSCs (58.18 ± 7.45 MFI) (Figure 4(d)). Finally, the intracellular and cell surface expression of HLA-G was assessed in both SHED and OOMDSCs treated with or without 25 ng/mL IFN-γ to verify whether the increase in HLA-G expression observed previously after treatment with 25 ng/mL IFN-γ was due to increased HLA-G surface or intracellular expression. The results demonstrated that while the intracellular expression of HLA-G increased significantly in the populations of both SHED (control = 21.5 ± 4.82 MFI, 25 ng/mL IFN-γ = 35.93 ± 3.81 MFI) and OOMDSCs (control = 24.2 ± 3.74 MFI, 25 ng/mL IFN-γ = 40.25 ± 4.74 MFI) treated with 25 ng/mL IFN-γ (Figure 4(f)), the expression of HLA-G on the cell surface of SHED treated with 25 ng/mL IFN-γ did not change (control = 12.90 ± 4.09 MFI, IFN-γ 25 ng/mL = 12.21 ± 6.39 MFI), but the expression of HLA-G on the cell surface of OOMDSCs increased when these cells were treated with 25 ng/mL IFN-γ (control = 20.15 ± 6.91 MFI, 25 ng/mL IFN-γ = 25.48 ± 8.79 MFI); however, this increase was not statistically significant (Figure 4(e)).

4. Discussion

The results obtained in the present study demonstrated that SHED and OOMDSCs behave in a similar way when considering the effect of a proinflammatory IFN-γ stimulus on proliferative capacity. The proliferation of both SHED and OOMDSCs was inhibited after the addition of IFN-γ to the medium used for culture. In addition, treatment with IFN-γ at higher concentrations resulted in greater inhibition of the proliferation of both SHED and OOMDSCs than treatment with IFN-γ at lower concentrations. However, varied results have been reported in relation to the effect of proinflammatory stimulation on the proliferative capacity of MSCs. For example, in a study by He et al. [27], it was shown that treatment of dental pulp stem cells with IFN-γ at low concentrations (0.05, 0.5, and 5 ng/mL) stimulated the proliferation and migration of this type of stem cell. On the other hand, Chan et al. [28] demonstrated that compared with untreated MSCs, MSCs treated with IFN-γ for 8 days had their proliferative capacity reduced by 50%. Similarly, Yazid et al. [29] demonstrated that MSCs isolated from healthy dental pulp have a greater proliferative capacity than MSCs isolated from inflamed dental pulp. In addition, in a study by Qin et al. [30], no statistically significant differences in

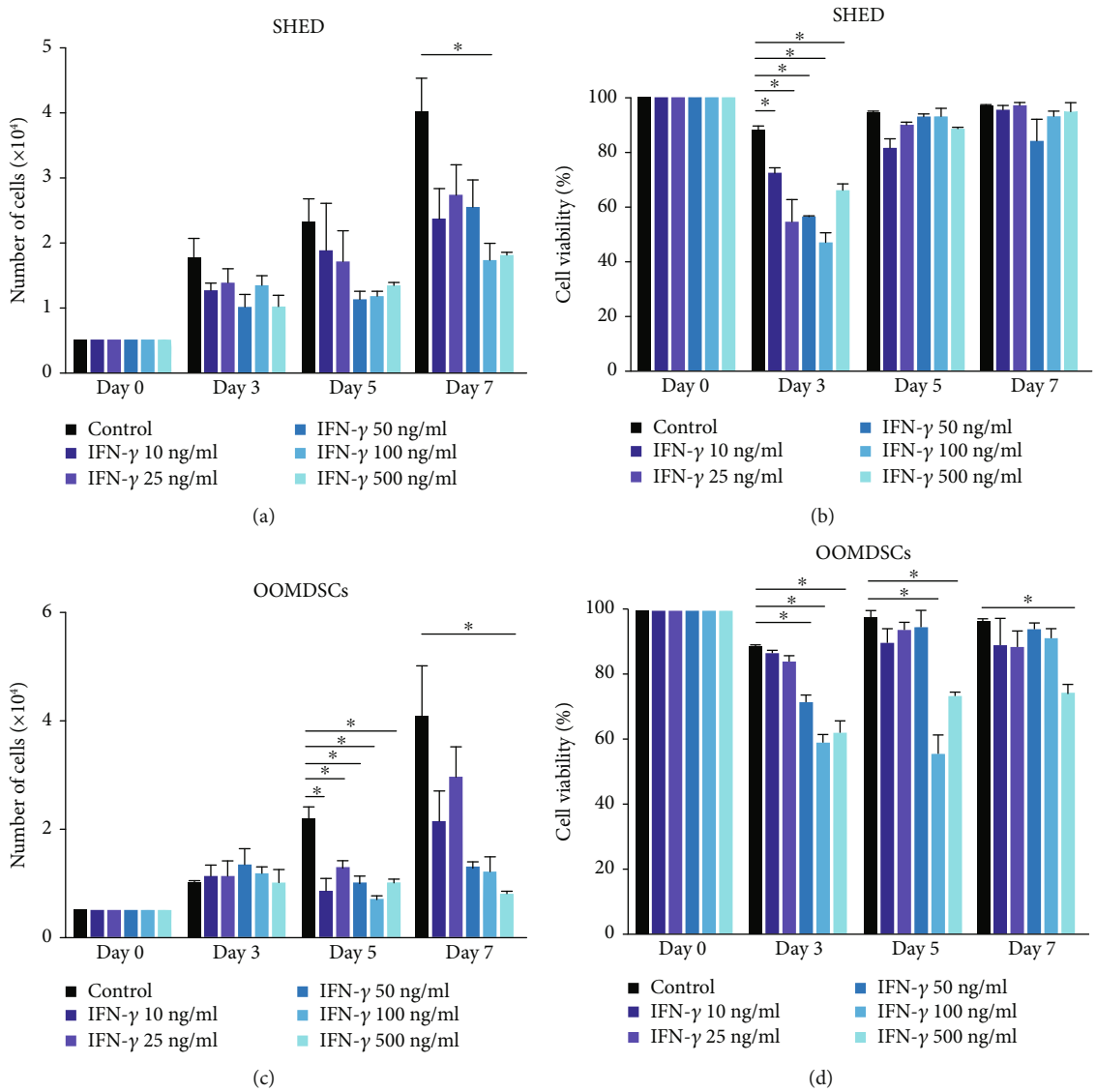
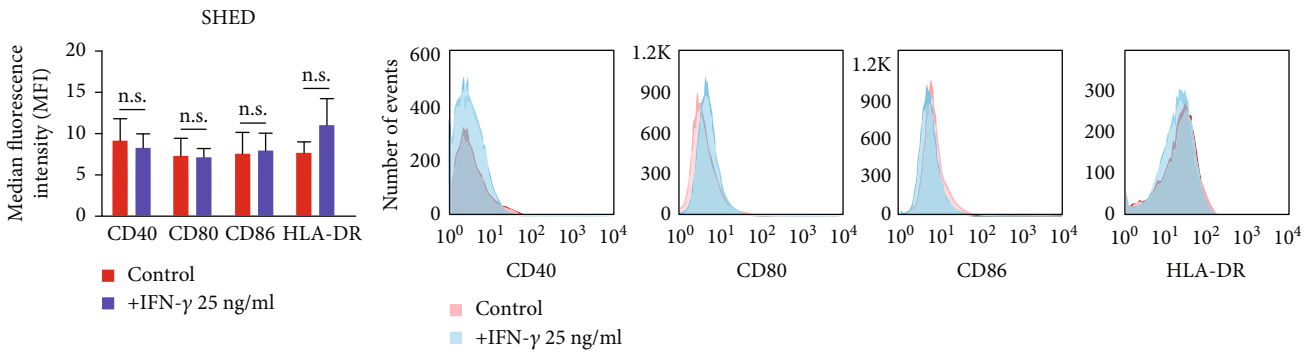
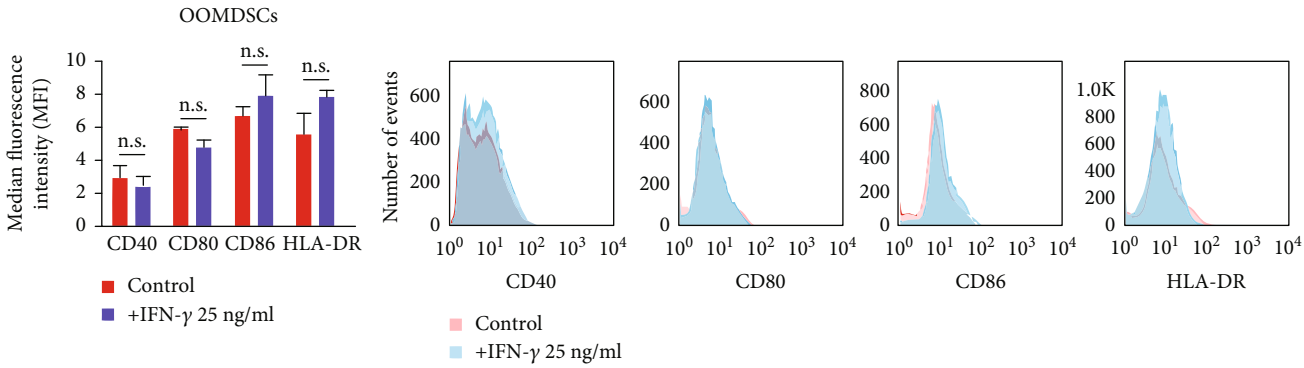


Figure 3: Effects of IFN-γ on the proliferative capacity and cell viability of SHED and OOMDSCs. (a) An inhibition of cell proliferation in SHED was observed after three days of culture in culture medium supplemented with IFN-γ at different concentrations. After the fifth day of culture, it was observed that the addition of IFN-γ at higher concentrations (100 ng/mL and 500 ng/mL) to the culture medium resulted in a greater inhibition of the proliferation of SHED than the addition of IFN-γ at lower concentrations (10 ng/mL, 25 ng/mL, and 50 ng/mL); however, this difference was not statistically significant. A similar effect was observed after seven days of cell culture; however, a statistically significant reduction in the cell proliferation of SHED treated with IFN-γ at the 100 ng/mL concentration compared with that of untreated SHED was observed. (c) An inhibition of proliferation in OOMDSCs was observed after five days of culture in culture medium supplemented with IFN-γ at different concentrations. Beginning on the fifth day of culture, it was observed that compared with no IFN-γ addition, the addition of IFN-γ to the culture medium resulted in a statistically significant inhibition of OOMDSC proliferation. After seven days of culture, the addition of IFN-γ at higher concentrations (50 ng/mL, 100 ng/mL, and 500 ng/mL) to the culture medium resulted in a greater inhibition of OOMDSC proliferation; however, compared with the OOMDSCs maintained in culture medium without IFN-γ, only the OOMDSCs treated with IFN-γ at 500 ng/mL had their proliferation significantly inhibited. (b) A statistically significant decrease in the cell viability of SHED was observed only on the third day of culture in culture medium supplemented with IFN-γ at distinct concentrations. Furthermore, a greater decrease in the viability of SHED was detected in the groups treated with IFN-γ at a 25 ng/mL, 50 ng/mL, or 100 ng/mL concentration than in the groups treated with lower concentrations of IFN-γ. No significant differences were observed among the populations of SHED after five or seven days of culture in culture medium supplemented with IFN-γ at distinct concentrations. (d) For OOMDSC populations, a statistically significant decrease in cell viability on the third day of culture was observed only when the cells were treated with IFN-γ at 50 ng/mL, 100 ng/mL, or 500 ng/mL. On the fifth day of culture, however, a statistically significant decrease in cell viability was observed only when OOMDSCs were treated with IFN-γ at 100 ng/mL or 500 ng/mL. Additionally, a statistically significant decrease in cell viability was observed on the seventh day of culture only when OOMDSC populations were treated with IFN-γ at a concentration of 500 ng/mL. $^{*}p < 0.05$, determined by two-way ANOVA followed by the Bonferroni post hoc test. Five experimental replicates were performed for each group for both SHED and OOMDSCs.



(a)



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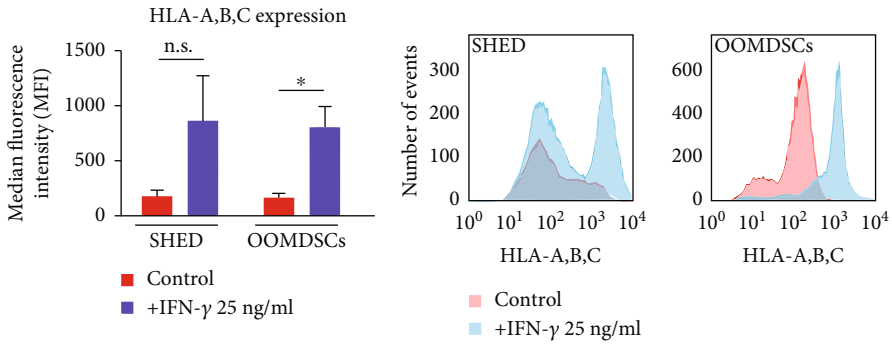
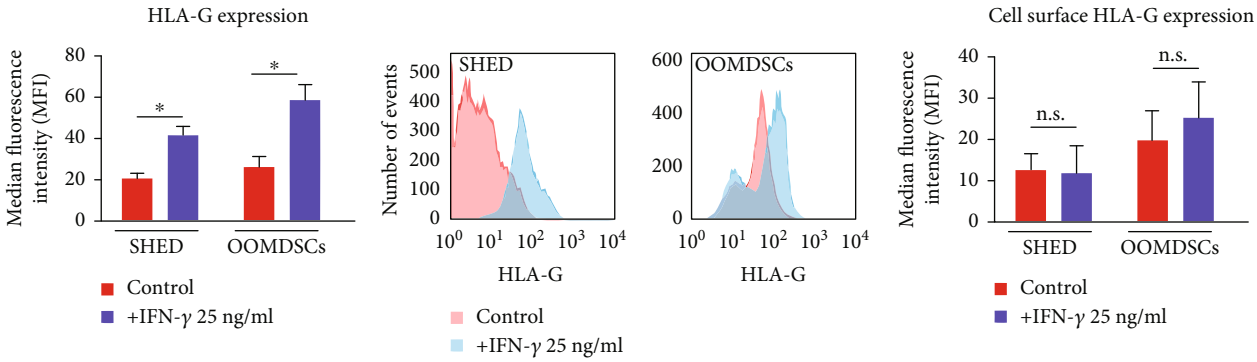


(c)



(d) (e)

Figure 4: Continued.

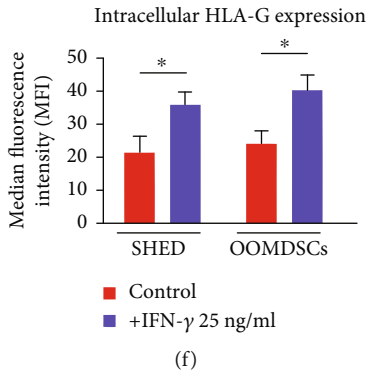


Figure 4: Effects of IFN-γ on the expression of immunogenic, costimulatory, and immunomodulatory molecules on the cell surface of SHED and OOMDSCs. (a, b) Neither SHED (a) nor OOMDSCs (b) expressed the costimulatory molecules CD40, CD80, and CD86 or the human leukocyte antigen class II HLA-DR on their cell surface, either in the absence of proinflammatory stimulation with IFN-γ or when stimulated with 25 ng/mL IFN-γ. (c) Higher expression of the human leukocyte antigen class I molecules HLA-A, HLA-B, and HLA-C was observed on the cell surface of both SHED and OOMDSCs treated with 25 ng/mL IFN-γ than on that of MSCs that were not treated with IFN-γ. However, this increase in the MFI value was statistically significant only when the OOMDSCs were treated with 25 ng/mL IFN-γ. (d) After treatment with 25 ng/mL IFN-γ, the expression of the immunomodulatory molecule HLA-G by both OOMDSCs and SHED was higher than that in the corresponding control-treated cells. This increase in the MFI value was statistically significant for both the SHED and the OOMDSCs treated with 25 ng/mL IFN-γ. (e, f) The effects of IFN-γ on the cell surface and intracellular expression of HLA-G were assessed. (e) Cell surface HLA-G expression remained unchanged in SHED treated with 25 ng/mL IFN-γ but increased in OOMDSCs treated with 25 ng/mL IFN-γ; however, this increase was not statistically significant. (f) Intracellular HLA-G expression increased significantly in both SHED and OOMDSCs treated with 25 ng/mL IFN-γ. Histograms demonstrate the binding of conjugated antibodies (in red) and isotype controls (in blue) to antigens. $^{*}p < 0.05$, determined by an unpaired Student t-test. Five experimental replicates were performed for each group for both SHED and OOMDSCs.

the proliferative capacity of dental pulp stem cells were reported when these stem cells were treated with the proinflammatory cytokine TNF-α, regardless of the concentration of the cytokine in the culture medium. Regarding the possible mechanism underlying the stimulation of proliferation in MSCs after treatment with IFN-γ, He et al. [27] demonstrated the occurrence of increases in PCNA and Ki-67 expression after IFN-γ treatment, indicating a high percentage of cells undergoing division. This study further demonstrated that the expression of cell cycle-promoting proteins such as Cyclin B1, Cyclin D1, and PCNA was increased, whereas the expression of proteins that inhibit cell cycle progression (such as P21) was reduced after stimulation of MSCs with IFN-γ [27]. Furthermore, Qin et al. [30] demonstrated that TNF-α was capable of stimulating the proliferation of human dental pulp stem cells through the Akt/Glycogen Synthase Kinase-3β/Cyclin D1 signaling pathways. Mechanisms related to the inhibition of proliferation in MSCs have also been observed in some studies after treatment of MSCs with proinflammatory cytokines. A study conducted by Croitoru-Lamoury et al. [31], for example, demonstrated that IFN-γ treatment was capable of activating indolamine-2,3-dioxygenase (IDO) in human and murine MSCs and that the activation of IDO could inhibit the proliferation of MSCs through the depletion of tryptophan, an essential amino acid required for protein biosynthesis. In addition, the production of tryptophan metabolites (e.g., kynurenine, 3-hydroxyanthranilic acid and quinolinic acid) by MSCs might be able, by a negative feedback mechanism, to inhibit the proliferation of these stem cells.

The present study also demonstrated that the osteogenic differentiation potential of populations of both SHED and OOMDSCs was maintained when these cells underwent a proinflammatory stimulation with IFN-γ. However, a study conducted by He et al. [27] demonstrated that low concentrations of the proinflammatory cytokine IFN-γ can inhibit the odontogenic and osteogenic differentiation of dental pulp stem cells through the nuclear factor kappa B (NF-κB) (p65) and MAPK (P38) signaling pathways. Sonoda et al. [26] also demonstrated that dental pulp stem cells isolated from teeth with irreversible pulpitis and treated with 100 ng/mL IFN-γ showed increased expression of osteoblast-specific genes, such as Runx2, alkaline phosphatase, and osteocalcin, after proinflammatory stimulation with IFN-γ. The effects of the addition of proinflammatory and immunomodulatory cytokines on the osteogenic differentiation potential of MSCs have also been reported in recent studies. A study conducted by Liu et al. [25] demonstrated that MSC-mediated bone regeneration was partially controlled by the host's local microenvironment and that the action of the host's immune cells and the production of inflammatory cytokines by host cells could considerably affect this process. This study showed that the osteogenic potential of autologous MSCs was adversely affected when these cells were transplanted into wild-type mice, while abundant bone formation was observed when the MSCs were transplanted into immunosuppressed mice.

In addition, studies conducted by Feng et al. [32] and Xing et al. [33] demonstrated that the addition of IGF-1 and TNF-α stimulated the osteogenic differentiation potential of dental pulp stem cells through the mammalian target of rapamycin (mTOR) and the NF-κB signaling pathways, respectively. The NF-κB pathway is active in many inflammatory diseases, such as arthritis, gastritis, and pulpitis

[34]. Previous studies have shown that NF-κB pathway signaling is involved in the regulation of odontogenic and osteogenic differentiation in dental pulp stem cells [35, 36]. Additionally, the proinflammatory cytokine TNF-α can stimulate the odontogenic and osteogenic differentiation of dental pulp stem cells via the NF-κB signaling pathway [32]. A study by He et al. [27] demonstrated that IFN-γ positively regulated P65 phosphorylation, which resulted in the inhibition of odontogenic and osteogenic differentiation in dental pulp stem cells, whereas pyrrolidinedithiocarbamate (PDTC), a specific inhibitor of the NF-κB signaling pathway, significantly suppressed the phosphorylation of the p-P65 protein and rescued the odontogenic and osteogenic differentiation capacities of dental pulp stem cells, indicating that the NF-κB pathway plays important roles in the odontogenic and osteogenic differentiation of dental pulp stem cells and that this pathway is regulated by IFN-γ. In addition, this study also demonstrated that the NF-κB signaling pathway was not the only pathway involved in the odontogenic and osteogenic differentiation of dental pulp stem cells induced by IFN-γ since IFN-γ also negatively regulates the phosphorylation of the p38 protein associated with the MAPK signaling pathway. It has been shown that the p38-MAPK signaling pathway is associated with odontoblastic stimulation during tertiary dentinogenesis by p38 phosphorylation and increased nuclear translocation [37]. In addition, TNF-α treatment is capable of activating the p38 pathway in dental pulp stem cells via p38 phosphorylation, while p38 inhibition decreases the expression of dentin phosphoprotein (DPP) and dentin sialoprotein (DSP) in these cells [37]. Finally, a study conducted by He et al. [27] showed that lipopolysaccharide (LPS) could promote odontogenic differentiation in human dental pulp stem cells via the MAPK signaling pathway. In addition, this study demonstrated that blockade of the p38-MAPK signaling pathway through the use of a specific inhibitor (SB203580) resulted in a significant rescue of the osteogenic differentiation potential in dental pulp stem cells induced by IFN-γ, indicating that the p38-MAPK signaling pathway plays an important role in bone regeneration responses.

The results obtained in this study also demonstrated that both SHED and OOMDSCs do not express HLA-DR or the costimulatory molecules CD40, CD80, and CD86 on their cell surface, even when stimulated with IFN-γ at 25 ng/mL for 48 hours. Similarly, a study conducted by Majumdar et al. [38] demonstrated that HLA class II molecules, such as HLA-DR, were not constitutively expressed by MSCs. HLA-DR is expressed by antigen-presenting cells and binds to T cell receptors during the immune response [39]. HLA-DR is not constitutively expressed in MSCs isolated from the bone marrow, but its expression has been shown to be positively regulated after proinflammatory stimulation [40]. However, in this study, we observed that neither SHED nor OOMDSCs expressed HLA-DR on their cell surface and that the expression of this protein remained unchanged in both types of MSCs even after stimulation with 25 ng/mL IFN-γ for 48 hours. In contrast, the costimulatory molecules CD40, CD80, and CD86, which are expressed by antigen-presenting cells [41], are not expressed by MSCs isolated from the bone marrow even after proinflammatory stimulation [42]. Therefore, both the SHED and the OOMDSCs used in this study should be incapable of stimulating the activation of lymphocytes, similar to the lack of stimulatory capacity observed in MSCs isolated from the bone marrow [43]. However, it was observed in this study that both SHED and OOMDSCs express the HLA class I molecules HLA-A, HLA-B, and HLA-C on their cell surface before treatment with IFN-γ. After 48 hours of proinflammatory stimulation with 25 ng/mL IFN-γ, HLA-A, HLA-B, and HLA-C expression on the cell surface of both SHED and OOMDSCs was increased. HLA class I molecules are responsible for the host response to intracellular pathogens and are important for the induction of cellular immunity [44]. A study conducted by Chan et al. [28] demonstrated that treatment of MSCs isolated from the bone marrow of healthy donors with IFN-γ increased the expression of HLA class I molecules on the surface of these cells after only 1 day, whereas increased expression of HLA class II molecules could only be observed 4 days after treatment with IFN-γ. Therefore, it is possible to hypothesize that the observed difference between the expression of HLA-DR and that of HLA-A, HLA-B, and HLA-C after two days of treatment with IFN-γ is due to slower intracellular transport of HLA class II molecules to the cell surface in both SHED and OOMDSCs. However, this hypothesis still needs to be confirmed. In addition, the present study demonstrated that both SHED and OOMDSCs express HLA-G at low levels. The expression of HLA-G increased in both SHED and OOMDSCs treated with 25 ng/mL IFN-γ for 48 hours compared with that in the corresponding untreated control cells. In particular, a significant increase in the intracellular expression of HLA-G was detected in both SHED and OOMDSCs after treatment with 25 ng/mL IFN-γ.

Similarly, previous studies have demonstrated that MSCs isolated from the bone marrow of baboons expressed HLA class I molecules such as HLA-A, HLA-B, HLA-C, and HLA-G at low levels on their cell surface [43]. HLA-G, in particular, is crucial to the maintenance of immunological tolerance during pregnancy, and its expression is indicative of the occurrence of strong immunosuppression [45]. HLA-G expression may also be upregulated in several tissues under "pathological" conditions, such as cancer [46]. In addition, a study conducted by Carosella et al. [47] reported that HLA-G expression was a prognostic indicator of graft tolerance in patients undergoing heart, kidney, liver, or hematopoietic stem cell transplantation. Phenotypic studies have demonstrated that both fetal and adult MSCs express HLA-G on their cell surface and intracellularly [48]. These studies detected the presence of HLA-G in protein extracts from fetal MSCs, whereas only HLAG mRNA was detected in adult MSCs. HLA-G can be expressed in seven different isoforms, depending on the alternative splicing of the primary transcript, and these isoforms include four membrane proteins (HLA-G1, HLA-G2, HLA-G3, and HLA-G4) and three soluble proteins (HLA-G5, HLA-G6, and HLA-G7) [47]. Regarding the biological activity of the HLA-G molecule, interaction of the HLA-G molecule with the specific HLA-G receptors KIR2DL4 and ILT-2 expressed in NK cells inhibits the

adhesive and migratory capacities of NK cells. The expression of HLA-G also inhibits the cytolytic potential of activated $CD8^+$ T lymphocytes and stimulates apoptosis in these lymphocytes when they are stimulated with phytohemagglutinin [49, 50]. In addition, the proliferation of allogeneic antigen-induced $CD4^+$ T lymphocytes is inhibited by both the soluble isoform HLA-G5 and the membrane-bound isoform HLA-G1 binding to their cognate receptors ILT2 and ILT4 expressed on the cell membrane. The HLA-G1 isoform also prevents the maturation of DCs, rendering them immunosuppressive as a result of the expression of TGF-β and IL-10 [51, 52].

The expression of HLA-G molecules by MSCs plays an important role in the immunosuppressive potential of these stem cells. This role has been demonstrated in studies in which the binding of the HLA-G molecule to its receptors was blocked by neutralizing antibodies. As a result, the abilities of MSCs to stimulate the expansion of $CD4^+CD25^{+-}$ $FoxP3^+$ regulatory T cells inhibit the proliferation of T lymphocytes and suppress the cytotoxic function of NK cells [53, 54]. In addition, a study conducted by Yazid et al. [29] demonstrated that dental pulp stem cells isolated from healthy dental pulp express HLA-G, HLA-A, HLA-B, and HLA-C in higher amounts than dental pulp stem cells isolated from inflamed dental pulp. It was suggested by Chan et al. [28] that upon the binding of IFN-γ to its cognate receptors (IFN-γR1 and IFN-γR2), signal transducer and activator of transcription 1 (STAT1) is phosphorylated by JAK1 and JAK2 and undergoes homodimerization. Dimerized STAT1 then translocates into the nucleus and binds to IFN-γ activation site (GAS) elements to initiate the transcription of interferon-stimulated genes (ISGs), including interferon-1 regulatory factor (IRF-1). IRF-1, in turn, regulates the transcription of other ISGs via IFN-stimulated response elements (ISRE), leading to the synthesis of HLA class I molecules (such as HLA-G, HLA-A, HLA-B, and HLA-C). Together, IRF-1 and dimerized STAT1 bind to the GAS promoter regions of the gene encoding the CIITA protein to promote gene transcription. The expression of the CIITA protein, on the other hand, induces the expression of HLA class II molecules (such as HLA-DR) [28].

5. Final Considerations

It is possible to conclude that SHED and OOMDSCs have their proliferation inhibited and their osteogenic differentiation maintained upon stimulation with IFN-γ. We also confirmed that the immunomodulatory potential of these cells is stimulated when they are treated with IFN-γ. Thus, our data suggest that both SHED and OOMDSCs are interesting stem cell populations that can be used for clinical applications and that the treatment of these cells with IFN-γ can enhance their immunomodulatory potential and further increase their therapeutic potential.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Review Article

Tissue Engineering Approaches for Enamel, Dentin, and Pulp Regeneration: An Update

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Stem/progenitor cells are undifferentiated cells characterized by their exclusive ability for self-renewal and multilineage differentiation potential. In recent years, researchers and investigations explored the prospect of employing stem/progenitor cell therapy in regenerative medicine, especially stem/progenitor cells originating from the oral tissues. In this context, the regeneration of the lost dental tissues including enamel, dentin, and the dental pulp are pivotal targets for stem/progenitor cell therapy. The present review elaborates on the different sources of stem/progenitor cells and their potential clinical applications to regenerate enamel, dentin, and the dental pulpal tissues.

1. Introduction

Dental caries is globally considered among the most prevalent bacterially induced diseases, resulting in enamel and dentin destruction. If untreated, the destruction will mostly lead to irreversible pulpal tissue damage [1]. Currently, the classical line of treatment involves the removal of the affected dental tissues and their subsequent replacement with artificial filling materials, with divergent physical and functional properties [1]. Due to various negative consequences of the restorative techniques and inherent deficiencies of the restoration materials, the ideal solutions to replace defective dental structures could be by biologically restoring/regenerating the lost dental tissues. The development of such new alternative treatment methods is currently considered as an important goal for the dental therapeutic researches.

Mesenchymal stem/progenitor cells (MSCs) are unspecialized plastic-adherent cells with the ability for self-renewal and multilineage differentiation [2] into multiple cell lineages [3]. They have been isolated from a variety of dental tissues, including dental pulp stem cells (DPSCs), stem/progenitor cells isolated from the human pulp of exfoliated deciduous teeth (SHED), periodontal ligament stem/-progenitor cells (PDLSCs), stem/progenitor cells from apical papilla (SCAP), alveolar bone-proper-derived stem/-progenitor cells (AB-MSCs), gingival mesenchymal stem/-progenitor cells (GMSCs), and dental follicle stem/progenitor cells (DFSCs) [4, 5]. The stem/progenitor cells derived from the oral cavity express several mesenchymal markers, including CD29, CD73, CD90, and CD105, as well as embryonic markers such as Sox2, Nanog, and Oct4 [6], but lack the expression of hematopoietic markers, including CD34, CD45, and HLA-DR. Relying on their remarkable

proliferative ability and differentiation potential, these stem/-progenitor cells are believed to be very promising in the development of future therapeutic approaches to regenerate the enamel, dentin, and pulpal tissues [7].

2. The Tissue Engineering Triad

Tissue engineering is an interdisciplinary field that applies the principles of engineering and life sciences towards the development of biological substitutes that could restore, maintain, or improve tissue and organ functions [8]. The concept of tissue engineering relies on the employment of a triad of stem/progenitor cells, scaffolds, and growth factors [8, 9] to regenerate functional biological tissues. Scaffolds have to be implemented with a suitable choice of cells and signaling molecules to initiate the formation of a new dental tissue that can homogenize with the surrounding tissues [10–12].

Numerous stem cell sources have been identified to play an essential role in tissue regeneration. Stem cells are either embryonic or adult stem cells [13]. Embryonic stem cells are immature, undifferentiated cells derived from the inner cell mass of blastocysts [14, 15], with the ability to undergo continuous self-renewal and differentiation. Adult stem/-progenitor cells are undifferentiated cells that are capable of differentiating into certain types of tissues [3]. They maintain the integrity of tissues they reside in such as blood, skin, bone, and dental pulp [16].

Scaffolds could be natural polymers (e.g., collagen, chitosan, alginate, and hyaluronic acid) or synthetic materials (e.g., polyglycolic acid, polylactic acid, and polylactic polyglycolic acid) and bioactive ceramics, with each category having its merits as well as limitations in use [17]. Scaffolds could be utilized as a cell support tool, upon which cells are cultured in vitro, prior to their transplantation together with their produced matrix in vivo. Scaffolds can further be employed as growth factor/drug delivery tools, to attract body cells to the scaffold site in vivo for new tissue formation [18]. In this context, scaffolds are essential to structurally support and transport growth factors, DNA, biologically active proteins, and cells as well as provide physical signals important for biological repair/regeneration processes [19, 20]. Aside from these, the topography, architecture, and composition of scaffolds can interact and affect cell response and subsequent tissue formation [18]. It is important for scaffolds to mimic the natural extracellular matrix of the tissue to be replaced [21, 22]. Optimum design for dental tissue regeneration should be made to achieve mechanical integrity and functionality and to help in cell adhesion and differentiation.

As a third important factor in the tissue engineering triad, growth factors were suggested to be crucial for the regenerative process. They are normally released from cells and are directly presented to cell surface receptors through their interaction with the neighboring extracellular matrix. Binding of growth factors to particular cell-membrane-linked receptors activates various mechanisms and pathways involved in tissue engineering such as cell migration, survival, adhesion, proliferation, growth, and differentiation into the desired cell type [23–27]. Especially, bone morphogenetic protein- (BMP-) 2 was shown to induce the differentiation of dental pulp stem/progenitor cells into odontoblasts [23]. It was also demonstrated that BMP-4 mediates the differentiation of human embryonic stem cells into dental epithelium with the ability for tooth formation [28]. In addition, transforming growth factor-β (TGF-β) promotes the differentiation of odontoblast-like cells and stimulates dental pulp stem cell-mediated mineralization [23]. Broadly speaking, these growth factor-mediated cell responses are crucial for growth, wound healing, and angiogenesis in repair/regeneration processes [26].

3. Enamel Regeneration

3.1. Enamel Structure and Amelogenesis. Enamel, the hardest tissue in the human body, is a highly organized dental tissue, covering the outer layer of the tooth crown. It possesses unique mechanical and structural properties [29–32], relying on its high hydroxyapatite content, the arrangement of apatite crystals into enamel prisms, and finally, the alignment of these prisms in a picket-fence appearance in a tissue of high physical resilience and great hardness [33–37]. Ameloblasts, the enamel-forming cells, are specialized epithelial cells differentiating from the inner cells of the enamel organ [38]. They exhibit polarization and elongation with a pronounced Golgi apparatus and endoplasmic reticulum to form and secrete enamel proteins and influx phosphate and calcium ions into the forming enamel matrix [39, 40]. Enamel proteins are necessary for enamel formation, with amelogenin, ameloblastin, and enamelin being the three major proteins observed in the developing teeth [41]. Recently, this list was amended by amelotin and odontogenic ameloblast-associated protein (ODAM), which were observed in the junctional epithelium and during the maturation stage of amelogenesis [42–45]. Once the enamel matrix is formed, ameloblasts reabsorb water and degrade enamel proteins during the maturation stage of amelogenesis [39, 40]. Finally, they undergo apoptosis and the mature enamel becomes acellular. As a result, once damaged, unlike other biomineralized hard tissues such as dentin and bone, the enamel cannot regenerate by itself [37, 46]. Therefore, a reparative healing of destroyed enamel depends mainly, if at all, on acellular remineralization of superficial demineralized defects [47].

To restore enamel defects either due to caries, trauma, or others, artificial materials were manufactured to resemble its hardness [48]. Unfortunately, most of the current materials do not possess the same mechanical, physical, and esthetic properties of the lost tissues [49]. Despite the urgent need for tooth enamel regeneration, enamel tissue engineering is facing many difficulties [50–53], including the complex posttranslational protein modifications required for crystal growth [54] and the recapitulation of the unique movements of ameloblasts during the organization of hydroxyapatite crystals into the enamel prisms [55]. Despite all these trials and findings, to date, there exists no scheme for viable cell-based in vivo enamel tissue engineering [37]. The main challenge remains to produce an artificial enamel that resembles the prismatic and interprismatic patterns of natural enamel,

has the proper anatomy, and can substitute for lost enamel-forming cells [56].

3.2. Cells. As enamel-forming cells are lost following tooth development, alternative cellular sources were needed to bring about a cellular-based regeneration. In this context, nondental epithelium-derived human cells, including gingival epithelial cells [57], induced pluripotent stem cells (iPSCs) [58], and human keratinocyte stem cells (hKSCs) [59, 60] were suggested to differentiate into enamel-forming ameloblasts when combined with mouse or human embryonic dental mesenchyme. Still, only a small percentage of these explants in subrenal cultures demonstrated the formation of dental enamel [60]. Embryonic stem/progenitor cells were similarly demonstrated to differentiate into oral ectoderm and dental epithelium, using variable concentrations of BMP-4 [61]. The formed dental epithelium, when mixed with mouse embryonic dental mesenchyme and transplanted into renal capsules for thirty days, subsequently generated teeth-like structures, including dentin and enamel, with an incisor-like appearance [28]. Similarly, human keratinocyte stem cells when combined with embryonic mouse dental mesenchyme, sonic hedgehog (SHH), and fibroblast growth factor 8- (Fgf8-) soaked agarose beads as reconstructed tooth germs [62] and transplanted into mice renal capsules, demonstrated ameloblastic differentiation with enamel deposition. Mouse induced pluripotent stem cells demonstrated a differentiation into ameloblast-like cells, using epithelial rests of Malassez cell-conditioned medium and gelatin-coated dishes, with high expression of amelogenin, ameloblastin, and keratin 14 [63].

Similarly, the Hertwig epithelial root sheath (HERS) and epithelial rests of Malassez (ERM) cells demonstrate a remarkable ability to produce enamel matrix proteins [64]. HERS cells entrapped in cementum produced amelogenin, ameloblastin, amelotin, and ODAM [45]. It was found that primary cultured HERS/ERM cells possess a primitive stem/-progenitor cell population that exhibits embryonic stem cell and epithelial markers [57, 65]. Ex vivo-expanded ERM exhibited both bone marrow mesenchymal stem/progenitor cell- (heat shock protein-90b, CD44 and CD29) and epithelial cell-markers (epithelial membrane protein-1, cytokeratin-8, and E-cadherin) proving their stem/progenitor cell-like properties [66]. An ERM cell line was further successfully generated from human periodontium to be used for future research [67]. ERM cells when cocultured with dental pulp cells were differentiated into ameloblast-like cells and produced enamel-like tissues [68]. Immortalized odontogenic epithelial cells isolated from ERM expressed stem-cell-related genes and generated calcification foci when transplanted into immunocompromised mice [69].

Odontogenic epithelial stem cells (OEpSCs) were first observed in the continuously growing rodent incisors. They are of epithelial origin, interact reciprocally with the mesenchymal stem/progenitor cells of ectomesenchymal origin [70], and possess the ability to generate all the epithelial tissues of the tooth, including the enamel-forming ameloblastic layer [71–73]. In postnatal life, OEpSCs were identified in the epithelial rests of Malassez (ERM) usually present near the incomplete root ends; the junctional epithelium (JE) which surrounds the neck of teeth; the reduced enamel epithelium (REE) covering the newly erupting tooth; the dental lamina (DL) and its remnants, known as cell rests of Serres, in the retromolar area; and the remnants of DL in the gubernaculum cord (GC), found above any erupting tooth [74]. Various genes were recognized in OEpSCs, including Bmi, Sox2, Yap, ABCG2, Lgr5, Oct3/4, Gli1, and Lrig1 [72, 73, 75–78]. It was demonstrated that Sox2+ odontogenic epithelial stem cells are able to produce teeth. The odontogenic epithelial stem cell niche was proved to be regulated by Fgf10 [79, 80].

3.3. Scaffolds and Biodegradable Materials. To successfully culture a patterned enamel organ, it was found that a proper three-dimensional scaffold such as a collagen sponge in combination with feeder cells such as NIH 3T3 mouse fibroblast cells should be present [81–83] to support and compensate for the epithelial-mesenchymal interactions that occur during early tooth formation [84]. Collagen sponge scaffolds and gels were demonstrated to help in cell attachment, proliferation, and differentiation as well as in the formation of calcified tissues [85]. Primary enamel organ cells cultured on feeder cells expressed many enamel proteins as kallikrein 4, ameloblastin, amelogenin, and matrix metalloproteinase (MMP) 20 [83]. Enamel organ epithelial (EOE) cells combined with dental pulp cells in scaffolds produced enamel with amelogenin expression in tall columnar epithelial cells found on enamel and dentin surfaces [48]. A three-dimensional multilayered macroscale biomimetic coculture system, using chitosan and type I collagen was similarly seeded with mesenchymal-derived dental pulp stem cells and HAT-7 dental epithelial cells to simulate epithelial-mesenchymal interactions. This system enabled the coculture of epithelial and mesenchymal cells, and the movement of the two cell types in various directions and Ca deposits were observed [86].

Still, available information is very limited and greatly diverse to distinguish the characteristics of each specific scaffold and its impact on possible stem/progenitor cell-mediated enamel regenerative outcomes [87].

3.4. Signaling Molecules in Amelogenesis. Various signaling molecules were proposed to be involved in the epithelial-mesenchymal interactions that occur during odontogenesis, including fibroblast growth factor (Fgf), sonic hedgehog (SHH), wingless (Wnt), bone morphogenic protein (BMP), and transforming growth factor β (TGF-β) [88, 89]. Activin, BMP, and Fgf signals in the epithelial stem cell niche regulate enamel deposition in mice incisors [90]. Mesenchymal signals involved in ameloblast induction include TGF-β1, BMP-4, and BMP-2 [91, 92]. SHH signaling preserves the stem cell niche present in the molar cervical loops [93]. FAK-YAP-mTOR signaling maintains the equilibrium between stem cell proliferation and differentiation towards ameloblast lineage [94]. BMP signaling was demonstrated to be crucial for ameloblast differentiation [91] and enamel formation [95]. Ectodysplasin (Eda), a signal found in primary and secondary enamel knots and the placodes of all ectodermal appendages, is considered a key regulator of

ectodermal organ development, including molecules from vital signaling pathways such as SHH and Fgf20 [96]. In addition, SHH found in epithelial stratum intermedium cells support ameloblastic differentiation and maturation [97, 98]. Other epithelial signaling molecules that regulate ameloblasts are Wnt3, TGF-β1, Follistatin, and Eda. Ameloblasts also express transcription factors such as Msx2 and Sp6 that have important roles in amelogenesis [99]. Regulating these molecules could help to generate ameloblast lineage precursors resembling odontogenic epithelial stem/progenitor cells that could be utilized in enamel regeneration approaches [6] (Table 1 and Figure 1).

4. Dentin Regeneration

4.1. Dentin Structure and Dentinogenesis. The pulp-dentin complex originates embryonically from the neural crest ectomesenchyme [100]. Odontoblasts are differentiated at the late bell stage of tooth development, and their major function is to secrete the extracellular dentin matrix components (ECM), followed by their mineralization, generating the primary dentin, the main bulk of the circumpulpal dentin matrix, and completing root formation. Secondary dentin is laid down as a physiological process throughout life, while tertiary dentin is generated at the pulp-dentin interface in response to environmental stimuli. Tertiary dentin might be reactionary (structurally similar to physiological dentin) or reparative (poorly organized, mainly atubular structure, with cells trapped within the matrix). Each type arises from two different populations of cells, original postmitotic odontoblasts and newly generated cells derived from the pulp (dental pulp stem/progenitor cells (DPSCs)), respectively [101, 102].

4.2. Cells. Aside from the attempts for enamel regeneration, stem/progenitor cell-based tissue engineering remains a promising modality for functional dentin regeneration [103–105]. A lineage-tracing study proved that new odontoblasts generated during reparative dentinogenesis in teeth come up from the perivascular cells identified by α-smooth muscle actin (αSMA) expression. Furthermore, it was demonstrated that the progeny of the αSMA+ population scarcely participated in physiological dentin deposition [106]. Coimplantation of MSCs and ECs accelerated pulp healing with a complete dentin bridge formation [107]. Swine autologous dental pulp stem/progenitor cells (sDPSCs) transferred via hydrogel and transplanted into a mini swine root model showed that vascularized pulp-like tissue and a layer of newly deposited dentin (reparative dentin) were deposited along the canal walls with the creation of a dentin bridge-like structure [108]. A further in vivo study showed that iPSCs generated a pulp-like tissue with functional odontoblasts capable of producing tubular dentin-like structures [109]. In vitro investigation demonstrated that utilizing SHED in combination with different pulp capping materials stimulated proliferation, migration, and odontogenic-like phenotype differentiation of the cells [110].

4.3. Scaffolds and Biodegradable Materials. Several successful in vitro studies tested variable biomaterials to promote dentin regeneration. A biomembrane composed of a chitosan/collagen matrix embedded with calcium-aluminate microparticles proved to induce the differentiation of HDPCs into odontoblast-like cells, with the deposition of a significant amount of mineralized matrix [111]. Culturing of DPSCs onto human-treated dentin (hTD) regenerated dentin-like tissues [112, 113]. Similarly, fibrin proved to enhance pulp-like tissue generation as well as odontoblast differentiation, with dentin sialoprotein expression [114].

An attempt to utilize a biodegradable collagen sponge as a delivery vehicle for molecules like MTA or other experimental small-molecule GSK3 inhibitors promoted tertiary dentin formation in deep dental lesions, following experimentally induced pulp exposure [115]. Ceramic scaffolds, such as calcium phosphates (Ca/P) and bioactive glasses or glass ceramics, were further tested. Ca/P scaffolds contain tricalcium phosphate (TCP) or hydroxyapatite (HA), which are notably related to the mineralization of the matrix of the tooth [116]. Calcium hydroxide, Mineral Trioxide Aggregate (MTA), and Biodentine were reported to aid in the formation of the tertiary dentin [117]. Another in vitro study tested three capping materials, namely, mineral trioxide aggregate (MTA), calcium hydroxide (CH), and Biodentine (BD), and proved that these materials are biocompatible and could stimulate proliferation, migration, and differentiation of SHED [110]. Nanofibrous spongy microspheres (NF-SMS), nanofibrous microspheres (NF-MS) without a pore structure, and conventional solid microspheres (S-MS) with neither nanofibers nor pore structure were further tested for dentin regeneration. The biodegradable and biocompatible poly(L-lactic acid) block-poly(L-lysine) were fabricated into the NF-SMS with interconnected pores, enhancing the proliferation and odontogenic differentiation of HDPSCs. NF-SMS provided superior dentin-like tissue formation compared to NF-MS or S-MS with a remarkable level of mineralization [118].

The application of biological printing combined with dental stem/progenitor cells employing clinical methods of 3D biofabrication and regeneration of dental tissues are the currently suggested alternative to classical dental restorations. The use of bioinks enabled the synthesis of scaffolds with precise, reproducible microarchitectures. Novel dentin-derived extracellular matrix (ECM) hybrid cell-laden hydrogel bioinks, synthesized from alginate and dentin matrix proteins were characterized and showed high printability and cell survival at different concentrations. Moreover, these hybrid hydrogels demonstrated the ability to be embedded with acid-soluble dentin molecules, enhancing odontogenic differentiation of SCAPs and effectively engineering the pulp-dentin complex [119].

4.4. Signaling Molecules. As previously mentioned, BMP-2 controls odontoblastic differentiation of dental pulp stem cells and transforming growth factor-β (TGF-β) can stimulate odontoblast-like cell differentiation and DPSC-mediated mineralization [23]. Also, platelet-derived growth factor (PDGFBB) and dentin-derived growth factors

Table 1: Summary of studies on enamel regeneration.

Enamel regeneration	Cells	Carrier/scaffold	Growth factors	Outcome
Angelova et al. 2013 [57]	Adult human gingival epithelial cells	—	—	Tooth-like structures Presence of enamel spaces and ameloblast-like cell populations
Hu et al. 2006	Mice bone marrow cells+mouse embryonic dental epithelial cells	—	—	Formation of nondividing, polarized, and secretory ameloblast-like cells without cell fusion
Cai et al. 2013 [58]	Integration-free human urine induced pluripotent stem cells	—	—	Tooth-like structures having elastic modulus and hardness similar to human tooth and containing enamel space and enamel organ Presence of ameloblasts with a ruffled border-like structure and papillary layer Expression of ameloblastin
Honda et al. 2005 [48]	Enamel organ epithelial cells	—	—	Production of enamel Expression of amelogenin in tall columnar epithelial cells found on enamel surface
Li et al. 2019 [28]	Human embryonic stem cells	—	Bone morphogenetic protein-4 (BMP-4)	Teeth-like structures Newly generated tooth-like structures contained enamel spaces similar to natural teeth
Shinmura et al. 2008 [68]	Quiescent porcine epithelial cell rests of Malassez from PDL of deciduous incisor teeth	Collagen sponge	—	Enamel-like tissues Positive staining for amelogenin in the enamel-like tissues Presence of well-developed ameloblasts
Wang et al. 2010 [60]	Human keratinocytes	—	Fibroblast growth factor 8 (Fgf8)	Epithelial cells became elongated and deposited enamel Immunohistochemical assays demonstrated the presence of ameloblastin and MMP-20
Hu et al. 2018 [62]	Human keratinocyte stem cells	—	Fibroblast growth factor 8 and Sonic hedgehog (Fgf8+SHH)	Tooth-like structures Intact prisms of the regenerated enamel
Liu et al. 2013	Rat skin epithelial cells	—	—	Enamel-dentin-like tooth germ-like structures
Yoshida et al. 2015 [63]	Mouse induced pluripotent stem cells (Mouse iPS)	—	—	Ameloblast-like cells Expression of high levels of amelogenin and ameloblastin

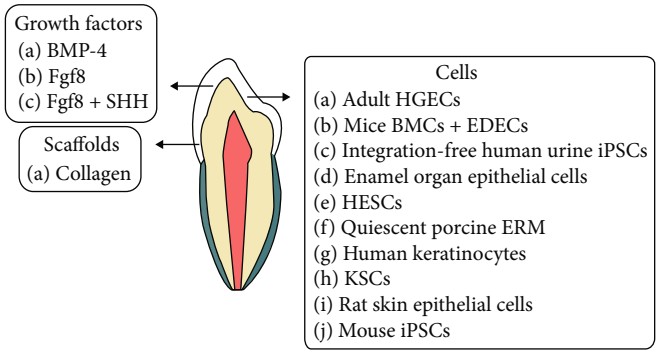

Figure 1: Diagram showing cells, growth factors, and scaffolds examined in the field of enamel regeneration.

(eDMP) proved to enhance HDPSC proliferation and odontoblastic differentiation, generating dentin-like mineralized tissues [120, 121]. G-CSF enhanced the proliferation and migration activity of stem/progenitor cells with dentin regeneration [122]. It was reported that the histone demethylation enzyme, lysine demethylase 1A (KDM1A), can regulate the directed differentiation in odontogenic MSCs by forming KDM1A and PLOD2 (procollagene-lysine2, oxoglutarate5-dioxygenase2) protein complex. It was reported that KDM1A in SCAP regulatory mechanisms of dynamic osteo/dentinogenic differentiation showed more diverse outcomes when applied in vitro than in vivo. However, in the final outcome of KDM1A inhibition, it promoted osteo/dentinogenesis in vivo [123]. Moreover, H2S proved to aid in the differentiation of DPSCs and dentin formation in vitro and in vivo via Ca^{2+} homeostasis and Ca^{2+} influx/GSK3β/(glycogen synthase kinase-3β) β-catenin cascade response. Also, it was evident that β-catenin signaling plays crucial roles in dentin formation [124].

Simvastatin (SIM), a drug commonly used to treat hyperlipidemia, was further reported to enhance odontogenic differentiation and accelerate mineralized tissue formation and de novo dentin formation [125, 126]. Combining SIM with canine DPSCs enhanced coronal pulp regeneration as well as dentin regeneration effectively and rapidly in beagle dogs. Small molecule inhibitors of glycogen synthase kinase 3 (GSK3) used in clinical trials for the treatment of neurological disorders such as Alzheimer's disease stimulated reparative dentine formation, with naturally generated new dentine at sites of damage [115, 127]. Smaphorin 3A (Sema 3A) and its receptor Nrp1, usually expressed in rat dental pulp tissue and human DPSCs, were thought to be potent factors capable of inducing differentiation of DPSCs into odontoblasts. Sema3A application to dental pulp exposure sites in a rat model induced effective reparative dentin reconstruction and promoted the formation of an odontoblastic layer, dental tubules, and predentin [128] (Table 2 and Figure 2).

5. Pulpal Tissue Regeneration

5.1. Pulpal Tissue Structure. Dental pulp is the soft tissue located in the center of the tooth, and it is surrounded by dentin. The primary function of the pulp is formative; it gives rise to odontoblasts that form dentin. Odontoblasts are the most distinctive cells of the pulp. They form a single layer at the periphery and synthesize the matrix, which becomes mineralized and form dentin. As previously discussed, the pulp-dentin complex originates embryonically from the neural crest ectomesenchyme and constitutes physiologically and functionally a single unit, providing vital functions for tooth homeostasis [100].

The dental pulp is a richly vascularized and innervated connective tissue comprising heterogeneous cell populations, among which stem/progenitor cells are anticipated to constantly replenish odontoblasts to form secondary and tertiary/reparative dentin throughout adult life [101, 102, 129, 130]. Mesenchymal stem/progenitor cell transplantation into endodontically treated root canals was attempted to regenerate the damaged dental pulp-dentin complex [131]. Although most of the research conducted on stem/progenitor cell-mediated reparative/regenerative endodontics used animal models, initial human clinical data are available now.

5.2. Cells. Coimplementation of endothelial cells with MSCs induced the acceleration of pulp tissue regeneration/healing and dentin bridge formation together with the upregulation of proangiogenic factors and the formation of a more organized dental pulp-like tissue and a thicker dentin bridge [107, 131–133]. Porcine deciduous pulp stem/progenitor cells (PDPSCs) transplanted to repair pulp chamber roof defects in the premolars of swine showed that after 16 weeks they regenerated dentin-like structures and nearly completely restored pulp chamber roof defects [134]. MDPSC (mobilized dental pulp stem cell) transplantation into pulpectomized teeth with G-CSF resulted in pulp/dentin regeneration as was evident by electric pulp testing, magnetic resonance imaging, and cone beam computed tomography [135]. Transplanted autogenous HDPSCs (human dental pulp stem cells), regenerated, innervated, and vascularized dental pulpal tissue in 26 patients with root length completion and apical foramen closure. Following up patients with the implanted HDPSCs for 24 months did not demonstrate any adverse events [136]. DPSCs (dental pulp stem cells) isolated from an inflamed third molar after being extracted and cultured then inoculated in another tooth of the same patient showed a normal periapical area after 3-year follow-up using cone beam computed tomography [137].

Table 2: Summary of studies on dentin/pulp regeneration.

Dentin/pulp regeneration	Cells	Carrier/scaffold	Growth factors	Outcome
Araujo et al. 2018 [110]	Stem cells from human exfoliated deciduous teeth (SHED)	Mineral trioxide aggregate (MTA) Calcium hydroxide (CH) Biodentine (BD)		The three tested materials maintained viability and stimulated proliferation, migration, and odontogenic-like phenotype differentiation
Athirasala et al. 2018 [119]	Human stem cells of apical papilla (human SCAP)	Bioink: printable alginate hydrogels with the soluble and insoluble fractions of dentin matrix		Odontogenic differentiation of SCAPs
Chen et al. 2017	Human dental pulp cells (HDPCs)	Human and porcine treated dentin matrix (TDM)		Complete dentin bridge formation Regeneration of reactionary dentin
El Ashiry 2018 [141]	Dental pulp stem cells (DPSCs)	Chitosan hydrogel scaffold	Vascular endothelial growth factor (VEGF-2) Basic fibroblast growth factor (bFGF) Platelet-derived growth factor (PDGF) Nerve growth factor (NGF) Bone morphogenetic protein-7 (BMP-7).	Periapical radiolucency healing Radicular lengthening Radicular thickening Apical closure
Iohara 2013 [148]	Dental pulp stem cells (DPSCs) Allogenic	Atelocollagen; collagen	G-CSF (granulocyte colony-stimulating factor)	Vascularization and neural regeneration in the DPSC group
Jia et al. 2016 [126]	Dental pulp stem cells (DPSCs)	—	—	Simvastatin stimulates DPSC-induced pulp and dentin regeneration after pulpotomy.
Kuang et al. 2015 [118]	Human dental pulp cells (HDPCs)	Nanofibrous spongy microspheres (NF-SMS)		Dentin-like tissue formation
Mangione et al. 2017	Dental pulp stem cells (DPSCs)	Hydrogel		Failure of partial pulp regeneration
Meza et al. 2019 [137]	Dental pulp stem cells (DPSCs) Autologous	Leukocyte-platelet-rich fibrin (L-PRF)		Six-month and 3-year follow-ups Periapical index (PAI) score of 1 Cone beam computed tomographic periapical index (CBCT PAI) score of 0

Table 2: Continued.

Dentin/pulp regeneration	Cells	Carrier/scaffold	Growth factors	Outcome
Nakashima 2017	Isolated human mobilized dental pulp stem cells (MDPSCs) Autologous	Atelocollagen scaffold	G-CSF (granulocyte colony-stimulating factor)	*CBCT evaluation*: Continued thickening of radicular walls (lateral dentin formation) Decrease in the volume of the dental pulp between 16 and 28 weeks *MRI evaluation of apical closure*: Relative signal intensity of apical part of root canal at 24 weeks *EPT (electric pulp tester) for evaluation of the sensibility of teeth*: Positive response in 4 cases out of the 5 cases
Sueyama et al. 2017 [107]	MSCs (mesenchymal stem cells) with ECs (endothelial stem cells)	Biodegradable hydrogel-made scaffolds		Pulp tissue regeneration/healing with complete dentin bridge formation
Tran et al. 2015 [112]	HDPCs	Human-treated dentin (hTD)		Regeneration of dentin-like tissues and expression of specific dentin markers
Wang et al. 2016 [105]	Human SCAP	NF-MS nanofibrous microspheres	Bone morphogenetic protein-2 (BMP-2)	Newly synthesized matrix and dentin-like tissues were present in BMP-2-treated groups
Xie et al. 2017	Induced pluripotent stem cells (iPSCs)	Poly-L-lactic acid (Boehringer Ingelheim) scaffold cast		Production of pulp-like tissue with functional odontoblasts capable of generating tubular dentin-like structures in vivo
Xuan et al. 2018 [136]	HDPSCs (human dental pulp stem cells) Autologous	—	—	Continued root lengthening and apical closure Increase in vascular formation At 12 months after treatment, laser Doppler flowmetry showed a mean increase in vascular formation
Zheng et al. 2012 [134]	Porcine deciduous pulp stem/progenitor cells (PDPSCs)	beta-Tricalcium phosphate (β-TCP)		Dentin-like structure Completely restored pulp chamber roof defects
Zu et al. 2018	Autologous swine dental pulp stem cells (sDPSCs)	Hydrogel		Dentin bridge formation

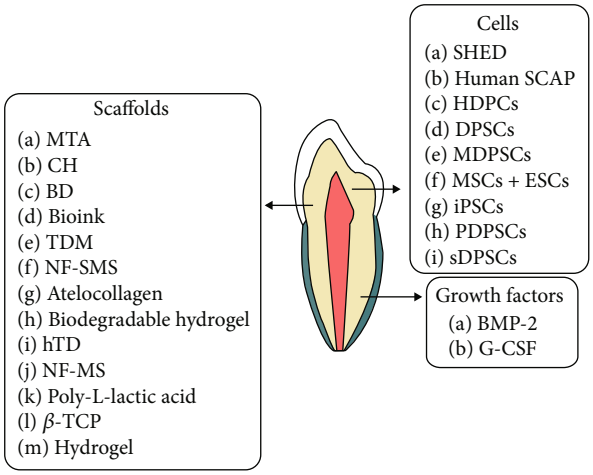

Figure 2: Diagram showing cells, growth factors, and scaffolds examined in the field of dentin regeneration.

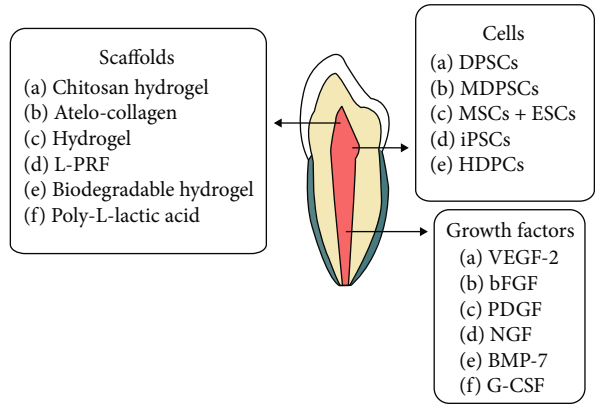

Figure 3: Diagram showing cells, growth factors, and scaffolds examined in the field of pulp regeneration.

Hence stem/progenitor cell transplantation holds a promising potential for dentin/pulp complex regeneration.

5.3. Scaffolds and Biodegradable Materials. Scaffolds harboring the appropriate growth/differentiation factors are very important for the success of pulpal tissue regeneration [132]. These scaffolds should mimic the natural pulpal microenvironment, providing the necessary structural signals, adhesion molecules, and pore sizes for homing, differentiation, and cellular phenotypic conversion, through permitting cell-matrix and cell-cell interactions [138]. Different scaffolds were used in different studies such as mineralized β-tricalcium phosphate carrier/scaffolds [134], injectable collagen [122, 139, 140] and hydrogel-chitosan carriers [141], and gelatin sponge [126]. Platelet-rich fibrin (PRF), centrifuged from the patient's own blood samples, was suggested as a natural scaffold for pulpal tissue regeneration. PRF introduced inside the root canal allows cellular migration, cytokine enmeshment, and slow continuous release of cytokines such as platelet-derived growth factor, transforming growth factor beta 1, fibroblast growth factor, and vascular endothelial growth factor from 7 to 28 days, achieving the peak level on day 14 [142]. In addition, it provides a strong firm architecture and a specific 3-dimensional distribution of platelets and leukocytes [137]. A novel transplant consisting of cell-sheet fragments of DPSCs and PRF granules proved to regenerate pulp-dentin-like tissues in the root canal, both subcutaneously in nude mice and in the roots of canines. It induced a favorable regeneration of compact pulp-like tissues, and a remarkable deposition of regenerated dentin along the intracanal walls at 8 weeks postoperation was observed [143]. Still, the impact of the characteristics of carrier/scaffolds on the transplanted stem/progenitor cell-mediated regenerative outcomes are currently only partly elucidated [87].

5.4. Signaling Molecules. Similar to enamel and dentin formation, cytokines or signaling molecules participate in pulp regeneration through their ability to mobilize endogenous cells and to regulate the proliferation and differentiation of the stem/progenitor cells [144]. Signaling molecules have been used and added to the scaffolds for proliferation, differentiation, and survival of stem/progenitor cells, with potentially important roles in signaling during pulp regeneration. Several studies suggested that many cytokines and growth factors were involved in promoting chemotaxis, proliferation, and differentiation of the stem/progenitor cells inside the root canal which led to generation of new tissues [145–147]. Transplantation of processed autologous dental pulp with growth factors (vascular endothelial growth factor-2 (VEGF-2), basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), nerve growth factor (NGF), and bone morphogenetic protein-7 (BMP-7)) embedded in a chitosan hydrogel scaffold was useful in regenerating pulp and dentin-like tissues in necrotic immature permanent teeth with apical periodontitis in dogs [141] with promising results. G-CSF in combination with DPSCs demonstrated pulpal tissue regeneration, vascularization, and nerve regeneration [148]. Basic fibroblast growth factor (bFGF) was demonstrated to be a potent homing/migration factor in pulp regeneration therapy similar to the influence of G-CSF [138]. Mobilized dental pulp stem cells and granulocyte colony-stimulating factor (G-CSF) with collagen transplanted into mature pulpectomized dogs' teeth completely filled the root canal with pulp-like tissue with large blood vessels and secondary dentin formation. However, MRI examination implied that the regenerated dentin might be undermineralized [149]. Another study revealed that stem cell factor (SCF) can accelerate cell homing and the maturation of the pulp-dentin complex in human immature teeth, as well as proliferation and odonto/osteogenic differentiation [150]. Still, the ideal constellation of growth/differentiation factors for functional pulpal regeneration remains largely unknown (Table 2 and Figure 3).

6. Conclusion

Stem/progenitor cell-based tissue engineering and bioprinting are promising approaches to protect the vitality and restore the integrity of dental tissues. Many attempts proved to be very promising, as reported in various in vitro studies, animal studies, and very few human trials. Despite the fact that the proposed biomaterials and techniques could be

promising for future dental tissues' regeneration, still the complexity and the multicellular interactions naturally existing in dental structures represent great currently unsolved challenges. A clear set of universally accepted markers for the isolation and characterization of stem/progenitor cells and the development of serum and animal product-free culturing media for cell expansion are further major hurdles prior to considering stem/progenitor cell-based transplantation therapies for routine clinical application. Finally, the side effects of stem/progenitor transplantation should be clearly investigated, prior to becoming a clinical therapeutic reality in restorative dentistry.

Abbreviations

BMP-4/2/7: Bone morphogenic protein-4/2/7
Fgf8: Fibroblast growth factor-8
SHH: Sonic hedgehog
HGECs: Human gingival epithelial cells
BMCs: Bone marrow cells
EDECs: Embryonic dental epithelial cells
iPSCs: Induced pluripotent stem cells
HESCs: Human embryonic stem cells
ERM: Epithelial rests of Malassez
KSCs: Keratinocyte stem cells
MTA: Mineral trioxide aggregate
CH: Calcium hydroxide
BD: Biodentine
TDM: Treated dentin matrix
NF-SMS: Nanofibrous spongy microspheres
hTD: Human-treated dentin
NF-MS: Nanofibrous microspheres
β-TCP: β-Tricalcium phosphate
SHED: Stem cells from human exfoliated deciduous teeth
SCAP: Stem cells from apical papilla
HDPSCs: Human dental pulp stem cells
DPSCs: Dental pulp stem cells
MDPSCs: Mobilized dental pulp stem cells
MSCs: Mesenchymal stem cells
ESCs: Endothelial stem cells
PDPSCs: Pig dental pulp stem cells
sDPSCs: Swine dental pulp stem cells
G-CSF: Granulocyte colony-stimulating factor
L-PRF: Leucocyte-platelet-rich fibrin
VEGF-2: Vascular endothelial growth factor-2
bFGF: Basic fibroblast growth factor
PDGF: Platelet-derived growth factor
NGF: Nerve growth factor.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Research Article

Effect of Inflammation on Gingival Mesenchymal Stem/Progenitor Cells' Proliferation and Migration through Microperforated Membranes: An In Vitro Study

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Background. In the field of periodontal guided tissue regeneration, microperforated membranes have recently proved to be very promising periodontal regenerative tissue engineering tools. Regenerative periodontal approaches, employing gingival mesenchymal stem/progenitor cells in combination with these novel membranes, would occur mostly in inflamed microenvironmental conditions intraorally. This in turn entails the investigation into how inflammation would affect the proliferation as well as the migration dynamics of gingival mesenchymal stem/progenitor cells. *Materials and Methods*. Clones of human gingival mesenchymal stem/progenitor cells (GMSCs) from inflamed gingival tissues were characterized for stem/progenitor cells' characteristics and compared to clones of healthy human GMSCs ($n = 3$), to be subsequently seeded on perforated collagen-coated poly-tetra-floro-ethylene (PTFE) membranes with a pore size 0.4 and 3 microns and polycarbonic acid membranes of 8 microns pore size in Transwell systems. The population doubling time and the MTT test of both populations were determined. Fetal bovine serum (FBS) was used as a chemoattractant in the culturing systems, and both groups were compared to their negative controls without FBS. Following 24 hours of incubation period, migrating cells were determined on the undersurface of microperforated membranes and the membrane-seeded cells were examined by scanning electron microscopy. *Results*. GMSCs demonstrated all predefined stem/progenitor cell characteristics. GMSCs from inflamed gingival tissues showed significantly shorter population doubling times. GMSCs of inflamed and healthy tissues did not show significant differences in their migration abilities towards the chemoattractant, with no cellular migration observed in the absence of FBS. GMSCs from healthy gingival tissue migrated significantly better through larger micropores (8 microns). Scanning electron microscopic images proved the migratory activity of the cells through the membrane pores. *Conclusions*. Inflammation appears to boost the proliferative abilities of GMSCs. In terms of migration through membrane pores, GMSCs from healthy as well as inflamed gingival tissues do not demonstrate a difference in their migration abilities through smaller pore sizes, whereas GMSCs from healthy gingival tissues appear to migrate significantly better through larger micropores.

1. Introduction

Periodontitis is an inflammatory degenerative disease associated with bacterial dysbiosis, leading if untreated to progressive loss of tooth-supporting tissues [1, 2]. Gingival mesenchymal stem/progenitor cells (GMSCs) exhibit multipotent differentiation capacities [3] and the potential for complete periodontal regeneration [3–5]. They further play a pivotal role in modulating the inflammatory response in their surrounding microenvironments [6–8].

Melcher was the first to describe guided tissue regeneration (GTR), with a promise for a complete regeneration of the periodontal apparatus [9]. Gamal and Iacono compared a traditional occlusive barrier membrane (OM) used in GTR to a perforated collagen membrane, concluding that the latter was associated with superior clinical outcomes

[10]. Recently, the ability of GMSCs from healthy gingival tissue origin to migrate selectively through microperforated membranes with suitable pore size in the presence of chemoattractants was clearly demonstrated [1]. The prospect of developing selective guided tissue regeneration membranes, allowing stem/progenitor cells to migrate through them, while being occlusive to unwanted cell lines, namely, epithelial and fibrous connective tissue cells, would represent a promising tool in the field of tissue engineering-mediated periodontal regeneration.

The objective of the present study was to determine and compare the potential of GMSCs extracted from healthy and inflamed gingival tissues to proliferate and migrate through novel microperforated membranes in vitro, additionally exploring role of the classical FBS chemoattractant factor in this process.

2. Materials and Methods

2.1. Sample Selection. Gingival connective tissue samples were extracted from discarded gingival specimens of patients with healthy and inflamed gingiva at the periodontal care clinic of Stony Brook University in Long Island, NY, in the course of regular periodontal therapy. Four subjects were included in this study, two for the healthy gingival tissue specimens and two for the inflamed ones. Experiments for each group were done in triplicate ($n = 3$). Informed consent was obtained from all the participants. The study was approved by the Committee of Research Involving Human Subjects at Stony Brook University and Ain Shams University scientific ethical committee (IRB number 575741).

2.2. Establishment of Cell Cultures from the Gingival Tissues. Gingival tissue samples were sliced and digested in 2 mg/ml Dispase II (Sigma-Aldrich, St. Louis, USA) at 4°C overnight, followed by 2 mg/ml collagenase IV (Thermo Fisher Scientific, Massachusetts, USA) for 40 minutes at 4°C. The resultant cellular suspension was filtered through a 40 μm cell strainer and centrifuged for 10 minutes at 1200 rpm. Single-cell suspensions were subsequently plated at a concentration of 60 cells/cm^2 in 10 cm tissue culture dishes for the isolation of single-cell-derived colonies in alpha minimal essential medium (alpha MEM 1×, Gibco) supplemented with 10% fetal bovine serum (FBS; Hyclone, Thermo Fisher Scientific), 50 U/ml penicillin G with 50 μg/ml streptomycin, and 2.5 μg/ml amphotericin B (fungizone, Thermo Fisher Scientific) in a humidified atmosphere (37°C, 5% CO_2). Cells were subcultured in P100 dishes for further passages. P10 plates were used for the colony-forming unit (CFU) assay.

2.3. Population Doubling Time Assay. Population doubling time was determined as previously described [11]. Briefly, GMSCs were seeded at 5×10^3 cells/cm^2 in 24-well plates, expanded to approximately 90% confluence, detached with 0.05% trypsin/EDTA, and counted. Subsequently, GMSCs were reseeded at 5×10^3 cells/cm^2 into another 24-well plate and cultured until in vitro cellular senescence was noted. Cells were counted at each passage and population doubling times were calculated using the following formula:

$$\frac{\log 2 \text{ final cell number}}{\log 2 \text{ seeding cell number}}. \quad (1)$$

Finally, the population doubling time value for the GMSC populations was calculated.

2.4. Flow Cytometry Expression of MSC-Associated Markers. GMSCs from the fourth and fifth passages were washed with PBS twice, detached with 0.05% trypsin/EDTA, and resuspended in blocking buffer 1% bovine serum albumin for half an hour. Approximately 1×10^5 cells were incubated for half an hour at 4°C in 2 μg/ml fluorescein isothiocyanate- (FITC-) conjugated mouse monoclonal antibodies specific for human CD73 and its isotype control (BD Pharmigen, San Jose, California, United States), APC-conjugated mouse monoclonal antibodies for CD90 and its isotype control (BD Pharmigen), Alexa 555 gout anti-mouse for primary unconjugated mouse monoclonal antibodies against CD105 (Dako) and its control Alexa 555 gout anti-mouse without primary mouse antibodies, and PE-conjugated mouse monoclonal antibodies for CD146 and its isotype control (BD Pharmingen). In terms of the hematopoietic markers, mouse monoclonal antibodies against CD14, CD34, CD45, and their isotype controls were used. After washing, centrifugation and resuspension twice, cells were analyzed flow cytometrically.

2.5. In Vitro Differentiation Capacity

2.5.1. Osteogenic Differentiation. GMSCs were seeded at 8×10^3 cells per cm^2 in six-well plates in osteogenic inductive medium (Gibco, Stem Pro), and the medium changed twice per week for 28 days [12] [13]. Subsequently, wells were washed twice with PBS, and the cells were fixed with 4% paraformaldehyde for 60 minutes at room temperature, washed twice with distilled water, stained by 2% Alizarin Red for 45 minutes in the dark, and finally washed four times with distilled water and twice in PBS.

2.5.2. Adipogenic Differentiation. GMSCs were seeded at 8×10^3 per cm^2 in six-well plates in adipogenic inductive medium (Gibco, Stem Pro), and the medium changed twice per week for 28 days [13, 14]. Subsequently, the wells were washed twice with PBS, and the cells fixed in 4% paraformaldehyde for 60 minutes at room temperature and washed twice in distilled water. After washing with 60% isopropanol for 5 minutes, the formation of lipid-laden fat cells was detected in 24-well plates by staining for 5 minutes with Oil Red O in isopropanol (300 mg oil red in 100 ml isopropanol) diluted in distilled water in a ratio of 3 : 2. Finally, the cultures were washed with tap water and stained with hematoxylin for 1 minute and then washed again with tap water and viewed under the phase-contrast inverted microscope.

2.5.3. Chondrogenic Differentiation. GMSCs were seeded at 8×10^3 per cm^2 in six-well plates and cultured in chondrogenic inductive medium (Gibco, Stem Pro), and the medium changed twice per week for 28 days. After 28 days, the wells were washed twice with PBS, and the cells fixed in 4% paraformaldehyde for 60 minutes at room temperature. The wells

were washed twice with distilled water, and the cells were stained with Alican blue (10 mg in 60 ml ethanol with 40 ml acetic acid) overnight in the dark to stain any formed cartilage glycoproteins blue. The wells were finally destained (120 ml ethanol with 80 ml acetic acid) for 20 minutes and washed twice with PBS, and the cultures examined under the microscope.

2.6. MTT Assay. GMSCs were seeded in a spectrophotometer tube with 500 μl alpha MEM (Gibco) and 10% FBS (Hyclone, Fisher Scientific). A cell-free tube was used as a control. The tubes were incubated in a humidified atmosphere (37°C, 5% CO_2) for a day. 100 μl of MTT was added to the tubes and they were incubated for four hours. The media were aspired, and 1000 μl of DMSO was added to each tube. The spectrophotometer read the absorbance of each sample at 595 nm wavelength.

2.7. Migration Assay

2.7.1. Microscopic Perforated Membranes. The cell migration assays were performed in a Transwell chemotaxis chamber with two types of membranes (Corning Life Sciences), namely, 12 mm collagen-coated poly-tetra-floro-ethylene (PTFE) membrane inserts with 0.4 μm and 3 μm pores and 6.5 mm polycarbonate membrane inserts with 8 μm pores. GMSCs were harvested using 0.05% trypsin/EDTA and resuspended in serum-free alpha MEM. 1×10^4 GMSCs were seeded in the upper compartments. The experimental groups received alpha MEM with 10% fetal bovine serum (Hyclon, Fisher Scientific), while in the control group, serum-free alpha MEM was used in the lower compartment. The plates were incubated in a humidified atmosphere (37°C, 5% CO_2). After 24 hours, the media were aspirated, and the inserts were washed twice in PBS. GMSCs on the upper surface of the membranes were removed with a cotton swab, and the cells that migrated to the lower side were fixed with 4% paraformaldehyde for 2 minutes, washed twice in PBS, permealized by 100% methanol for 20 minutes and stained with crystal violet stain (1% in 80% ethyl alcohol, Sigma-Aldrich). The washing was performed again twice in PBS, and the membranes were visualized under light microscopy at 40x magnification.

2.7.2. Scanning Electron Microscopy. The Transwell membranes were cut off the inserts, fixed in 4% PFA, and left to dry. The membrane specimens were dehydrated in a series of 50%, 70%, 80%, 90%, and 100% ethyl alcohol for 10 minutes for each concentration. Finally, the specimens were left overnight at -80°C in a closed box and examined at the electron microscope.

2.7.3. Statistical Evaluation. Differences in the outcomes between the groups were done using the Mann–Whitney *U* test (SPSS v20 program, IBM) assuming equal variance and a nonparametric distribution, with value of significance set at $p < 0.05$. Experiments were conducted in triplicates. Graphs were plotted using Microsoft Excel 2007 (Figure 1).

3. Results

3.1. Colony-Forming Unit Assay. Gingival cell suspensions (1000 cells/ml) formed distinctive colonies with typical fibroblastic morphology in P10 dishes after 14 days of culturing in vitro. Experiments for each group were done in triplicates. No significant differences were noted regarding the number of colonies between the healthy and inflamed gingival tissue groups ($p > 0.05$; Mann–Whitney *U* test; Figure 2).

3.2. Population Doubling Assay. Both GMSC groups demonstrated remarkable proliferative capacity. The population doubling time was however significantly less in inflamed than in healthy GMSC groups ($p < 0.05$; Mann–Whitney *U* test; Figure 2).

3.3. Flow Cytometry Expression of MSC Markers. At passages 4 and 5, cultured GMSCs expressed MSC-associated markers CD105, CD73, CD90, and CD146 and lacked the expression of hematopoietic markers CD14, CD34, and CD45 (Figure 2).

3.4. Multilineage Differentiation Capacity. Culturing of GMSCs in osteogenic, chondrogenic, and adipogenic inductive media for 28 days showed remarkable multilineage differentiation ability, which was proved by using Alizarin Red, Alican Blue, and Oil Red, respectively. Using the same stains on the control group grown in 10% serum alpha MEM media did not demonstrate any signs of cellular differentiation (Figure 2).

3.5. MTT Assay. The viability and metabolic activities of GMSCs demonstrated no significant differences between experiment and control groups in health and inflamed groups, respectively, using MTT at passage 5 ($p > 0.05$; Mann–Whitney *U* test).

3.6. Transwell Migration Assay

3.6.1. Microscopic Perforated Membranes

(1) 0.4 μm and 3 μm Perforated Collagen-Coated PTFE Membranes. GMSCs significantly migrated through 3 μm and 0.4 μm pores in the chemoattractant as compared to the control group. The migration was lower than the one noted through the membranes with 8 μm pores. No significant difference was found in the migration patterns of the GMSCs isolated from healthy versus inflamed tissues. Comparing the median of the cells from the healthy as well as inflamed clones migrating through the 0.4 μm as well as the 3 μm pores showed no significant differences with a mean rank of 12.6 for the healthy and 18.40 for the inflamed as well as a mean rank of 14.30 for healthy and 16.70 for the inflamed, respectively ($p > 0.05$, Mann–Whitney *U* test; Figure 3).

(2) 8 μm Perforated Polycarbonate Membrane. 40x magnification and flow cytometry assay of the media in the lower compartment could not detect any cells floating in both serum and serum-free groups. Significantly higher migration

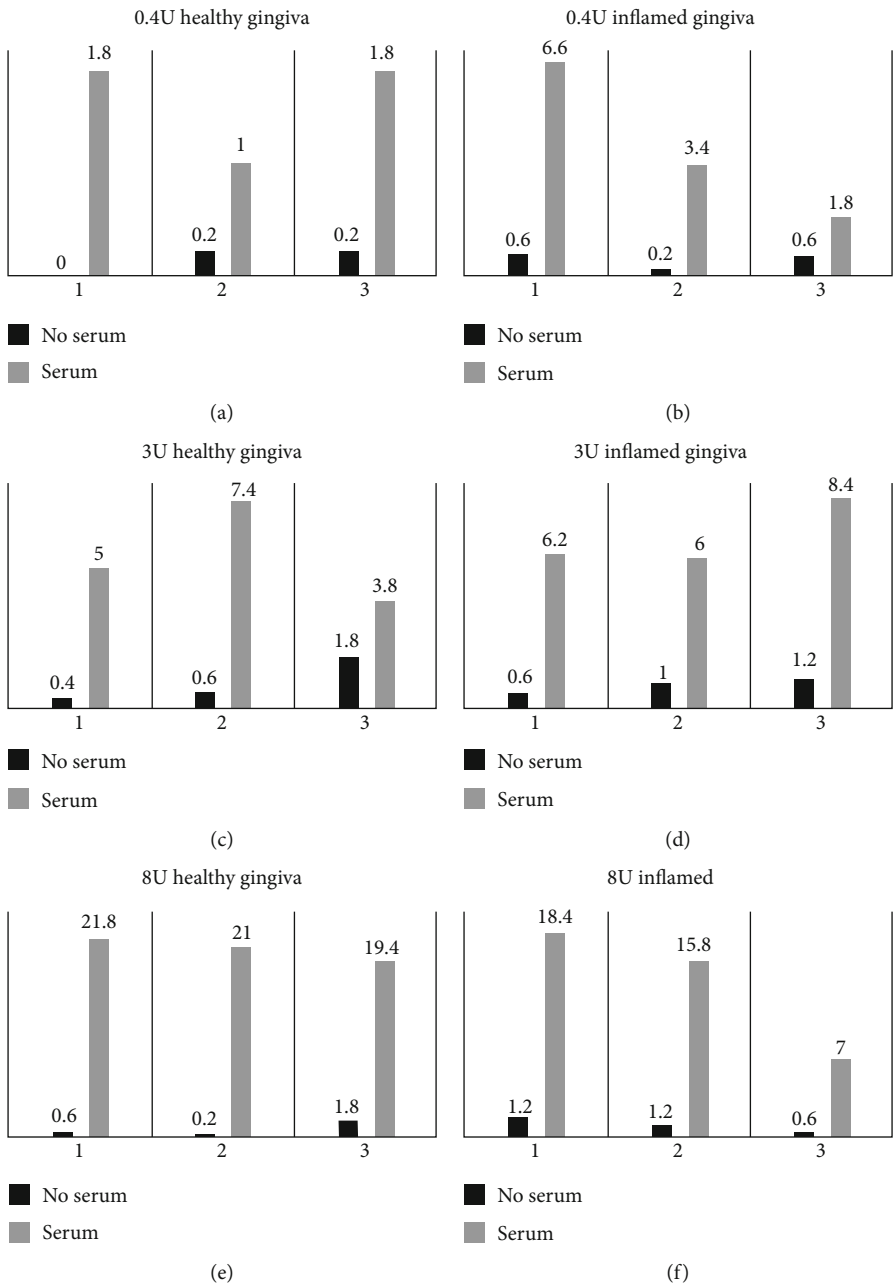


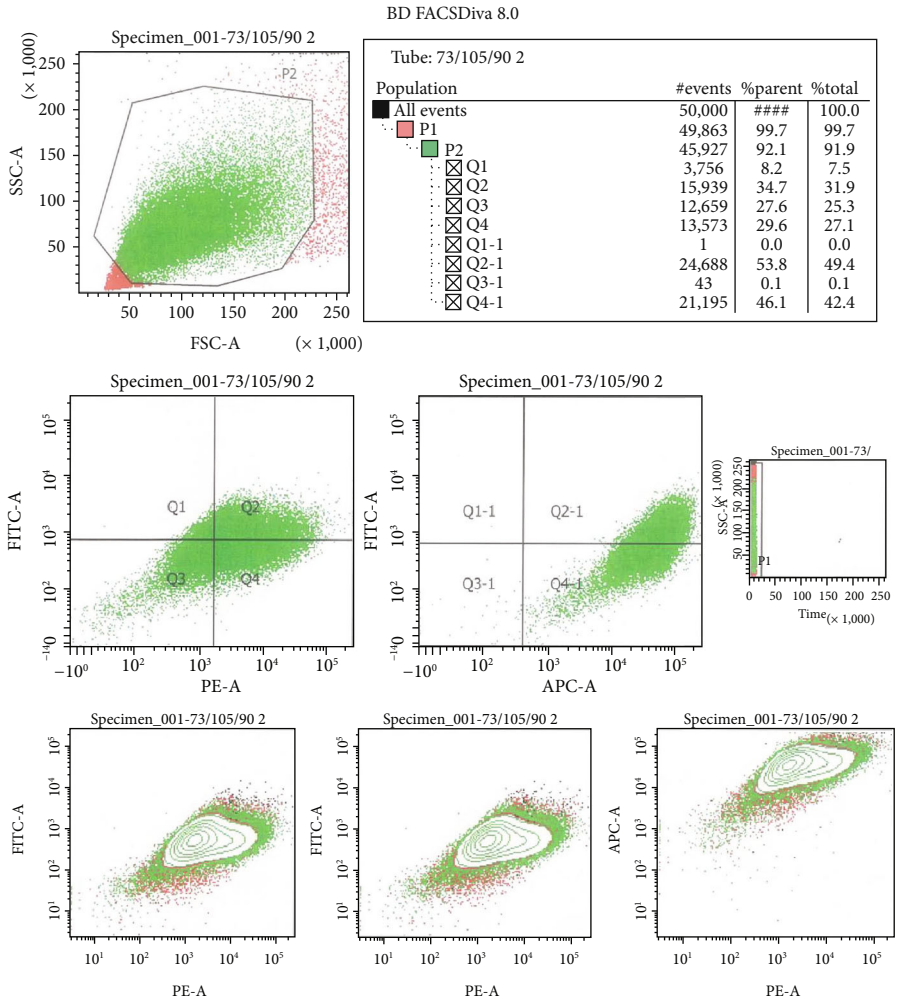
Figure 1: Mean counts of migrated GMSCs isolated from healthy and inflamed gingival tissues through the different pore sizes.

was notable in favor of GMSCs from healthy gingival tissues as compared to GMSCs from inflamed ones with a mean rank of 19.53 for healthy and 11.47 for the inflamed, respectively ($p = 0.011$; Mann–Whitney U; Figure 4).

3.7. Scanning Electron Microscopic Examination. No differences were detectable between GMSCs from inflamed and healthy tissues. GMSCs migrating through polycarbonate membrane seemed to look flatter in shape and spread over the membrane, in contrast to cells migrating through the collagen membrane, which looked more bulbous, and confined to the strands of the collagen (Figures 4(d) and 3(c)).

4. Discussion

Periodontitis is an inflammatory disorder of the tooth-supporting structures associated with bacterial dysbiosis [15]. In the course of the inflammatory periodontal disease as well as in the initial phases of any periodontal healing, GMSCs interact with their inflammatory microenvironment, affecting their cellular attributes [6]. The present study investigated the proliferative and migratory potentials of GMSCs isolated from healthy and inflamed gingival tissues in the presence and absence of FBS as chemoattractant through membranes with different pore sizes (0.4 μm, 3 μm, and 8 μm) in vitro. The hypothesis was that



(a)

Figure 2: Continued.

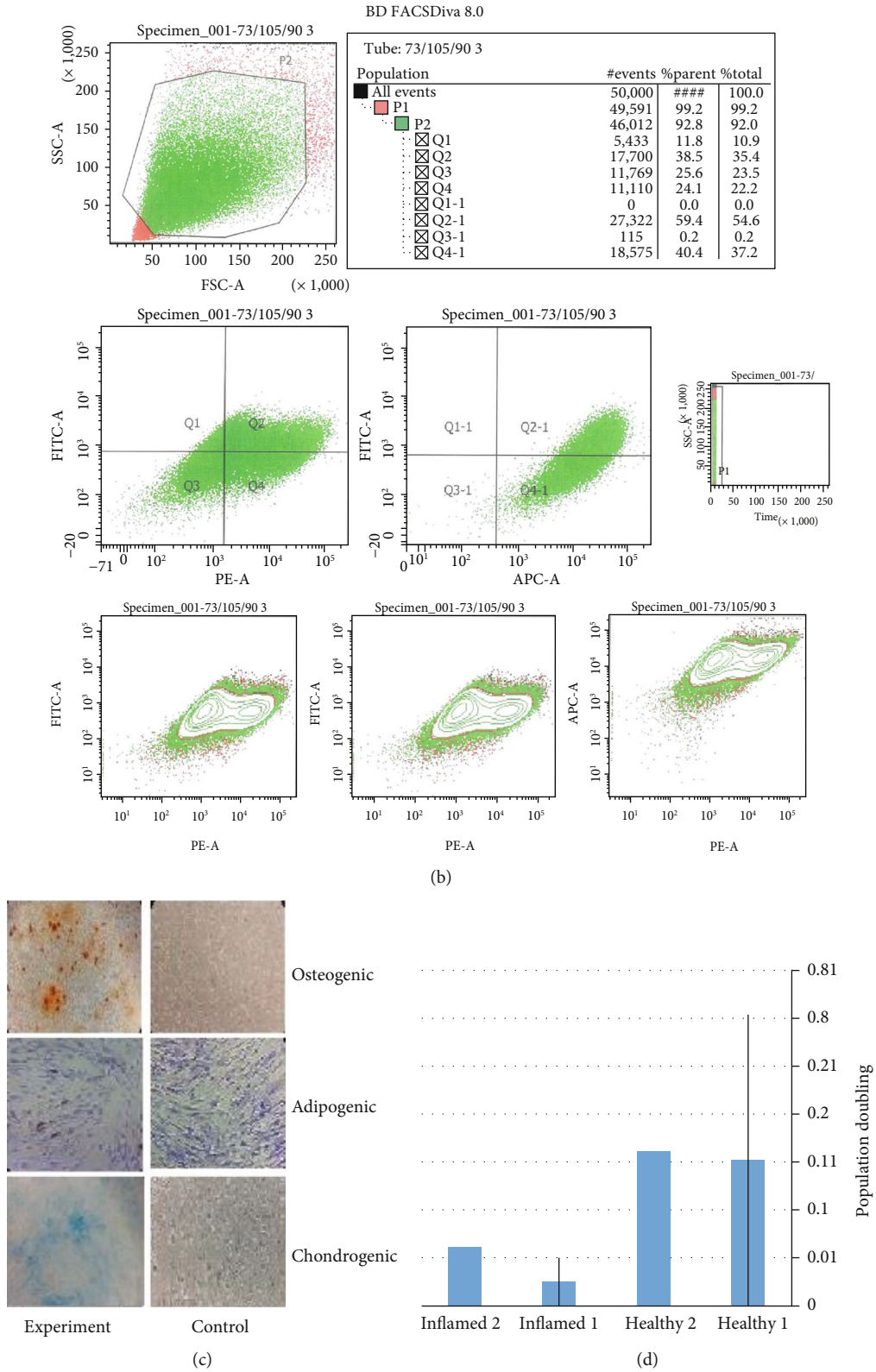
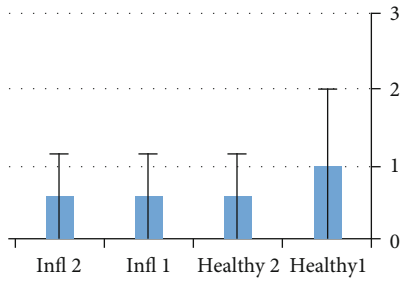


Figure 2: Continued.



(e)

Figure 2: (a) Flow cytometric analysis of the surface marker expressions CD90, CD73, and CD105 in healthy gingival tissues. (b) Flow cytometric analysis of the surface marker expressions CD90, CD73, and CD105 in inflamed gingival tissues. (c) Alizarin red staining of calcium deposits of GMSCs in osteogenic medium, Oil Red staining of oil droplets of GMSCs in adipogenic medium, and Alican blue staining of cartilage glycoprotein of GMSCs in chondrogenic medium and their respective controls. (d) Population doubling time assay with means and standard deviation of the of GMSCs from healthy and inflamed gingival tissues, with significantly shorter population doubling time in the inflamed group. (e) Graph showing colony-forming unit assay with means and standard deviation of the of GMSCs from healthy and inflamed gingival tissues.

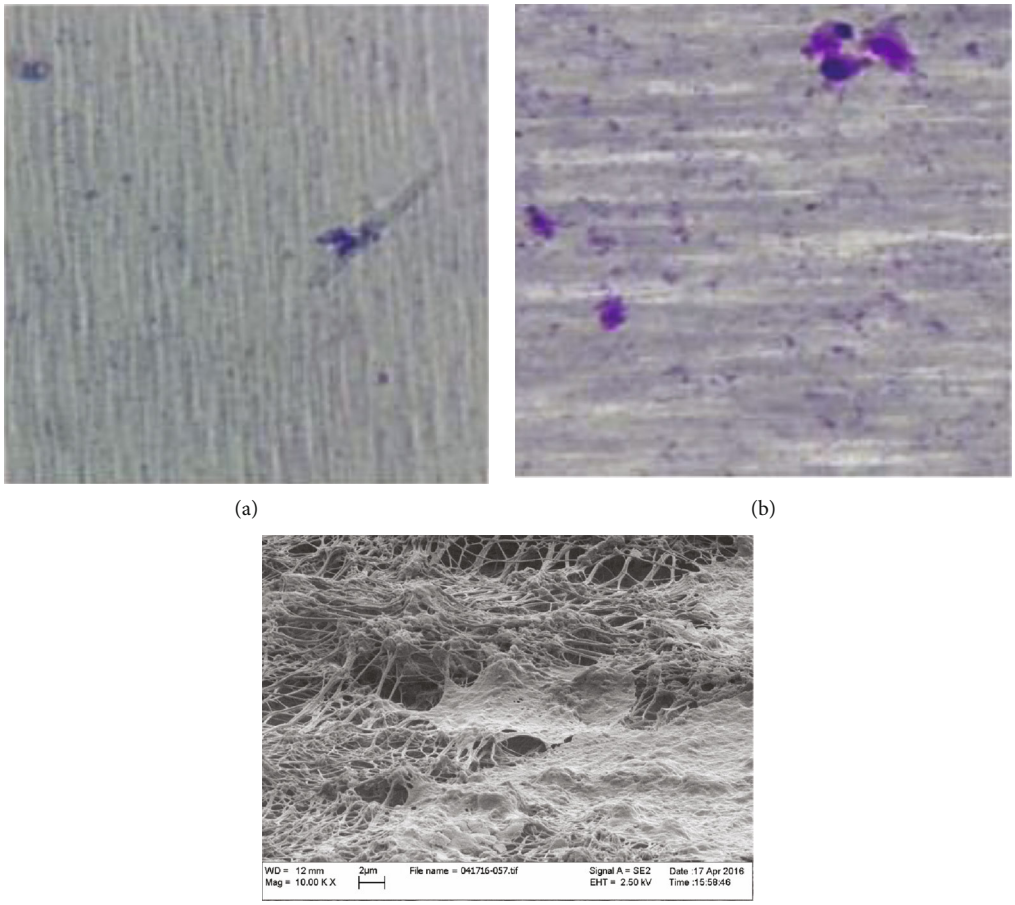


Figure 3: Representative image of migrated GMSCs in lower compartment of 3 microns pores perforated collagen-coated PTFE membranes with GMSCs from (a) inflamed tissues and GMSCs from (b) healthy tissues. (c) SEM image showing migrated GMSCs passing through 3 microns pores of perforated collagen-coated PTFE membranes.

inflammation would exert an effect on the proliferation and migration of GMSCs.

The investigated GMSCs demonstrated all predefined MSCs' markers, namely, CD105, CD90, and CD73, as well as CD146, CFUs, and a remarkable multilineage differentiation potential into osteoblasts, chondroblast, and adipocytes [8, 14, 16, 17]. Interestingly, in comparison to GMSCs from healthy gingival tissue, GMSCs from inflamed one, similar

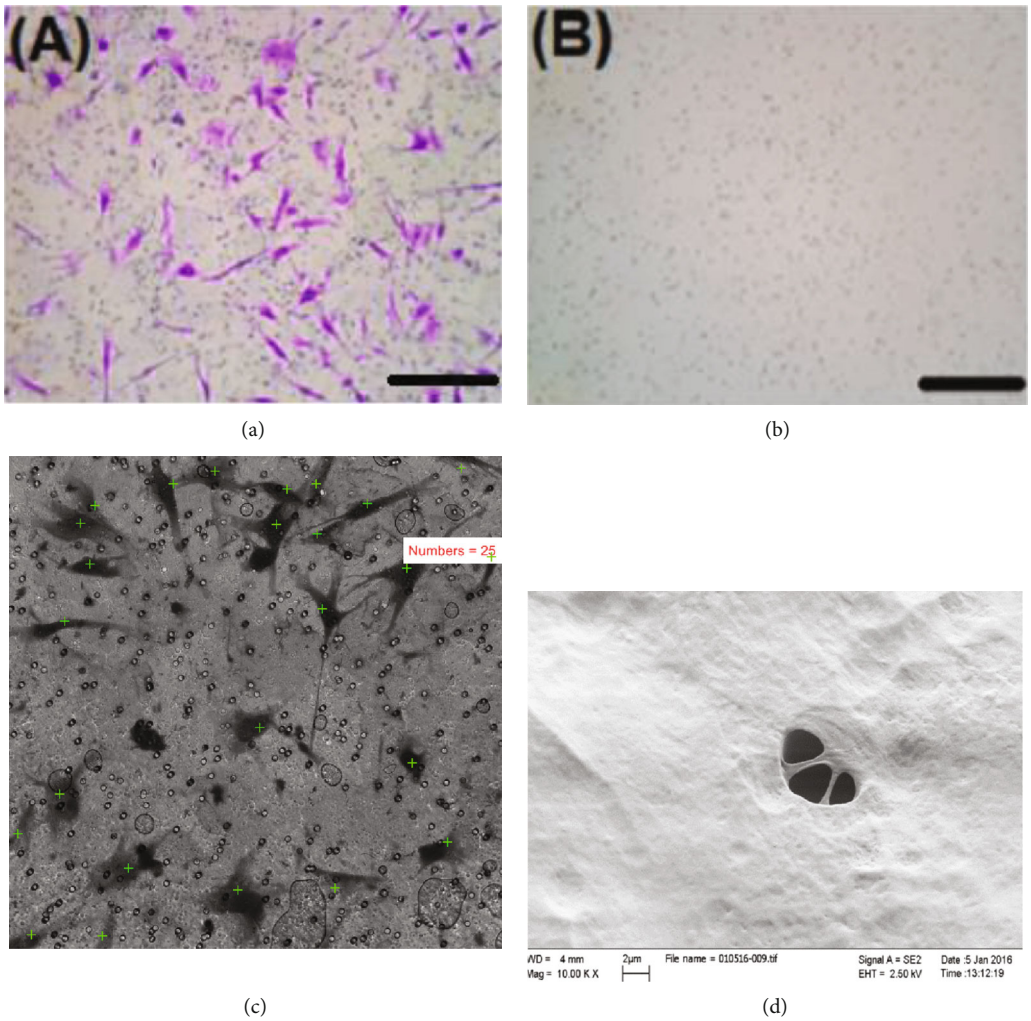


Figure 4: (a) Representative images of 8 μm perforated polycarbonate membranes with migrated cells in the lower compartment of membrane when bovine serum was used as a chemoattractant (cells stained with crystal violet; 10,000 cells seeded in the upper compartment). (b) Lower side of the membrane in the serum-free control group, showing no cells migrated. (c) Cells counted in the lower compartment in 5 random fields, 40x magnification (serum group). (d) SEM image of GMSCs aggregating over 8 microns pores of perforated polycarbonate membrane, with a cell process still inside one of the membrane pores.

to earlier investigations [18–21], demonstrated significantly faster proliferation, with a markedly shorter population doubling time.

In the present study, FBS was employed as a chemoattractant to assess the migratory activity of GMSCs through ultrafine pores of the examined membranes. The seeding of 10,000 GMSCs in the upper compartment was deemed suitable to easily identify migrated cells in the lower parts of the membranes. The migration rates in a 24-hour interval varied depending on the pore sizes. A significant difference was demonstrated in the serum-driven migration groups compared to control groups, where GMSCs actively migrated through membrane pores towards the serum in the lower compartment irrespective of pore size, gravity effects, or fluid diffusion.

GMSCs from healthy as well as inflamed tissue origins migrated through the 0.4 μm and 3 μm pores with no significant difference. However, there was a significant difference in cells migration in case of larger 8 μm pores, where cells originating from healthy tissues migrated more actively. This peculiar finding suggests that 8 μm pores might have, despite its larger more permissive diameter, a selective migratory effect on the GMSCs according to their inflammatory status. Furthermore, the observed difference may be attributed to structural characteristics of collagen membranes, with healthy tissue-derived cells sticking more readily to collagen than to polycarbonate, facilitating its migrating through the polycarbonate membrane.

SEM analysis could not determine any morphological differences between the GMSCs derived from healthy and inflamed tissues. GMSCs demonstrated a fibroblast-like morphology under the SEM. However, GMSCs attached on the polycarbonate membranes looked flat and showed more pseudopodia, while the GMSCs adherent on collagen-coated PTFE membranes had a rougher surface and conformed to the shape of collagen strands. The observed

morphological differences of the attached GMSCs could be largely attributed to the variability of membrane roughness [22–24] and are consistent with previous investigations on the effect of substrates on the morphology of the attached cells [25, 26] . These findings are further consistent with previous investigations [27], displaying different GMSC morphologies on polycarbonate versus collagen membranes.

5. Conclusion

Inflammation of the gingival tissue does not affect the existence of multipotent mesenchymal stem/progenitor cells in them. Although inflammation appeared to boost proliferation as was evident through a shorter population doubling time, regarding the migration dynamics, there was no significant difference in the number of migrated GMSCs through different membrane micropore sizes in the healthy and the inflamed groups, except with large micropore sizes, where GMSCs from healthy tissue demonstrated a higher migratory activity. No migration would occur in the absence of chemoattractant. The present results shed new light on the effect of inflammation and GTR membrane pore size on different attributes of GMSC pivotal for periodontal repair/regeneration and could represent an initial step in the formulation of a novel concept for membrane-driven periodontal guided tissue regeneration.

Data Availability

The data used to support the findings of this study are available in the study findings, and more details or photos are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

Al Bahrawy M and El-Sayed K contributed equally in editing this manuscript.

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Research Article

Highly Proliferative Immortalized Human Dental Pulp Cells Retain the Odontogenic Phenotype when Combined with a Beta-Tricalcium Phosphate Scaffold and BMP2

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Human dental pulp cells (HDPCs) play a vital role in dentin formation and reparative dentinogenesis, which indicated their potential application in regenerative medicine. However, HDPCs, which can only be obtained from scarce human pulp tissues, also have a limited lifespan *in vitro*, and stem cells usually lose their original characteristics over a large number of passages. To overcome these challenges, we successfully immortalized human dental pulp cells using the piggyBac system which was employed to efficiently overexpress the SV40 T-Ag, and we then comprehensively described the cell biological behavior. The immortalized human dental pulp cells (iHDPCs) acquired long-term proliferative activity and expressed most HDPC markers. The iHDPCs maintained multiple differentiation potential and could be induced to differentiate into chondrogenic, osteogenic, and adipogenic cells *in vitro*. We also proved that the iHDPCs gained a stronger ability to migrate than the primary cells, while apoptosis was inhibited. Furthermore, highly proliferative iHDPCs displayed no oncogenicity when subcutaneously implanted into athymic nude mice. Finally, iHDPCs exhibited odontogenic differentiation ability and secreted dentin sialophosphoprotein (DSPP) when combined with a beta-tricalcium phosphate scaffold and bone morphogenetic protein-2 (BMP2) *in vivo*. Conclusively, the established iHDPCs are a valuable resource for mechanistic study of dental pulp cell differentiation and dental pulp injury repair, as well as for applications in tooth regeneration.

1. Introduction

The stem cells in teeth have prospects for broad application in regenerative medicine. Human dental pulp cells (HDPCs), comprising a cell mixture of undifferentiated dental pulp stem cells, fibroblasts, dendritic cells, and macrophagocytes, are endowed with multiple differentiation potential. Among the multitudinous tooth-related stem cells, HDPCs have great potential for both dentin formation and regeneration [1]. Previous studies have employed multiple scaffolds for tooth and bone regeneration [1–5]. When HDPCs are recombined with biological scaffolds and implanted into a root canal, they can generate dentin pulp-like tissues *in vivo* [1, 2]. Indeed, osteogenic differentiation of HDPCs will be induced by bECM hydrogel without the addition of growth factors [4]. Meanwhile, HDPCs are regarded as the ideal cell source for many areas of regenerative medicine beyond the tooth due to the fact they can be easily isolated from discarded teeth. However, HDPCs have a limited lifespan *in vitro* and stem cells usually lose their original characteristics after many passages [1]. Consequently, it is necessary to seek efficient methods to harvest enough HDPCs from a lim-

ited supply of pulp tissue and to expand the cells sufficiently to provide an adequate number of cells *in vitro* to cater to these urgent needs.

Immortalization of various cells without losing their stemness has been adopted to solve these problems thanks to developments in technology. Although some attempts have been made to obtain human dental pulp cells and culture them *in vitro*, few *in vivo* applications of immortalized human dental pulp cells have been reported, and there is also a lack of comprehensive descriptions of their biological behavior [6–8]. Immortalization of cells can be achieved by means of oncogene overexpression and tumor suppressor gene inhibition [9]. The SV40 T-Ag has been recognized as the most widely used gene for immortalization [10–12]. Unfortunately, due to the low viral titers of a retrovirus when a long gene fragment is transduced, the primary cells have a low immortalization efficiency when a retroviral vector is employed to overexpress the SV40 T-Ag [11, 13–17]. Thus, the major challenge to immortalization of cells is the search for a simple, efficient, and convenient method to transfer the immortalizing genes into cells [18].

The piggyBac (PB) system is composed of mutant baculovirus strains derived from the cabbage looper moth *Trichoplusia ni* [19]. PB transposition, endowed with host factor-independent characteristics, is regarded as the most popular nonviral gene delivery tool [20]. The SV40 T-Ag gene located between two flippase recognition target (FRT) sites and delivered by the vector pMPH86 has contributed to the immortalization of various human and mouse cells [16].

The aim of this research was to immortalize human dental pulp cells efficiently and safely using a PB-based gene delivery system. In addition, experiments were carried out to test the characteristics of immortalized human dental pulp cells (iHDPCs) and explore the feasibility of applying these iHDPCs in tooth regeneration. In this study, we elucidated the biological behavior changes of iHDPCs which hinted at some superior characteristics in pulp regeneration. Meanwhile, cells combined with beta-tricalcium phosphate (β-TCP) were transplanted subcutaneously to test the odontogenic feasibility of iHDPCs *in vivo*. Our results demonstrated that iHDPCs, which have superior biological performance, might be a valuable resource to explore the mechanism of dental pulp cell differentiation and dental pulp injury repair, as well as for application in the future study of pulp regeneration.

2. Materials and Methods

2.1. Ethics Statement. Human pulp tissues were collected from patients at West China Hospital of Stomatology under approved guidelines. The research was approved by both the Ethical Committees of West China School of Stomatology, Sichuan University, and the State Key Laboratory of Oral Diseases.

2.2. Establishment of Reversibly Immortalized HDPCs. Dental pulp was collected from donors (aged 18–22 y), and human primary dental pulp cells were isolated and cultured in Dulbecco's modified Eagle's medium (DMEM) (HyClone, Logan, UT, USA) supplemented with 10% fetal bovine serum and 100 U/mL penicillin and 100 U/mL streptomycin [21]. To establish the iHDPCs, early passages of human dental pulp cells (passage 3) were transfected with the PB vector pMPH86 and the PB transposase expressing the adenoviral vector AdpBase. Then, hygromycin B (4 mg/mL, Gibco/Life Technologies, Carlsbad, CA, USA) was added for 3 days to select stably immortalized HDPCs. To establish deimmortalized human dental pulp cells (dHDPCs), iHDPCs were infected with Ad-FLP which effectively recognized the FLP site and removed the SV40 T-Ag. Cell morphology and growth features were observed under an inverted microscope. Total RNA was extracted, and real-time reverse transcription polymerase chain reaction (qRT-PCR) and agarose gel electrophoresis were performed to test the SV40 T-Ag gene expression level.

2.3. Surface Antigen Expression Assay. IHDPCs were cultured in 60 mm dishes until 70% confluence. Cell surface antigens were detected by immunofluorescence. Primary antibodies against CD90, CD105, CD34, CD45 (all at 1 : 100, Abcam, Cambridge, MA, USA), and CD73 (1 : 100, BioLegend, San Diego, CA, USA), a goat anti-mouse lgG secondary antibody fluorescent labeled with Alexa Fluor 555 (1 : 1000, Invitrogen, Carlsbad, CA, USA), or a goat anti-rabbit lgG secondary antibody fluorescent labeled with Alexa Fluor 488 (1 : 1000, Invitrogen) was used. Flow cytometry of HDPCs and iHDPCs was performed as described previously [22].

2.4. Cell Counting Kit-8 (CCK8) Proliferation Assay. Three thousand cells per well were seeded into 96-well plates. Cells were continuously observed for 5 days to determine cell viability, and 10 μL of CCK8 solution (Dojindo Laboratories, Kumamoto, Japan) was added into the medium daily followed by 1.5 h incubation. The absorbance of the medium at 450 nm was measured with a microplate reader (BioTek, Winooski, VT, USA).

2.5. Colony Formation Assay. HDPCs, iHDPCs, dHDPCs, and iHDPCs infected with Ad-GFP were seeded into 6-well plates at a density of three hundred cells per well. Cells were fixed in 4% paraformaldehyde for 15 min after culture in growth medium for 3 weeks. Then, crystal violet staining (Beyotime Institute of Biotechnology, Jiangsu, China) was performed and recorded using a bright-field microscope.

2.6. Crystal Violet Assay. Subconfluent HDPCs, iHDPCs, dHDPCs, and iHDPCs infected with Ad-GFP were seeded into 6-well plates. At the indicated time points, all of the cells were fixed and subjected to crystal violet staining. The stained cells were evaluated by quantitative measurement after dissolving the stain in 10% acetic acid for 20 min with agitation and then measuring the absorbance value at 570 nm.

2.7. Flow Cytometric Analysis. Before analysis of cell cycle distribution, cells were fixed in ice-cold 70% ethanol for 2 hours. After washing with PBS, cells were incubated with RNase (KeyGen Biotech Co. Ltd., Nanjing, China) for

30 min at 37°C followed by incubation with propidium iodide (PI) (KeyGen Biotech) for 30 min at 4°C. The results were examined on a Guava easyCyte HT flow cytometer (Merck-Millipore, Darmstadt, Germany) and analyzed with InCyte 2.7 software (Millipore). For analysis of apoptosis, cells were stained with Annexin V and 7-AAD (both from KeyGen Biotech) according to the manufacturer's instructions. Apoptotic fractions were analyzed using a FACScan cytometer (Becton-Dickinson, Franklin Lakes, NJ, USA). In our studies, the early apoptotic cells (Q4: Annexin V+/7-AAD-staining) and the late apoptotic cells (Q2: Annexin V+/7-AAD+staining) were considered to be undergoing apoptosis, and the numbers of these apoptotic cells as a proportion of total cells were analyzed.

2.8. Scratch Wound Healing Assay. Cells were cultured in 60 mm dishes at a density of 1×10^6 cells per well until 90% confluence. A 10 mL plastic pipette was used to create a wound across the diameter of the plate. After washing with medium to remove debris, cell migration was observed under a microscope after a 24 h interval.

2.9. In Vitro Multidifferentiation of iHDPCs. Cells were cultured in twelve-well plates at a density of 1×10^5 cells per well. To induce adipogenic differentiation, the medium was replaced with basal α-modification of Eagle's medium plus 100 nM dexamethasone, 50 μg/mL ascorbic acid 3-phosphate, and 50 μg/mL indomethacin (Sigma-Aldrich, St. Louis, MO, USA) for 2 weeks. The differentiated cells were fixed with 4% polyoxymethylene for 15 min before staining with 0.3% Oil Red O (Sigma-Aldrich) solution to evaluate adipogenesis. To induce chondrogenic differentiation, the cells were incubated in the presence of chondrogenic differentiation medium (Lonza, Basel, Switzerland) with recombinant transforming growth factor beta-3 protein (R&D Systems, Minneapolis, MN, USA) for 2 weeks. The induced cells were fixed with 4% polyoxymethylene for 15 min, followed by staining with 1% Alcian blue (Sigma-Aldrich). To induce osteogenic differentiation, the cells were incubated in the presence of 10 nM dexamethasone, 50 mg/mL ascorbic acid 2-phosphate, and 10 mM β-glycerophosphate (all from Sigma-Aldrich) for 5 days. Then, the differentiated cells were fixed with 4% polyoxymethylene for 15 min, followed by alkaline phosphatase staining (Beyotime) to assess mineral deposition according to the manufacturer's instructions.

2.10. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR). To detect multipotential differentiation of HDPCs, iHDPCs, and dHDPCs, total RNA was extracted from cells using the RNeasy mini kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The isolated RNA was reverse transcribed using the PrimeScript RT Reagent Kit (Takara). The complementary DNA samples were used as templates in SYBR Premix Ex Taq (Takara) PCR reactions. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as an internal control.

2.11. Subcutaneous Tumor Formation in Nude Mice. Seven-week-old nude mice ($n = 5$) were inoculated subcutaneously with cells in 0.20 mL of PBS per mouse at the level of the scapulae. SCC-4, an established human-origin head and neck squamous cell carcinoma tumor cell line, was used as the positive control. The nude mice were observed up to 3 weeks, when there was obvious tumor formation in the positive control group. After imaging, tissues near the injection sites were collected and examined by hematoxylin and eosin (H&E) (Solarbio, Beijing, China) staining.

2.12. Subcutaneous Transplantation of IHDPCs. The β-TCP blocks were purchased from the Biological Materials Manufacturing Core, Sichuan University. Cells were infected with Ad-BMP2 in advance. The composites of β-TCP blocks and cells were prepared as previously described [23] and implanted into the left and right subcutaneous dorsal pockets of six-week old BALB/c immunodeficient nude mice ($n = 5$). One month after implantation, the composites were harvested and fixed with 4% formalin, followed by decalcification with 10% ethylenediaminetetraacetic acid (EDTA) for one week.

2.13. H&E Staining and Masson's Trichrome Staining. The tissues were embedded in paraffin. Serial sections of the embedded specimens were stained with H&E and Masson's trichrome stains (both from Solarbio) according to the manufacturer's protocol.

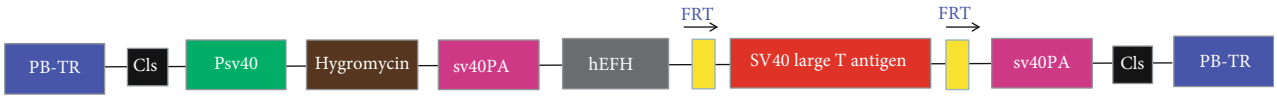
Images were obtained by a Nikon Eclipse 300 fluorescence microscope (Compix Media Inc., Irvine, CA, USA).

2.14. Immunofluorescence Staining. Paraffin sections were incubated overnight with the primary antibody anti-dentin sialophosphoprotein (DSPP) (1 : 20; Santa Cruz, CA, USA) at 4°C. This procedure was followed by secondary antibody fluorescent labeling with Alexa Fluor 555 (1 : 1000, Invitrogen, Carlsbad, CA, USA) for 60 minutes at room temperature. The cell nuclei were also labeled with diamidinophenylindole (DAPI; Invitrogen). Images were obtained using a Nikon Eclipse 300 fluorescence microscope (Compix Media Inc.).

2.15. Statistical Analysis. Experimental data are presented as means ± SD. Significance was determined by the one-way analysis of variance test. Each assay condition was repeated in triplicate for all quantitative assays. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. HDPCs Can Be Effectively Immortalized by the PiggyBac Transposon System. HDPCs were transduced with the piggyBac vectors pMPH86 (Figure 1(a)) and AdpBase and incubated in DMEM supplemented with normal serum for 3 days; then, hygromycin B was added for the next 3 days. On day 8, the positive cells were transferred to DMEM for further expansion and maintained in this medium thereafter. A schematic of the protocol is shown in Figure 1(b). After hygromycin selection, the surviving immortalized human dental pulp cells maintained a high proliferation rate and were passaged consecutively for more than 70 generations over 210 days, well beyond the Hayflick limit without any



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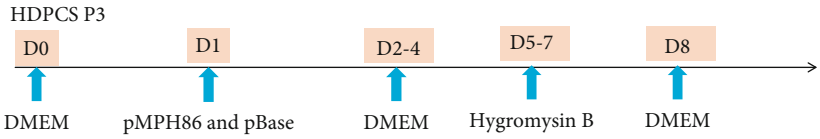

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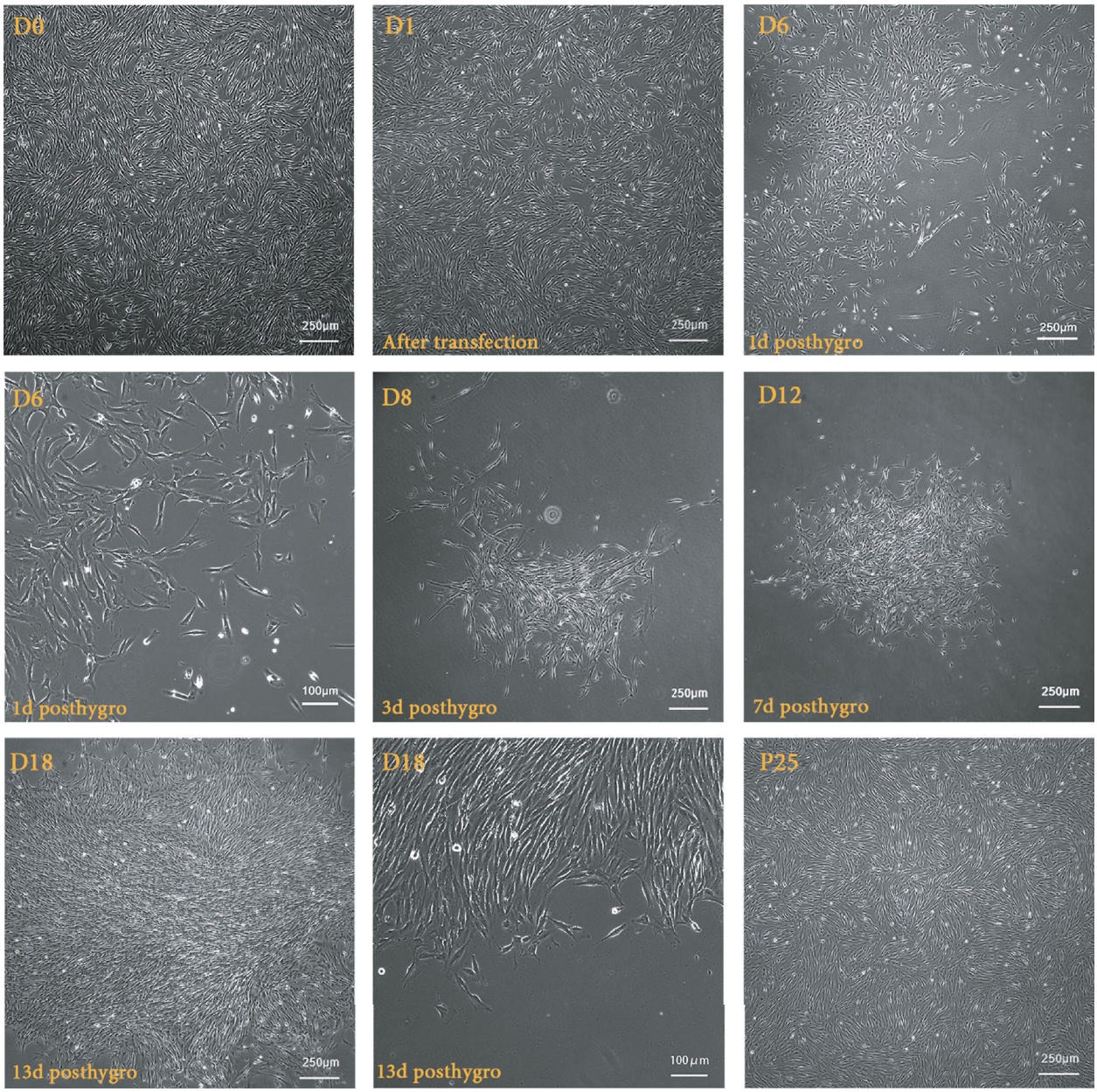

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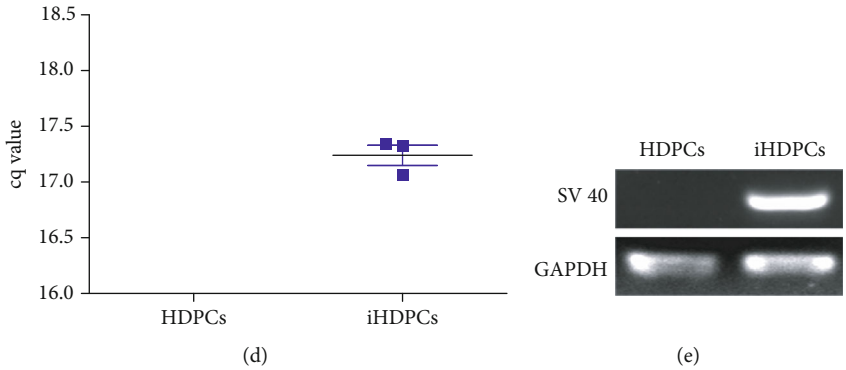


Figure 1: Immortalization of human dental pulp cells using the piggyBac transposon system. (a) Schematic diagram of pMPH86. The SV40 T-Ag gene is located between two flippase recognition target (FRT) sites. (b) Schematic of the experimental approach. (c) IHDPC establishment. The adenoviral vectors AdpBase and pMPH86 transfected the primary human dental pulp cells, and cells were selected in medium with hygromycin for 3 days. Surviving cells were observed at day 3, day 7, and day 13 postselection. The iHDPCs passaged consecutively for 25 passages (p25) (d and e). The mRNA expression of SV40T-Ag was upregulated after immortalization.

change in their fibroblast-like morphology (Figure 1(c)). Cells infected with pMPH86 exhibited a higher integration rate of SV40 T-Ag (Figures 1(d) and 1(e)).

3.2. IHDPCs Have a Greater Expansion Capacity Than HDPCs. The CCK8 cell proliferation assay suggested that iHDPCs had stronger proliferation ability than HDPCs, especially within the first 4 days (Figure 2(a)) which was also indicated by the colony formation assay (Figure 2(b)) and the crystal violet staining assay (Figure 2(c)). Quantitative assessment supported the staining results, confirming that iHDPCs had significantly higher cell proliferation activity at day 2 than the HDPCs (Figure 2(d)).

3.3. Biological Behavior Changes of IHDPCs. IHDPCs ranging from passage 10 to passage 70 were collected for cell cycle analysis by flow cytometry. We observed higher G2-M phase fractions in the iHDPCs, and the cell cycle distribution of iHDPCs was not affected by passaging (Figures 3(a) and 3(b)). Meanwhile, the percentage of apoptotic cells among iHDPCs was significantly lower than that among HDPCs and was also not affected by passaging (Figures 3(c) and 3(d)). Furthermore, compared with HDPCs, iHDPCs had superior migration ability which was quantified by the wound healing assay (Figures 3(e) and 3(f)). The nude mouse tumorigenicity assay was used to test the oncogenicity of iHDPCs, taking the human head and neck squamous cell carcinoma cell line SCC-4 as the positive control. There were no abnormal mitoses or tumor cell formation in the iHDPC group compared with the SCC-4 group (Figure 3(g)). Thus, we concluded that iHDPCs did not have tumorigenic properties. Our data suggested that the biological behavior changes of iHDPCs might contribute much to their application in pulp tissue engineering.

3.4. The IHDPCs Express the Majority of Marrow Stromal Cell Markers. It has been reported that consensus human dental pulp stem cell markers consist of CD37, CD90, and CD105. Negative cell surface markers include CD34 and CD45. Results of immunofluorescence staining showed that iHDPCs expressed the antigens CD37, CD90, and CD105 but did not express antigens CD34 and CD45, indicating that they were identical to primary human dental pulp cells (Figures 4(a)–4(c)).

3.5. The IHDPCs Are Capable of Differentiating into Adipogenic, Chondrogenic, and Osteogenic Lineages. IHDPCs were positive for Oil Red O staining, Alcian blue staining, and alkaline phosphatase (ALP) staining (Figure 4(d)). IHDPCs exhibited higher expression of peroxisome proliferator-activated receptor gamma (PPARγ), CCAA T/enhancer binding protein alpha (C/EBPα), and fatty acid-binding protein 4 (FABP4) (genes involved in adipogenic differentiation) (Figure 4(e)) and exhibited higher expression of collagen 2a1, collagen 10a1, and aggrecan (genes involved in chondrogenic differentiation) compared with control groups (Figure 4(e)). After culture in osteogenic/odontogenic medium for 5 days, IHDPCs showed weaker osteogenic differentiation compared with HDPCs (Figure 4(d)). IHDPCs exhibited expression of ALP, runt-related transcription factor 2 (RUNX2), collagen-1 (COL1), DSPP, and dentin matrix protein-1 (DMP1) after culture in osteogenic/odontogenic medium for 5 days or 14 days (Figure 4(e)).

Taking these results together, iHDPCs were multipotent like HDPCs. However, the results demonstrated that iHDPCs were less easy to differentiate compared to HDPCs. We suspected that this may be attributable to the high proliferative ability of iHDPCs.

3.6. Removal of the SV40 T Antigen Mediated by FLP Recombinase Contributes to the Recovery Effect of the IHDPCs. IHDPCs were efficiently infected with Ad-GFP or Ad-FLP (Figure 5(a)). The SV40 T antigen was efficiently removed from Ad-FLP-infected iHDPCs, compared with the Ad-GFP group (Figures 5(b) and 5(c)). The proliferative activity of the dHDPCs was reduced, as assessed by the colony formation assay, CCK8 cell proliferation assay, and crystal violet staining (Figures 5(d)–5(g)). Flow cytometric analysis showed that the cell cycle distribution of dHDPCs was similar to that of HDPCs but not iHDPCs

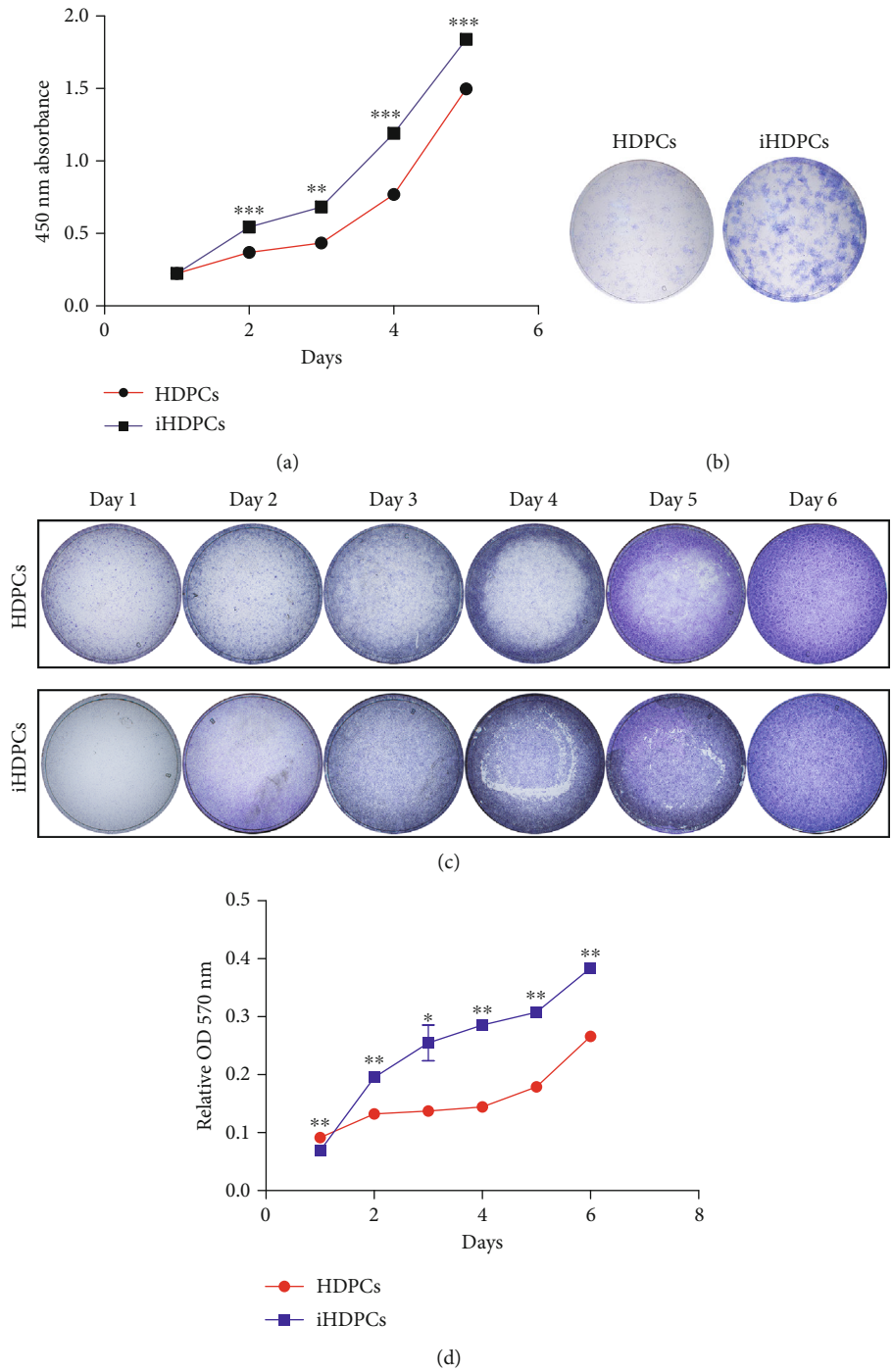
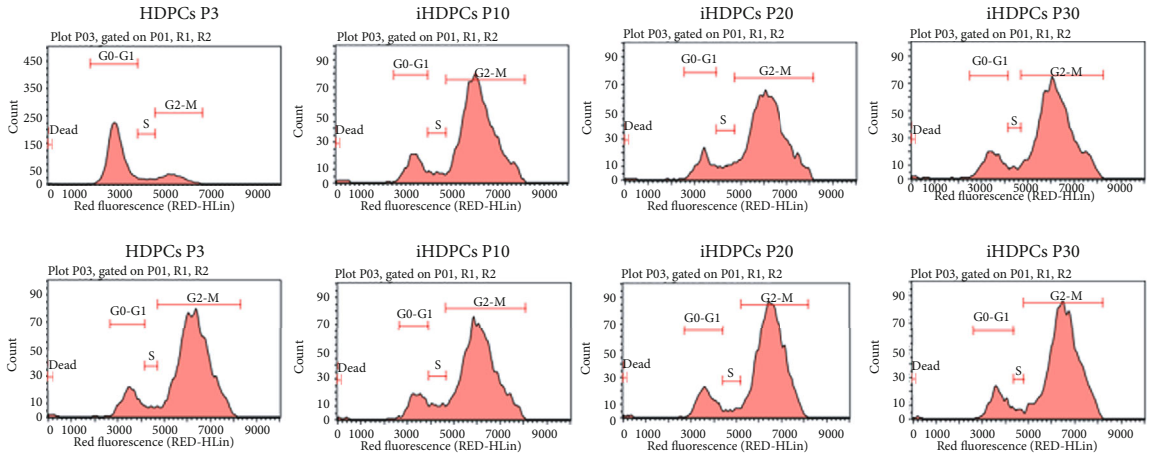


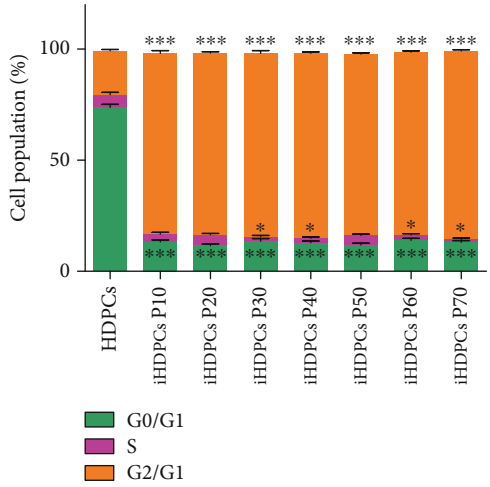
Figure 2: IHDPCs acquired strong self-renewal and proliferation ability. (a) Cell proliferation was observed by the CCK8 assay (**$P < 0.01$, ***$P < 0.001$). (b) Colony formation assay using crystal violet staining. (c) Cell proliferation assessed by the crystal violet staining assay. (d) The stain extracted from the cells was dissolved for quantitative determination at A570 nm (*$P < 0.05$, **$P < 0.01$).

(Figures 5(h) and 5(i)). Meanwhile, analysis of apoptosis by flow cytometry revealed that dHDPCs maintained a lower apoptosis rate (Figures 5(j) and 5(k)). As with the iHDPCs, the nude mouse tumor transplantation experiment revealed that dHDPCs had satisfactory characteristics without tumorigenesis compared with SSC4 cells (Figure 5(l)). Collectively, these results showed that dHDPCs which retain superior characteristics can be successfully established by reversing the immortalization of iHDPCs.

3.7. IHDPCs Can Be Differentiated into Odontoblasts When Combined with a Beta-Tricalcium Phosphate Scaffold and the Growth Factor BMP2. As previously confirmed, the ALP activity of iHDPCs was decreased compared to that of



(a)



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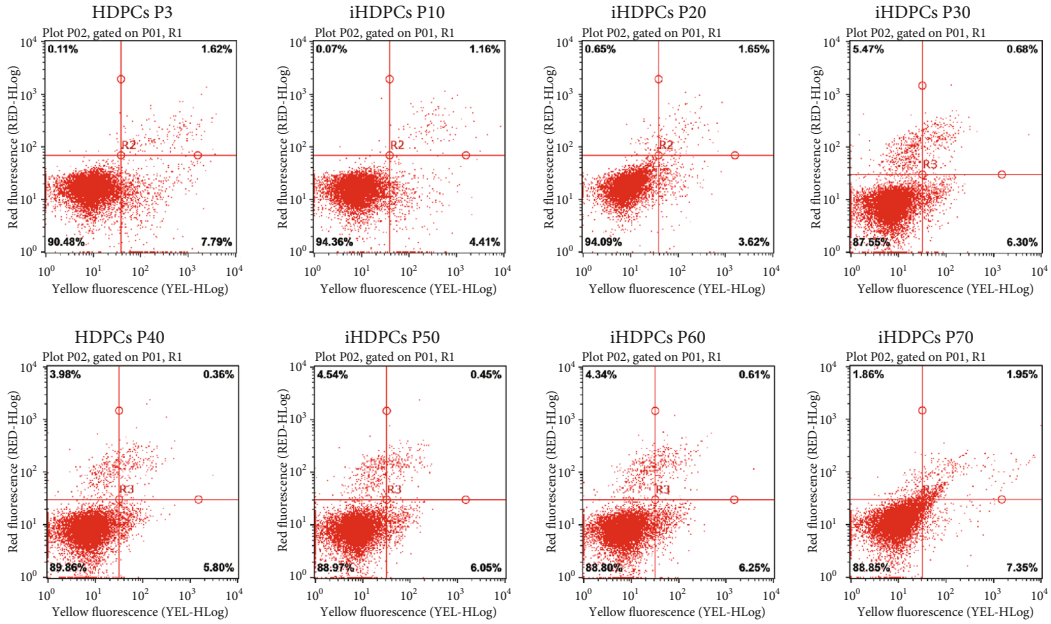

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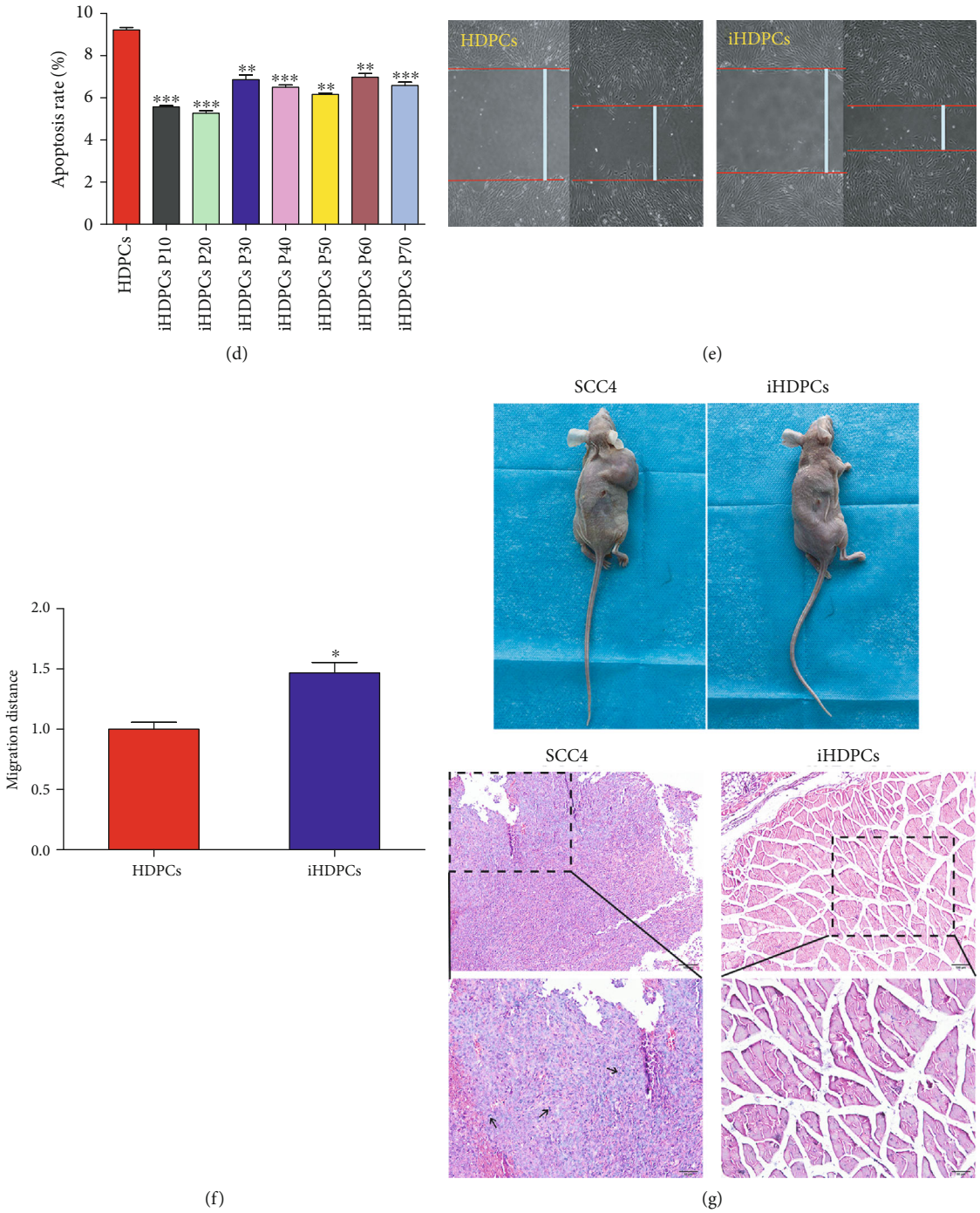
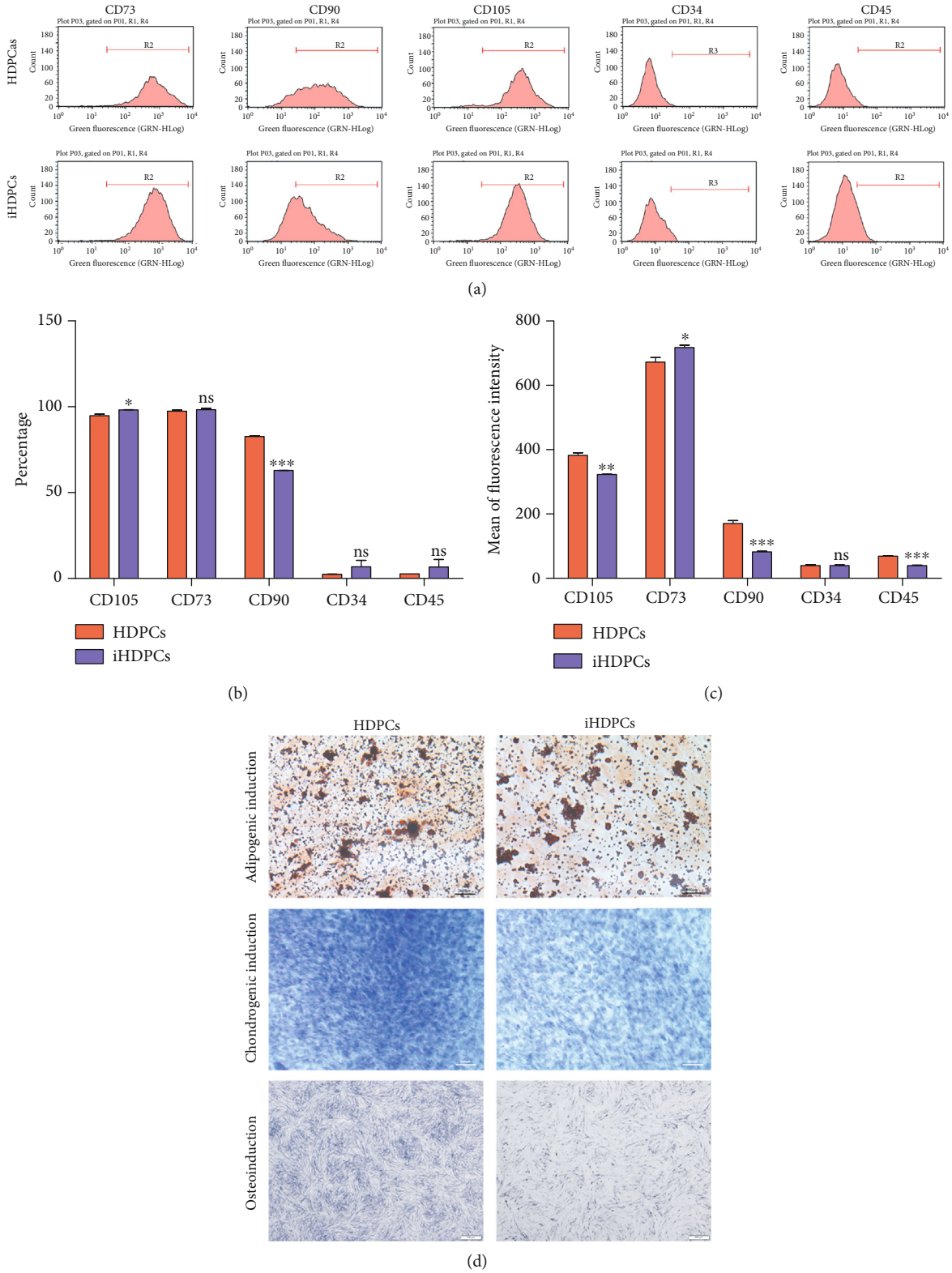


Figure 3: Biological behavior changes of iHDPCs. (a) Flow cytometric analysis showed the cell cycle distribution of HDPCs and iHDPCs. (b) Cell cycle analysis by flow cytometry showed increased G2-M phase fractions in the iHDPCs while the cell cycle distribution of iHDPCs was not affected by passaging ($^{***}P < 0.001$). (c) Flow cytometric analysis showed the levels of apoptosis in HDPCs and iHDPCs. (d) The percentage of apoptotic cells among iHDPCs was significantly lower than that among HDPCs and was not affected by passaging ($^{**}P < 0.01$, $^{***}P < 0.001$). (e and f) The migration rates of HDPCs and iHDPCs were quantified using the wound healing assay ($^{*}P < 0.05$). (g) The nude mouse tumorigenicity assay was used to test the oncogenicity of iHDPCs, with SCC-4 used as the positive control.

HDPCs *in vitro* (Figure 4(d)). In order to achieve better osteogenic or odontogenic differentiation, the growth factor BMP2 was added to the iHDPCs or dHDPCs. Odontogenic differentiation of cells was assayed by ALP staining after culture in odontogenic medium for 5 days (Figure 6(a)). Finally, BMP2 effectively rescued the differentiation and mineralization of iHDPCs and dHDPCs which were impaired by immortalization *in vitro* (Figure 6(a)). To evaluate the feasibility of applying iHDPCs and dHDPCs for pulp regeneration *in vivo*, the β-TCP/cell composites were subcutaneously transplanted into BALB/c nude mice (Figure 6(b)). Four weeks later, we found that cells were able to grow well



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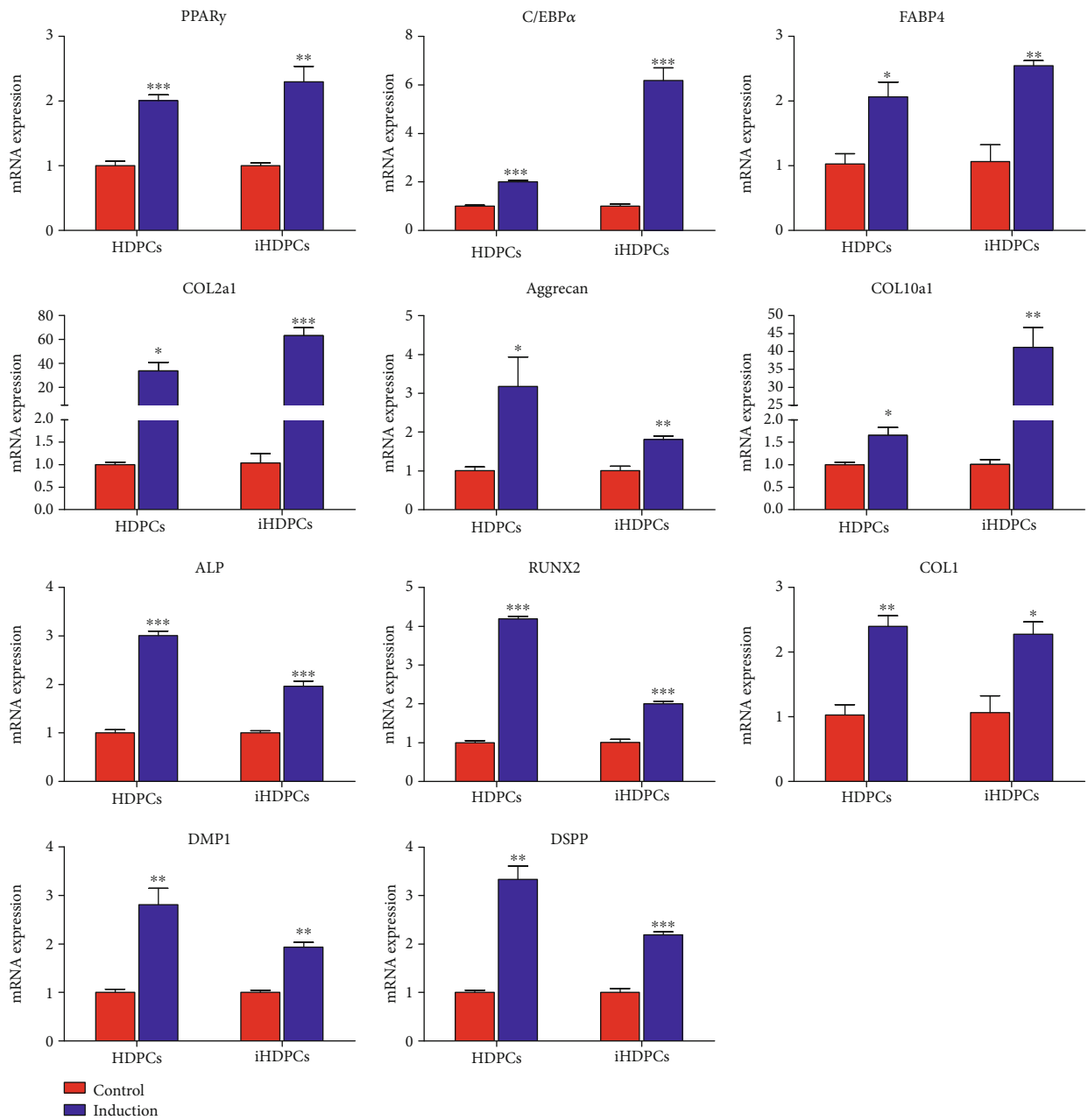


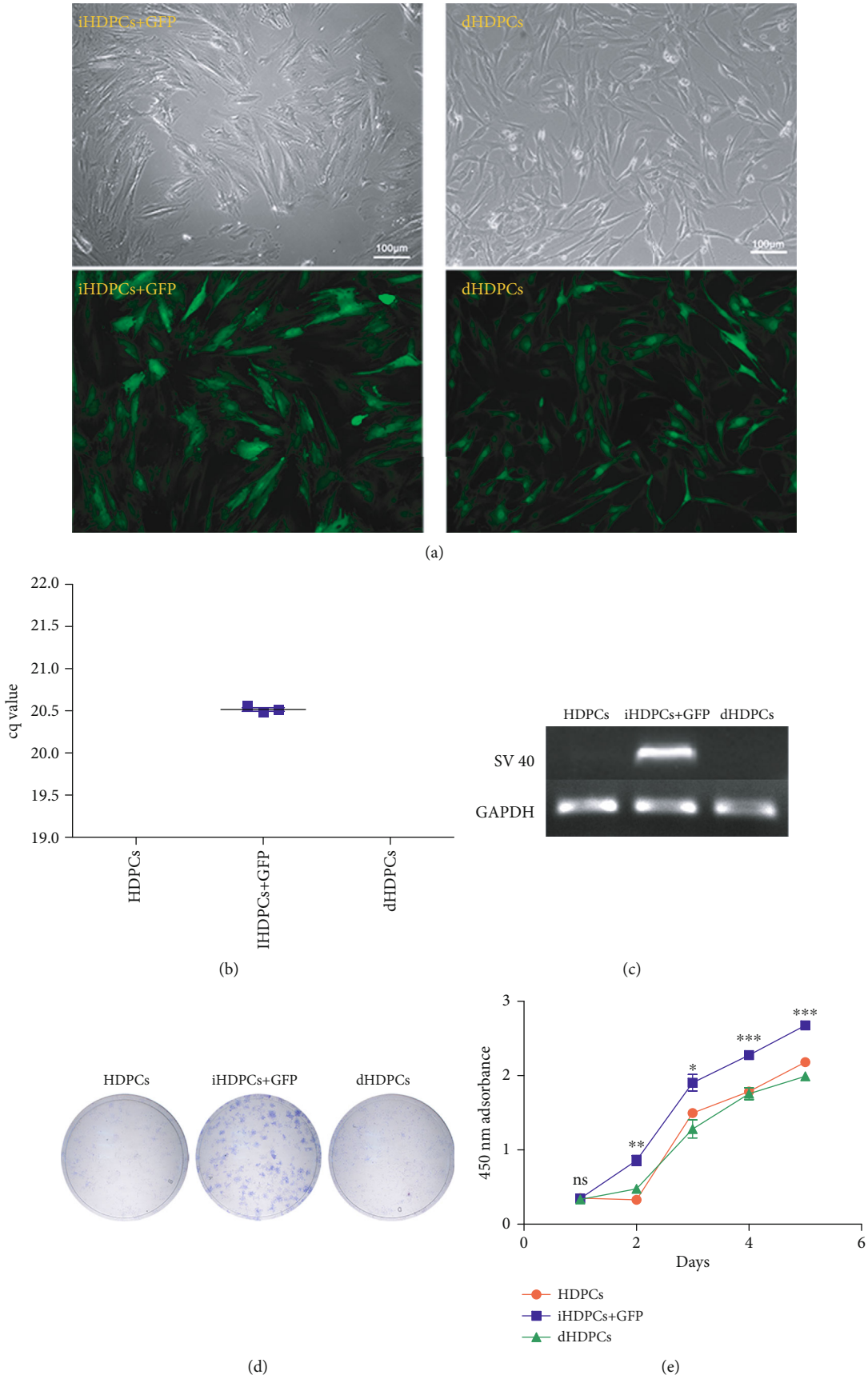
(e)

Figure 4: Multilineage differentiation of HDPCs and iHDPCs. (a) Flow cytometric analysis of the antigens expressed by HDPCs and iHDPCs. (b) Percentages of positive cells. (c) Mean fluorescence intensity of cells stained with the different antigens. (d) Adipogenic differentiation of HDPCs and iHDPCs determined by Oil Red O staining after culture in adipogenic medium for 14 days. Chondrogenic differentiation of HDPCs and iHDPCs determined by Alcian blue staining after culture in chondrogenic medium for 14 days. Osteogenic/odontogenic differentiation of HDPCs and iHDPCs determined by ALP staining after culture in osteogenic medium for 5 days. (e) qRT-PCR analysis of PPARγ, C/EBPα, EABP4, collagen 2a1, aggrecan, collagen 10a1, ALP, RUNX2, COL1, DMP1, and DSPP expression ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$).

in the scaffold (Figure 6(c)), and more mature mineralized nodules (dark blue) were observed in the BMP2 groups compared with the GFP group (Figure 6(d)). In addition, the Ad-BMP2-infected groups showed increased expression of the odontoblast-specific marker DSPP (Figure 6(e)). Consistently, these results suggested that iHDPCs can differentiate into odontoblasts especially when used in combined applications with BMP2.

4. Discussion

Caries and dental trauma are high-incidence diseases leading to hard tissue injuries and pulp inflammation. Many adult stem cell sources have been applied to tissue regeneration in the oral and maxillofacial region, such as HDPCs, stem cells derived from root apical papilla (SCAP), stem cells from human exfoliated deciduous teeth (SHED), stem cells from



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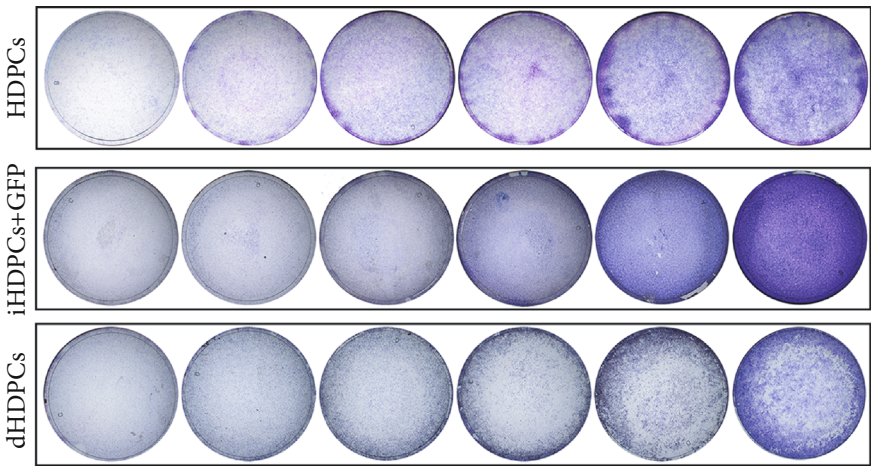

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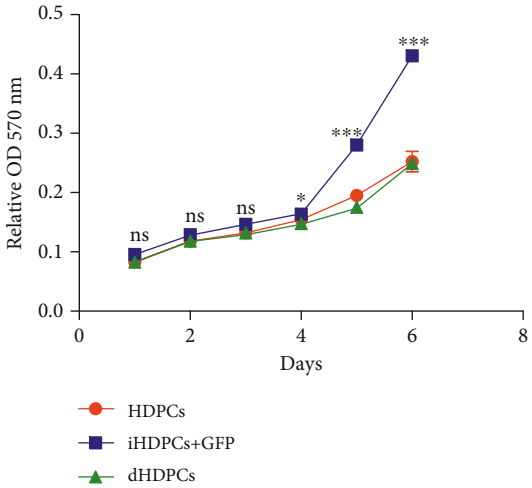

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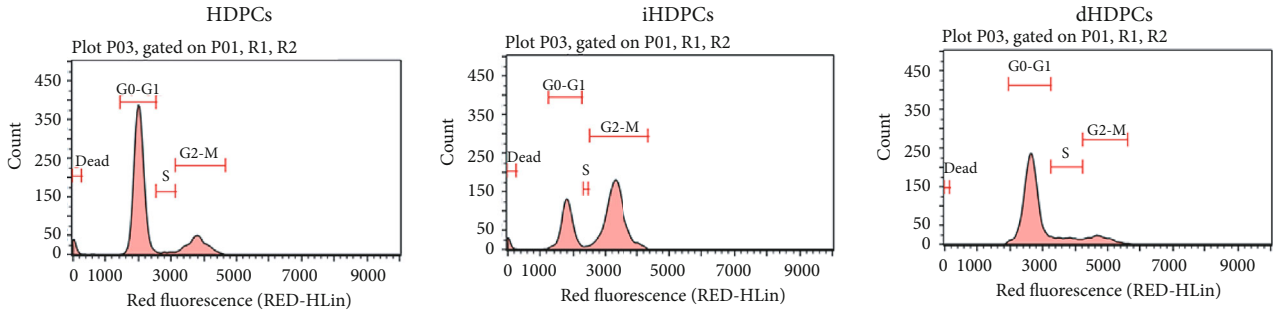

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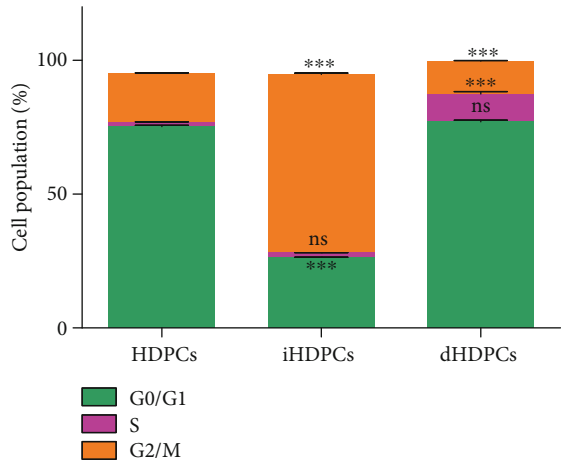

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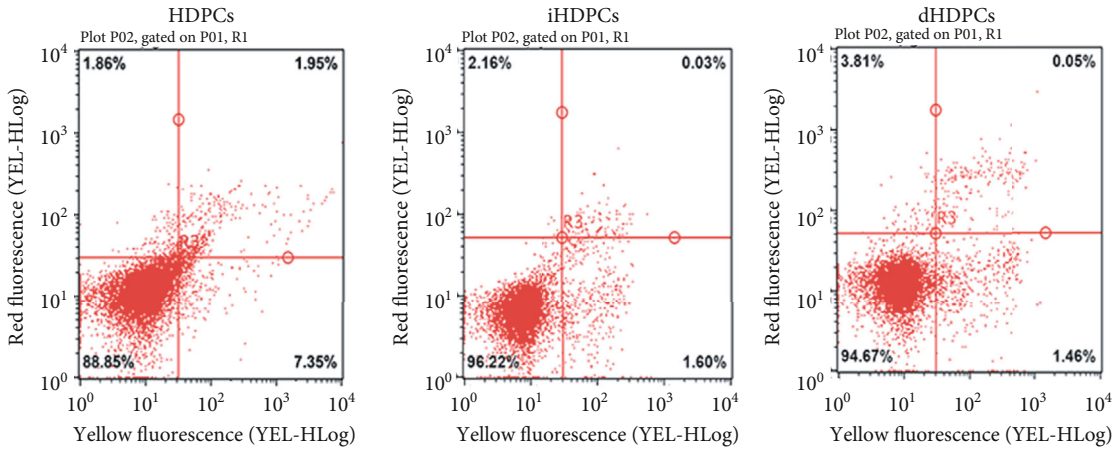

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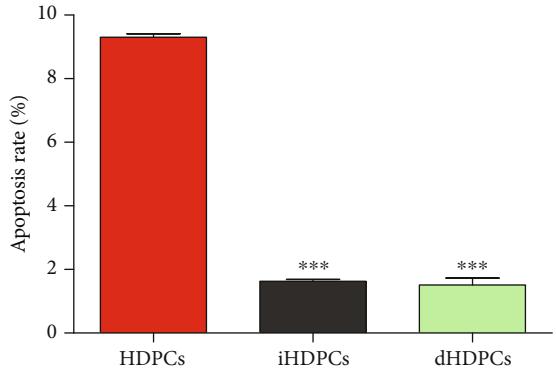

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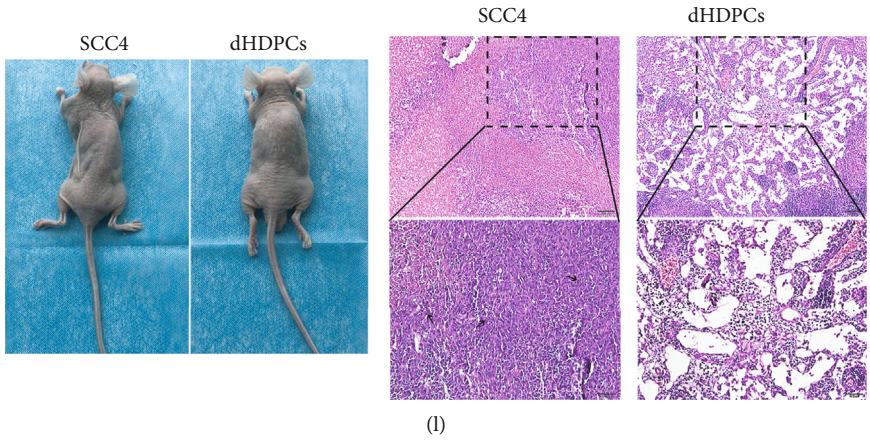
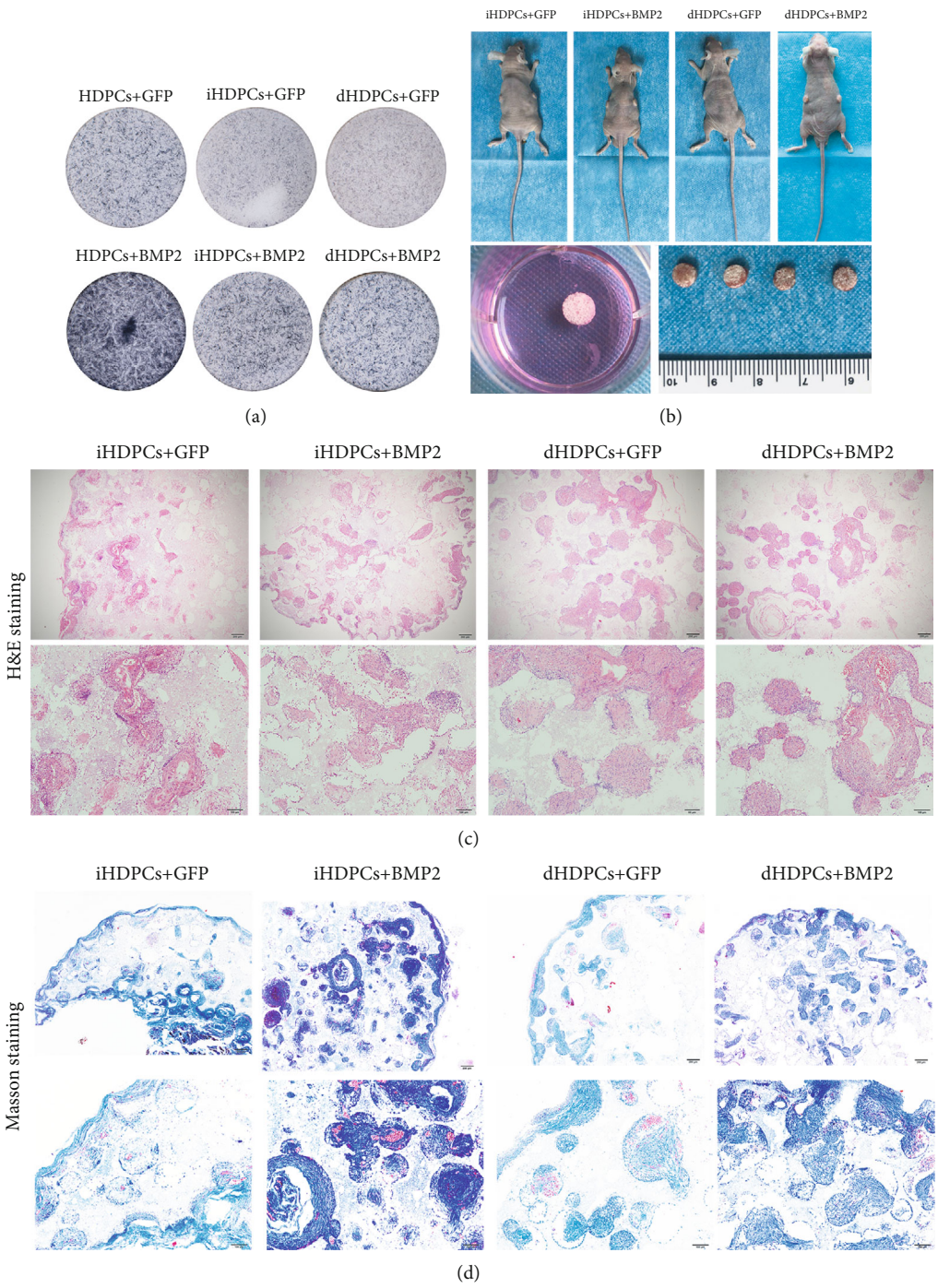


Figure 5: Deimmortalized human dental pulp cells. (a) IHDPCs transduced efficiently by Ad-FLP to establish deimmortalized human dental pulp cells (dHDPCs). (b) mRNA expression of SV40T-Ag was downregulated after deimmortalization ($^{***}P < 0.001$). (c) Expression of SV40T-Ag in dHDPCs. (d) The viability of dHDPCs was tested by the colony formation assay. (e) Cell proliferation assessed by the CCK8 assay ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). (f) Cell proliferation assessed by the crystal violet staining assay. (g) The stained cells were dissolved for quantitative determination at A570 nm ($^{*}P < 0.05$, $^{**}P < 0.01$). (h and i) Flow cytometric analysis showed the cycle distribution of HDPCs, iHDPCs, and dHDPCs ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$). (j and k) Flow cytometric analysis showed the apoptosis of HDPCs and iHDPCs ($^{***}P < 0.001$). (l) The nude mouse tumorigenicity assay was used to test the oncogenicity of dHDPCs in comparison with SCC-4 cells as the positive control. There were no developed tumors in the dHDPC group after three weeks. In contrast, there were many abnormal mitotic images in the SCC-4 group (arrows).

cryopreserved periodontal ligament (PDLSCs), human periapical cyst mesenchymal stem cells (hPCy-MSCs), and oral mucosal progenitor cells [22, 24] among which HDPCs are the most common and easily obtained from extracted human teeth. HDPCs successfully regenerate a dentin pulp-like structure *in vivo* when transplanted with hydroxyapatite/tricalcium phosphate (HA/TCP) powder [25]. Based on the aforementioned properties, HDPCs not only are promising cells for tooth regeneration owing to their ability to differentiate into odontoblasts and create reparative dentin but also have attracted attention because of their ability to repair multitudinous tissues outside of the tooth [1, 26, 27]. However, isolation of adult stem cells from the human body will cause ethical discussion despite their potential applications in regeneration. Meanwhile, it is difficult to isolate enough cells from the limited human pulp tissue. Establishment of immortalized human dental pulp cells has been put forward to overcome this problem. Cell immortalization has been realized by means of spontaneous random mutagenesis of primary cells, overexpression of oncogenes, and tumor suppressor gene inhibition [18]. According to the literature, immortalized human dental pulp cells have been established with the help of a retroviral vector involved in the immortalization system [6–8]. In our research, we utilized the piggyBac transposon-mediated system (Figure 1(a)) to stably express SV40 T-Ag which has proven to be more effective than the retroviral vector-based reversible immortalization system related to the efficient immortalization of various cells including mouse embryonic fibroblasts, mouse hepatic cells, mouse cardiomyogenic cells, and mouse melanocytes [11, 14, 15, 17, 28, 29]. There are many striking features of the piggyBac transposon-mediated system [30]. Briefly, it can insert foreign fragments of DNA of up to 100 kb into the genome of human and mouse cells [28, 31] and efficiently integrate DNA elements at multiple locations [32]. Moreover, there is little evidence of mutations associated with the piggyBac transposon system owing to its infrequent integration near active genes or cancer-related genes [33]. In addition, piggyBac transposase can reversibly immortalize cells by removing the immortalization gene SV40 T-Ag with the help of FLP recombinase [18]. Thus, the piggyBac transposon system is a superior DNA transposon to deliver genes compared with other transposons [33].

The seed cells involved in tissue engineering should possess potentiality to proliferate and differentiate *in vivo*. In our research, we performed more detailed testing and description of cell characteristics involved in tooth regeneration compared with previously established immortalized HDPCs. As the results showed, iHDPCs were endowed with superior abilities of proliferation, migration, and apoptosis without tumorigenesis (Figures 2 and 3) and have been proven to express the same pattern of stem cell surface markers as primary HDPCs (Figures 4(a) and 4(b)) indicating that they contain the same amount of stem cells. Meanwhile, we were able to reversibly immortalize cells by removing the immortalization gene SV40 T-Ag. Those deimmortalized cells still retained the superior characteristics including a high rate of proliferation, low apoptosis, and odontogenic differentiation ability (Figure 5). These results suggested that iHDPCs may be an alternative to primary dental pulp cells in the field of pulp and dentin regeneration.

However, their osteogenic and odontogenic differentiation ability is not satisfactory (Figure 4(d)). There is extensive evidence in the literature that cell proliferation and differentiation are negatively correlated. Cells maintain a high proliferation rate when they are in the cell cycle, and the cell cycle



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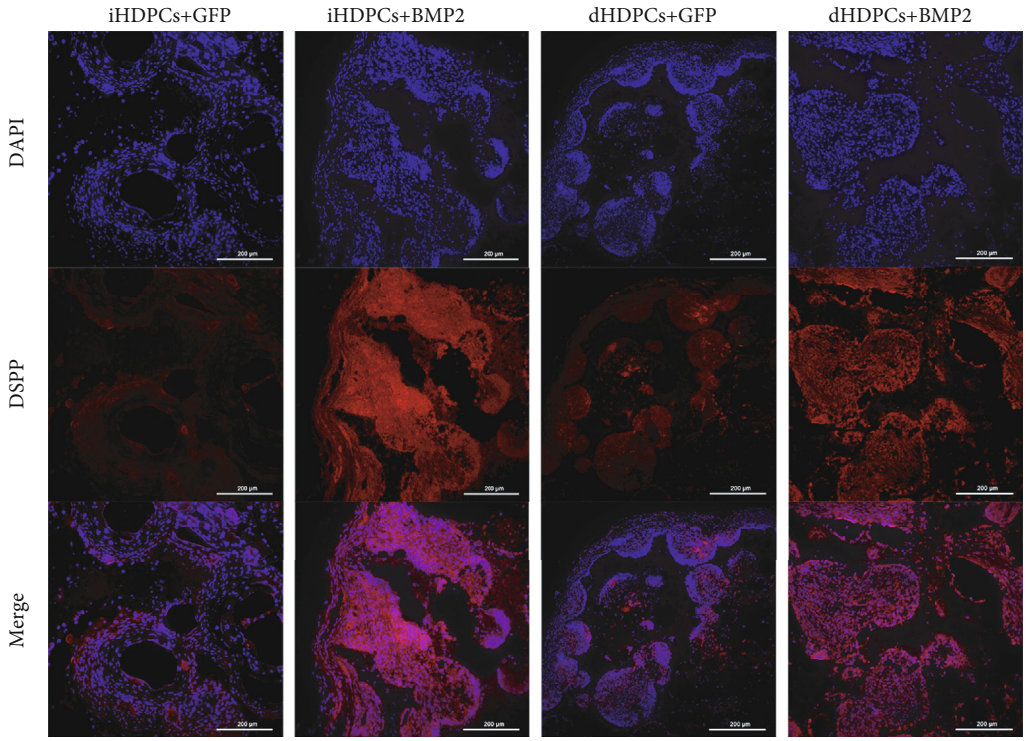

(e)

Figure 6: IHDPCs can differentiate into odontoblasts when combined with a beta-tricalcium phosphate scaffold and growth factor *in vivo*. (a) BMP2 promotes the differentiation and mineralization of iHDPCs and dHDPCs *in vitro*. ALP staining of iHDPCs and dHDPCs after culture in odontogenic medium for 5 days. (b) Composites of scaffolds and cells were carefully implanted into the dorsal subcutaneous region of nude mice for 4 weeks. (c) H&E staining of the composites of β-TCP scaffolds and iHDPCs/dHDPCs treated with BMP2 after subcutaneous implantation for 4 weeks. (d) Masson's trichrome staining of the composites. (e) The levels of DSPP in the β-TCP scaffold and iHDPC/dHDPC composites were analyzed by immunocytochemical staining.

exit is closely coordinated with cell differentiation [34, 35]. Therefore, we estimated that impaired odontogenic differentiation ability may be attributed to the high cell proliferative ability (Figure 2) and large G2/M-phase fraction (Figures 3(a) and 3(b)). BMP2 is one of the most commonly used BMPs and can induce odontoblast differentiation and dentin formation and the expression of DSPP and DMP1 by enhancing the activity of transcription factors [36, 37]. Thus, we opted to use BMP2 as the growth factor in our research. As expected, it achieved ideal effects in that BMP2 effectively promoted the differentiation and mineralization of iHDPCs and dHDPCs *in vitro* and *in vivo*.

Although the results support the potential use of the iHDPCs in pulp and dentin engineering, their osteogenic and odontogenic differentiation ability is not satisfactory without growth factors. So, future experiments are needed to improve cell performance to cater to superior tissue regeneration. In addition, due to the multiple factors affecting tissue homeostasis and regeneration, such as inflammatory conditions and types of growth factors, more experiments will be necessary to detect the inflammation conditions during the tissue regeneration and identify the optimum conditions for the therapeutic application of iHDPCs [38, 39].

5. Conclusion

We demonstrated that the established immortalized human dental pulp cells were stable, reversible, highly proliferative, and nontumorigenic cells, which should be valuable for studying the mechanisms of pulpitis, reparative dentin formation, and odontogenic differentiation of cells.

Data Availability

The data used to support the findings of this study are included within the article.

Conflicts of Interest

The authors declared no potential conflicts of interest.

Authors' Contributions

X. Li contributed to conception, design, data acquisition, analysis, and interpretation and drafted and critically revised the manuscript. L. Wang contributed to data acquisition, analysis, and interpretation and critically revised the manuscript. Q. Su, L. Ye, and X. Zhou were involved in conception

and design and critically revised the manuscript. D. Song and D. Huang contributed to conception, design, data analysis, and interpretation and also drafted and critically revised the manuscript. All authors gave the final approval and agree to be accountable for all aspects of the work.

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