STEM CELLS IN ORAL CAVITY: FROM DEVELOPMENT TO REGENERATION

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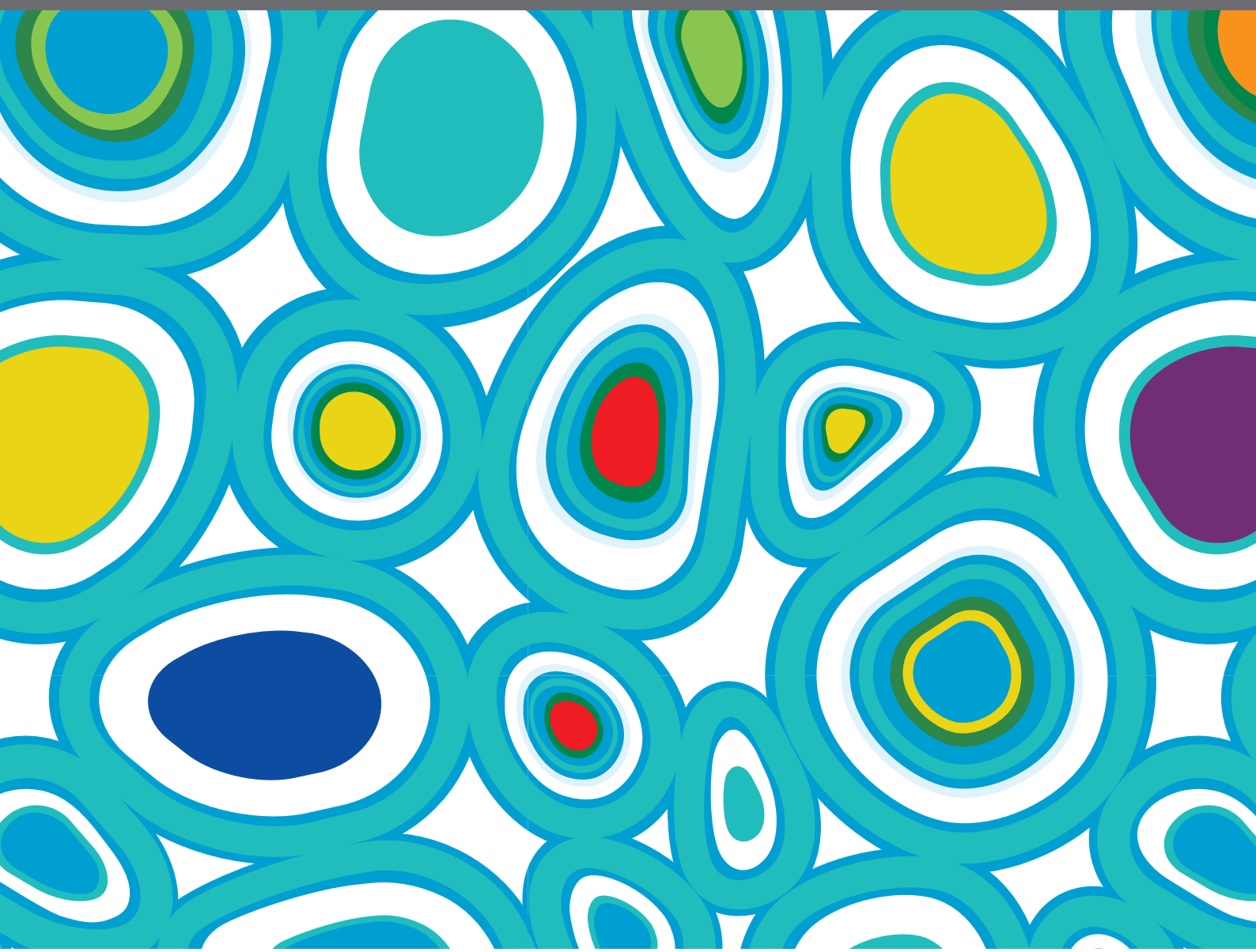

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STEM CELLS IN ORAL CAVITY: FROM DEVELOPMENT TO REGENERATION

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EDITORIAL
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Editorial: Stem Cells in Oral Cavity: From Development to Regeneration

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Editorial on the Research Topic

Stem Cells in Oral Cavity: From Development to Regeneration

Craniofacial stem/progenitor cells are central research topics in the dental field (Yu et al., 2021; Fan et al.). A tool for tissue engineering and regenerative therapy has received medical and scientific attention. Several studies confirmed that intraoral mesenchymal stem/stromal cells (MSCs) derived from craniofacial neural crest cells (NCCs) are useful cell sources. The application of such oral tissue stem cells appears promising for the regeneration of periodontal tissue, alveolar bone, and mucosal structures lost due to congenital abnormalities, trauma, and infections (Li et al.; Cui et al.). Cranial NCCs populate the future facial region and produce ectomesenchyme-derived tissues, such as cartilage, bone, dermis, smooth muscle, adipocytes, and so on.

Kaucka et al. (2016) reported a great degree of similarity in clonal dynamics between neural crest–and paraxial mesoderm–derived mesenchyme in the face and branchial arches. Their results support a profound similarity between vertebrate face and limb development and a deep homology between these seemingly unrelated structures.

In the long bone, which is a typical model of skeletal stem cells (SSCs), SSCs are generally defined as self-renewing cells with the trilineage potential to differentiate into osteoblast, chondrocyte, osteoblasts, and marrow stromal cells or adipocytes. Markers for skeletal progenitor cell populations identified in postnatal growing bones are expressed by growth plate chondrocytes and undifferentiated marrow stromal cells, particularly those located immediately below the growth plate. So far, many markers are reported for SSCs, including chondrocyte-specific genes (Mizoguchi and Ono, 2021). On the other hand, in the craniofacial region, Longaker's group reported that mechanoresponsive stem cells acquire neural crest fate in jaw regeneration by using lineage tracing of NCC/chondrocyte responsible gene, Sox9 (Ransom et al., 2018). Authors developed a dissectible model of mandibular distraction osteogenesis and used this model to show that newly formed bone is clonally derived from skeleton resident stem cells. Cell lineage tracing to clarify determination fates and non-biased single cell RNA sequence (scRNA-seq) is a strong tool in cellular and developmental biology (Matsushita et al., 2020). In calvaria, Farmer et al. used scRNA-seq to reveal cell diversity within mouse coronal sutures. The authors generated a single-cell transcriptome and performed extensive expression validation. They found preosteoblast features between the bone front and the periosteum, ligament-like populations on sutures that persist into adulthood, and chondrogenic-like populations in the dura mater under sutures (Farmer et al., 2021). Holmes reported that *Hhip*, an inhibitor of hedgehog signaling, is required for normal coronal suture development (Holmes et al., 2021). These reports strongly support the importance of our deeper understanding of single cell level transcriptomics which in turn are influenced by microenvironment and signaling pathway.

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In the dental mesenchyme, the combination of lineage tracing and scRNA-seq is an especially strong tool to visualize complicated and heterogeneous tissue cell types (Krivanek et al., 2020; Pagella et al., 2021). Zhang et al. reported a global mapping of open chromatin regulatory elements during dentinogenesis and illustrated how cells are regulated via dynamic binding of different transcriptional factor families, resulting in odontoblast terminal differentiation. Dental stem cells are generally characterized by their tissue origins (Mattioli-Belmonte et al., 2015). Functionally, dental pulp stem cells (DPSCs) have the ability to proliferate and give rise to several lineages including odontoblasts (Gronthos et al., 2000). Paduano et al. demonstrated the control for dedifferentiation of DPSCs and reported that the rerouting of cell fate could potentially be used to enhance their osteogenic therapeutic potential under physiological conditions. Cell-cell communication and interaction are pivotal to perform biological roles and activate functional abilities. N-cadherin-mediated cell-cell interactions are involved as important factors in controlling cell fate decisions in MSCs. Deng demonstrated that N-cadherin acted as a negative regulator via controlling β-catenin activity in the odontogenic differentiation of DPSCs by pharmacological intervention and gene silencing (Deng et al.). Stem cells from human exfoliated deciduous teeth (SHED) have higher proliferation ability compared with adult DPSCs (Miura et al., 2003). Dental pulp cells exist under hypoxia condition in the tooth. Hypoxia inducible factor-1α (HIF-1α) is well known to mediate adaptive functions to ischemic stress. Han et al. revealed that HIF-1α plays an essential role in post-implantation survival and angio-/vasculogenic properties of SHED by maintaining cellular and mitochondrial reactive oxygen species levels, homeostasis, inducing metabolic adaptations, and vascular endothelial growth factor (VEGF) secretion. Janebodin et al. revealed that VEGF receptor plays an important role in dentin regeneration by gene silencing and *in vivo* studies. Thus, ligand and receptor axis such as growth factor and its receptor, hormone and its receptor are always important in cellular and molecular biology (Lyu et al.). As other dental stem cells, stem cells from root apical papilla (SCAP) (Sonoyama et al., 2006; Driesen et al.) and periodontal ligament stem cells (PDLSCs) (Seo et al., 2004) are well studied. Such dental stem cell exerts its therapeutic effect mainly by the secretion of exosomes via the paracrine mechanism as well. Stem cell derived exosomes have special advantages such as high drug loading potentials, high specificity, low immunogenicity, excellent biocompatibility, readily available, low side effects, and nanoscale size. In addition, exosomes regulate many important biological processes such as cell-cell communication, anti-inflammatory, bone formation, angiogenesis, immunoregulation, neuronal growth, and promotion of tumor cell apoptosis and so on (Mai et al., 2021).

In regard to oral epithelium, the keratinized epithelial cells of the tongue are responsible for squamous cell carcinoma. However, little is known about the mechanisms of tissue maintenance and regeneration of these cells. Ueno's group revealed that stem cells positive for Bmi1 rapidly entered the cell cycle and regenerated the tongue epithelium after irradiation-induced damage. And the removal of Bmi1-positive stem cells significantly suppressed regeneration (Tanaka et al., 2013). These results suggested that the $Bmi1^+$ stem cells are important for tissue maintenance and tongue epithelial regeneration. Stratified squamous epithelial stem cells are generally thought to attach to a non-hierarchical single progenitor cell model. Byrd et al. (2019) demonstrated lineage tracing and genetic label retention assays in order to show that the hard palate epithelium of the oral cavity is unique in exhibiting marked proliferative heterogeneity. They showed that stratified epithelia of the oral cavity display unusual proliferative heterogeneity, particularly in the hard palate region. A slow-cycling population residing in the junctional zone niche self-renews through planar symmetric divisions, responds to masticatory stress, and promotes wound healing. To help understanding tissue-specific pathophysiology in oral mucosa, Williams et al. (2021) provided the single-cell transcriptome atlas of the human oral mucosa in healthy individuals and patients with periodontitis. It revealed the existence of a complex cellular landscape in oral mucosal tissue and identified a population of epithelial and stromal cells with inflammatory signature that promote antibacterial defense and neutrophil recruitment.

In the craniofacial and dental region, multiple kinds of tissue types, including mesenchymal and epithelial cells, interact together. To prove their hierarchy and fate commitment, lineage tracing technology is one of the strong tools (Orsini et al., 2015). Evaluation of protein expression patterns and gene ablation based on non-biased scRNA-seq provide new insights into genetic regulation in stem cells and their development. Major regulatory mechanisms that control the transcriptional networks of stem cells are mediated through various types of transcriptional factors. Posttranscriptional regulation is essential for stem cell maintenance and cell fate determination. Important players in posttranscriptional control include RNA-binding proteins and noncoding RNAs (i.e., miRNAs, piRNAs, and lncRNAs). Modification of the translated protein influences a large variety of dental cell activities that regulate stem cell maintenance and differentiation throughout all mammalian species (Bian et al.; Wang et al.).

Lastly, further studies through multifacet evaluations, by combining functional analysis under physiological and pathological conditions, will definitely strengthen this research field.

AUTHOR CONTRIBUTIONS

TO, GO, AG, and MK wrote the editorial and invited authors to participate in the research topic. All authors contributed to the article and approved the submitted version.

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ORIGINAL RESEARCH
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Upregulating the Expression of LncRNA ANRIL Promotes Osteogenesis via the miR-7-5p/IGF-1R Axis in the Inflamed Periodontal Ligament Stem Cells

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Background: Long non-coding RNA (lncRNA) antisense non-coding RNA in the INK4 locus (ANRIL) is a base length of about 3.8 kb lncRNA, which plays an important role in several biological functions including cell proliferation, migration, and senescence. This study ascertained the role of lncRNA ANRIL in the senescence and osteogenic differentiation of inflamed periodontal ligament stem cells (iPDLSCs).

Methods: Healthy periodontal ligament stem cells (hPDLSCs) and iPDLSCs were isolated from healthy/inflamed periodontal ligament tissues, respectively. The proliferation abilities were determined by CCK-8, EdU assay, and flow cytometry (FCM). The methods of Western blot assay (WB), quantitative real-time polymerase chain reaction (qRT-PCR), alizarin red staining, alkaline phosphatase (ALP) staining, ALP activity detection, and immunofluorescence staining were described to determine the biological influences of lncRNA ANRIL on iPDLSCs. Senescence-associated (SA)-β-galactosidase (gal) staining, Western blot analysis, and qRT-PCR were performed to determine cell senescence. Dual-luciferase reporter assays were conducted to confirm the binding of lncRNA ANRIL and miR-7-5-p, as well as miR-7-5p and insulin-like growth factor receptor (IGF-1R).

Results: HPDLSCs and iPDLSCs were isolated and cultured successfully. LncRNA ANRIL and IGF-1R were declined, while miR-7-5p was upregulated in iPDLSCs compared with hPDLSCs. Overexpression of ANRIL enhanced the osteogenic protein expressions of OSX, RUNX2, ALP, and knocked down the aging protein expressions of p16, p21, p53. LncRNA ANRIL could promote the committed differentiation of iPDLSCs by sponging miR-7-5p. Upregulating miR-7-5p inhibited the osteogenic differentiation of iPDLSCs. Further analysis identified IGF-1R as a direct target of miR-7-5p. The direct binding of lncRNA ANRIL and miR-7-5p, miR-7-5p and the 3′-UTR of IGF-1R were verified by dual-luciferase reporter assay. Besides, rescue experiments showed that

knockdown of miR-7-5p reversed the inhibitory effect of lncRNA ANRIL deficiency on osteogenesis of iPDLSCs.

Conclusion: This study disclosed that lncRNA ANRIL promotes osteogenic differentiation of iPDLSCs by regulating the miR-7-5p/IGF-1R axis.

Keywords: lncRNA ANRIL, inflamed periodontal ligament stem cells, miR-7-5p, IGF-1R, differentiation

INTRODUCTION

Periodontitis is a chronic infectious disease of periodontal support tissue, during which osteoclast activates and leads to bone resorption (Hienz et al., 2015). Until now, the reconstruction and repair of periodontal support tissue have been an unsolved problem in oral clinic treatment. Thus, periodontal tissue regeneration has become a hotspot of research in the region of oral medicine research in recent years. Increasing studies about periodontal ligament stem cells (PDLSCs) emerged as PDLSCs are considered to be the important seed cells of periodontal tissue regeneration and repair (An et al., 2016). PDLSCs are a type of tissue−specific mesenchymal stem cells (MSC) and have corresponding specialties, such as self−renewal, multipotency, immunosuppressive response. They can differentiate into chondrogenic, adipogenic, and osteogenic lineages *in vivo* and *in vitro* (Wada et al., 2009; Tsumanuma et al., 2011; Nunez et al., 2019). However, under the condition of inflammation, the osteogenic differentiation capacity is reduced along with the accelerated process of cell senescence of remaining iPDLSCs, and then iPDLSCs cannot achieve effective periodontal support tissue regeneration (Yi et al., 2017; Rotini et al., 2018). The methods to facilitate the directional differentiation ability of iPDLSCs might provide a new strategy for clinical treatment of periodontitis.

LncRNAs are a class of non-coding RNAs longer than 200 nt in length. They have received extensive attention as burgeoning regulators involved in diverse biological processes (Liu Y. et al., 2019). Huang et al. (2015) reported that lncRNA H19 may be involved in osteogenesis, invasion, and migration of human MSCs. As a kind of lncRNA with a base length of about 3.8 kb, lncRNA ANRIL has been discovered to play an important role in many cancers. A lot of studies revealed that lncRNA ANRIL could regulate cell growth or proliferation in several cancers including retinoblastoma, colorectal cancer, and cervical cancer (Naemura et al., 2016; Zhang J. J. et al., 2018; Wang X. et al., 2019). Besides, lncRNA ANRIL also takes part in the senescence of vascular smooth muscle cells (Tan et al., 2019). However, the effects of lncRNA ANRIL on osteogenesis and senescence of iPDLSCs and underlying mechanisms remain elusive.

MicroRNAs (miRNAs) are a highly conserved group of short non-coding RNAs found in most tissues, and they can regulate post-transcriptional gene expression (Lu and Rothenberg, 2018; Zhang J. J. et al., 2018). Meanwhile, miRNAs play an indispensable role in developmental and cellular processes such as metabolism (Rottiers and Näär, 2012), cell cycle (Bueno and Malumbres, 2011), differentiation (Ivey and Srivastava, 2010), and signal transduction (Inui et al., 2010). Increasing evidence has indicated that miRNAs are involved in multiple physical performances of iPDLSCs, including proliferation and differentiation (Li X. B. et al., 2018). Mir-7-5p is the most studied miRNA sequence in the microRNA-7(miR-7) family, which is a crucial miRNA that plays a variety of roles in physiological and pathological processes (Pogribny et al., 2010; Kalinowski et al., 2014). Studies have shown that miR-7-5p can inhibit cell proliferation, promote apoptosis by regulating the EGFR/Akt/mTOR and RelA/NF-κB signaling pathways and play the role of "tumor suppressor gene" in various malignant tumors (Giles et al., 2016; Song et al., 2016). By regulating the STAT3/miRNA-7-5p, the osteoblast capacity of MSCs can be influenced (Tang et al., 2020). Nevertheless, clarification of the role of miR-7-5p on the regulatory mechanism of osteogenic differentiation of iPDLSCs is still unclear.

Insulin-like growth factor-1 receptor (IGF-1R) is a pervasive growth receptor and has been found on the surface of many kinds of cells, including hepatocytes, myocytes, and osteocytes (Wang Z. et al., 2019; Zhang et al., 2019). It is also involved in the regulation of proliferation, apoptosis, differentiation, and malignant transformation of cancer cells (Zhang et al., 2019). Insulin-like growth factor (IGF-1) is the most affluent growth factor in the bone matrix, which can combine with IGF-1R to maintain bone mass and form new bone (Xian et al., 2012). Our previous studies demonstrated that IGF-1R has a crucial impact on the osteo/odontogenic differentiation of DPSCs and SCAPs (Shu et al., 2016; Liu et al., 2018). Similarly, numerous studies have shown that the activation of IGF-1/IGF-1R can regulate cell senescence and osteogenesis, participate in maintaining intracellular homeostasis, and promote tooth-related tissue regeneration (Sergi et al., 2019; Zhou et al., 2019). This study explores the role of lncRNA ANRIL/miR-7-5p/IGF-1R axis in the osteogenic differentiation of inflamed periodontal ligament stem cells for the first time.

MATERIALS AND METHODS

Cell Culture

IPDLSCs were isolated from teeth with periodontitis from periodontitis patients (n = 20, aged 28–45 years) with their informed consent referred to the Jiangsu Provincial Stomatological Hospital. At the same time, the Medical Ethics Committee of the Stomatological School of Nanjing Medical University approved this study. The patients diagnosed with severe chronic periodontitis at active inflammatory stage and teeth needed to be removed were defined as alveolar bone loss no less than 2/3 and more than 1 pocket (depth 5 mm), and

hPDLSCs of human healthy impacted third molars were acquired from patients aged from 28 to 45 years.

The teeth were washed thrice with phosphate-buffered saline (PBS, Gibco, Life Technologies, United States), then PDL was separated from the middle third of the root using a surgical scalpel, digested in medium containing 3 mg/ml collagenase type I (Sigma, St. Louis, MO, United States) and 4 mg/ml trypsin (Beyotime, Haimen, China) for 30 min at 37°C. After digestion, we centrifuged at 1,000 r/min for 5 min, and tissue clumps were collected. The hPDLSCs and iPDLSCs were grown in alpha minimum essential medium (α-MEM; Gibco, Life Technologies, United States) with 10% fetal bovine serum (FBS, Gibco, Life Technologies, United States), 100 mg/ml streptomycin, and 100 units/ml penicillin at 37°C in a humidified incubator containing 5% CO_2. The medium was replaced every 3 days. When the cells grew to 70–80% confluence, trypsin was used to digest and the cells were collected for passage in a 1:3 radio. The cells from passage 3 to 5 were used for following experiments in the present study.

Characterization and Differentiation Assay

To identify the phenotypes of hPDLSCs and iPDLSCs, the surface markers of mesenchymal stem cells were detected by flow cytometry. Cells at passage 3 were incubated with primary antibodies for human CD105, CD90, CD73, CD29, CD34, and CD45 (all from BD Pharmingen, San Diego, CA, United States) according to the manufacturer's instructions. The incubation procedure was carried out at 4°C in the dark for 1 h, then cells were rinsed twice with PBS and subjected to flow cytometric analysis.

HPDLSCs and iPDLSCs were seeded in six-well plates with adipogenic induction medium and cultured in 15 ml conical−bottomed sterile tubes with chondrogenic induction medium, respectively. The cells were then cultured in a 5% CO_2 incubator at 37°C for 25 days. The adipogenic medium was changed every 3 days, and the chondrogenic medium was changed every 2 days. After 25 days, the cells were analyzed for adipogenesis and chondrogenesis by Oil Red O staining and Alcian Blue staining. HPDLSCs and iPDLSCs were also seeded in six-well plates with osteogenic induction medium, and the cells were analyzed by Alizarin red staining after 14 day culturing. The stained cells were photographed using a microscope.

Flow Cytometry

Cell cycle analysis was operated according to previous study's protocol (Li et al., 2019). In brief, hPDLSCs and iPDLSCs were collected by trypsin and fixed with 70% cold ethanol overnight at 4°C in dark. Washed with PBS, samples were measured using FAC Scan flow cytometer (BD Biosciences, San Jose, CA) and independently analyzed three times.

Western Blot Analysis

After being cultured for 7 days, transfected cells were rinsed with PBS two times and total protein was extracted using RIPA buffer (Beyotime) containing phenylmethylsulfonyl fluoride on ice. After centrifugation, proteins were collected and denatured by boiling for 10 min. Proteins were separated by 10% SDS-PAGE and transferred to 0.22 μm PVDF membranes (Millipore, Shanghai, China). After incubated with primary antibodies including OSX (ab22552), RUNX2 (ab76956), ALP (ab95462), IGF-1R (ab182406) from Abcam, P16 (#80772), P21 (#2947), P53 (#2527) from Cell Signaling Technology, GAPDH (Protein Tech Group) overnight at 4°C, the membranes were incubated with secondary antibodies for another 1 h at room temperature, followed by TBST wash for 30 min and 10 min each. The band density was quantified by Image J software.

Quantitative Real-Time-Polymerase Chain Reaction (RT-PCR) Analysis

After 7 days of induction, total cellular RNA was extracted by Trizol reagent (Invitrogen, United States), and using a PrimeScript RT Master Mix kit (TaKaRa, Otsu, Japan) to reverse transcribe into cDNA according to conventional protocols. qRT−PCR was conducted using the ABI 7300 real−time PCR system. Triplicate reactions (20 μl volume) were performed. Two internal normalized controls including *GAPDH* and *U6* were used for mRNA and miRNAs, respectively. The expression levels of *LncRNA ANRIL*, *miR-7-5P*, *OSX*, *ALP*, *RUNX2*, *P16*, *P21*, *P53*, and *IGF-1R* were detected with qRT-PCR. The data were analyzed using the $2^{-\Delta\Delta Ct}$ relative expression method. The PCR primer sequences are listed in **Table 1**.

Immunofluorescence Staining

The transfected cells were inoculated into a 12-well plate with a coverslip in each hole and cultured in mineralized medium

TABLE 1 | Primer sequences.

Genes	Primes	Sequences (5′–3′)
ANRIL	Forward	CTAAGGAGCAGAAGACATC
	Reverse	GTAGAATCTCTCAGACGGTTG
OSX	Forward	CCTCCTCAGCTCACCTTCTC
	Reverse	GTTGGGAGCCCAAATAGAAA
RUNX2	Forward	TCTTAGAACAAATTCTGCCCTTT
	Reverse	TGCTTTGGTCTTGAAATCACA
ALP	Forward	ACCTGAGTGCCAGAGTGA
	Reverse	CTTCCTCCTTGTTGGGTT
P16	Forward	CCCCGATTGAAAGAACCAGAGAG
	Reverse	TACGGTAGTGGGGGAAGGCATA
P21	Forward	AGCGACCTTCCTCATCCACC
	Reverse	AAGACAACTACTCCCAGCCCCATA
P53	Forward	AGCTTTGAGGTGCGTGTTTGTG
	Reverse	TCTCCATCCAGTGGTTTCTTCTTTG
IGF-1R	Forward	AGGATATTGGGCTTTACAACCTG
	Reverse	GAGGTAACAGAGGTCAGCATTT-T
GAPDH	Forward	GAAGGTGAAGGTCGGAGTC
	Reverse	GAGATGGTGATGGGATTTC
miR-7-5p	Forward	CAGGGAGGCGTGGATCACTG
	Reverse	CGTCGGGGGCTCATGGAGCGG
U6	Forward	CTCGCTTCGGCAGCACA
	Reverse	AACGCTTCACGAATTTGCGT

for 7 days. After washing with PBS twice, the cells were fixed with 4% paraformaldehyde for 30 min. The cells were then penetrated with 0.25% Triton-100 at room temperature for 12 min, washed several times with PBS, and sealed with goat serum (DCS/BioGenex, Hamburg, Germany) at 37°C for 45 min. The cells were then incubated with an antibody against RUNX2 and ALP at 4°C overnight, and then incubated with a fluorescence-labeled secondary antibody at room temperature for 45 min, and the nuclei were dyed with 4′,6-diamidino-2-phenylindole (DAPI; Beyotime) for 90 s. Images were observed using an inverted fluorescent microscope (Olympus, Shanghai, China).

Cell Counting Kit-8 Assay and EdU

Cell proliferative activity was measured using the Cell Counting Kit-8 (CCK-8, Dojindo, Tokyo, Japan). Transfected cells were seeded in 96-well culture plates at a density of 2×10^3 cells/well in complete culture medium. CCK-8 reagent (10 μl) and 90 μl of α-MEM were added to each well at the indicated time points (days 0, 1, 3, 5, 7, 9). After incubation for 2 h, the cells were assessed at 450 nm absorbance by a microplate reader.

Transfected iPDLSCs were inoculated on 12-well plates with 4×10^5 per well and incubated with EdU medium for 4 h at 37°C. Then the cells were fixed with 4% paraformaldehyde for 30 min and incubated with 0.25% triton-100 for 10 min. After rinsing with PBS three times, 1 × Apollo staining solution was added to incubate for 30 min, and the DNA was stained with Hoechst 33342 for 20 min in darkness, subsequently observed under a fluorescence microscope and EdU analysis was quantified using Image J software.

Alkaline Phosphatase Staining and Activity

At 7 days after transfected iPDLSCs were seeded in six-well plates and cultured in osteogenic medium, BCIP/NBT staining kit (Beyotime, China) was used to assess the osteogenic differentiation according to the instructions. The cells were washed with PBS and fixed with 4% paraformaldehyde for 30 min, then rinsed three times with PBS and the alkaline solution was added to each well. ALP quantitative analysis was performed using an alkaline phosphatase assay kit (Jian Cheng, Nanjing, China) following the protocol.

Alizarin Red Staining

Cell mineralization was evaluated by ARS staining, and transfected iPDLSCs were cultured in osteogenic induction medium for 14 days. The cells were washed with PBS three times, and 4% paraformaldehyde was added to fix the cells for 30 min. The alizarin red solution was added, 1 ml/well, for 30 min at room temperature. Then the mineralized nodules were photographed by using an inverted microscope.

Senescence-Associated β-Galactosidase Staining (SA-β-Gal)

The cellular senescence of transfected iPDLSCs was measured by SA-β-Gal staining kit (GenMed Scientifcs Inc., Shanghai, China). Briefly, the cells were washed with GENMED cleaning fluid, then covered with fixed fluid at room temperature for 5 min and incubated with SA-β-Gal staining solution at 37°C without CO_2 for 24 h. After incubation, the sections were washed twice in PBS and mounted in glycerol and observed under a microscope. Image J software was used to quantify the senescent cells.

Cell Transfection

LncRNA ANRIL overexpression and knockdown were conducted via lentiviral transfection. IPDLSCs were transfected into the following groups: NC group, ANRIL group, sh-NC group, and sh-ANRIL group. Recombinant lentiviruses were synthesized by Gene Chem (Shanghai, China). The miR-7-5p mimics and inhibitor transfected to overexpress and inhibit miR-7-5p in iPDLSCs were purchased from Ribobio (Guangzhou, China). The transfected cells were divided into groups: NC group, mimics group, iNC group, and inhibitor group.

Fluorescence *in situ* Hybridization

Ribo^{TM} Fluorescent *in situ* Hybridization Kit (RiboBio) was used to perform FISH experiments according to the protocols. Briefly, the cells were grown on the slides, and when grown 60–70% confluence, we rinsed the cells with PBS and fixed them with 4% paraformaldehyde. Then, the cells were incubated with hybridization mixed with FISH probe overnight at 37°C in dark. After washing with washing buffer, the cells were counterstained with 4,6-diamidino-2-phenylindole and visualized using a confocal microscope.

Dual-Luciferase Reporter Gene Assay

LncBase Predicted v.2 was performed to determine the binding sites between ANRIL and miR-7-5p, as well as potential targets of miR-7-5p and IGF-1R were predicted by TargetScan. MiR-7-5p mimics or normal control was co-transfected with IGF-1R-MUT, IGF-1R-WT, lncRNA ANRIL-MUT, or lncRNA ANRIL-WT into 293T cells following the manufacturer's instructions. Luciferase activity was detected using a Dual-Luciferase Reporter Assay System (Promega, Madison, United States).

Statistical Analysis

All consequences were presented as the mean and standard deviation (mean ± SD). and the experiments were performed in triplicates. GraphPad Prism 5.0 software and SPSS 20.0 software were utilized for statistical analyses. Statistical significance was established at $P < 0.05$.

RESULTS

Characterization of Periodontal Ligament Stem Cells

PDLSCs were successfully isolated from the collected healthy teeth and the teeth with periodontitis, respectively. The adherent cells had spindle-like morphology (**Supplementary Figures 1A, 2A**). Flow cytometry assay described that these cultured cells were positive expressions of CD29, CD73,

CD90, and CD105, and hardly express CD34 and CD45 (**Supplementary Figures 1C, 2C**). The results illustrated that the isolated cells were mesenchymal stem cells. Chondrogenic and adipogenic differentiation was tested by Alcian Blue staining and Oil Red O staining. At the same time, osteogenic differentiation was verified by Alizarin Red Staining (**Supplementary Figures 1D, 2D**). Furthermore, **Supplementary Figures 1B, 2B** showed that MSC surface molecule STRO-1 was observed on the cell surface of the periodontal ligament stem cells.

Long Non-coding RNA (LncRNA) Antisense Non-coding RNA INK4 Locus Is Down-Regulated in Inflamed Periodontal Ligament Stem Cells Compared With Healthy Periodontal Ligament Stem Cells

HPDLSCs and iPDLSCs were cultured in complete medium. We performed qRT-PCR to assess the expression of lncRNA ANRIL, miR-7-5p, IGF-1R, ALP, OSX, and RUNX2 in hPDLSCs and iPDLSCs. We found that expressions of ANRIL, IGF-1R, ALP, OSX, and RUNX2 were significantly decreased while miR-7-5p was increased in iPDLSCs compared with hPDLSCs (**Figure 1A**) ($P < 0.05$). The results indicated that iPDLSCs had lower osteogenic activity compared with hPDLSCs, and lncRNA ANRIL might play a positive role in osteogenic differentiation potential.

Long Non-coding RNA Antisense Non-coding RNA INK4 Does Not Markedly Affect the Proliferative Ability of Inflamed Periodontal Ligament Stem Cells

To explore the vital role of lncRNA ANRIL in iPDLSCs, we first studied the impact on the proliferative ability of iPDLSCs *in vitro*. Cells were transfected with lentivirus and transfection efficacy was confirmed by qRT-PCR analysis (**Figures 1B,C**) ($P < 0.05$). CCK-8 assay indicated that lncRNA ANRIL had no significant difference in cell viability between ANRIL-group and NC-group ($P > 0.05$), and sh-ANRIL group and sh-NC group also showed the same result (**Figure 1D**). Meanwhile, flow cytometry analysis revealed that the proliferation index (PI = G2M + S) has no statistical significance in the ANRIL group and NC group as well as in the sh-ANRIL group and sh-NC group (**Figure 1E**). The above conclusions were further confirmed by EdU experiment (**Figures 1F,G**).

Long Non-coding RNA Antisense Non-coding RNA INK4 Locus Affects the Osteogenesis and Senescence of Inflamed Periodontal Ligament Stem Cells

The cells were transfected with lentivirus and transfection efficacy was confirmed by qRT-PCR (**Supplementary Figure 3A**) ($P < 0.05$). Cultured with mineralized induced medium for 7 days, western blot confirmed that the expression levels of osteogenic differentiation proteins (ALP, RUNX2, and OSX) were significantly up-regulated, and qRT-PCR analysis further revealed that lncRNA ANRIL overexpression significantly increased *ALP*, *OSX*, *RUNX2* mRNA levels (**Figures 2A,B**) ($P < 0.05$). The above results suggested that overexpression of lncRNA ANRIL promoted osteogenic differentiation of iPDLSCs. ALP activity, ALP staining, alizarin red staining, and immunofluorescence staining further confirmed the above conclusion (**Figures 2E–H**). On the contrary, sh-ANRIL group showed that down-regulating lncRNA ANRIL could inhibit osteogenesis the opposite trend (**Figures 2C,D,G,I**). These results indicated that lncRNA ANRIL enhanced the osteogenic differentiation of iPDLSCs.

Then we investigated the effects of lncRNA ANRIL on cell senescence of iPDLSCs. After 7 days cultured in complete culture medium, the protein levels of p16, p21, and p53, as well as mRNA expression of *p16*, *p21*, and *p53* were downregulated in the ANRIL group while upregulated in the sh-ANRIL group as shown in **Figures 3A–D** ($P < 0.05$). At the same time, the positive expression of SA-β-gal in ANRIL group was significantly decreased compared with NC group, while it was increased in the sh-ANRIL group compared with the sh-NC group (**Figures 3E,F**). Thus, our data suggested that lncRNA ANRIL postponed the cell senescence of iPDLSCs.

MiR-7-5p Is the Direct Target of Long Non-coding Antisense Non-coding RNA INK4 Locus

To study the molecular mechanism of how lncRNA ANRIL regulate the osteogenic differentiation of iPDLSCs, existing studies have shown that lncRNAs can act as a ceRNA or sponge miRNAs to regulate gene expression (Zhang L. M. et al., 2018; Li et al., 2020). To determine whether lncRNA ANRIL plays a regulatory role through the ceRNA mechanism, we first assured its localization in the iPDLSCs, and FISH results showed that lncRNA ANRIL was distributed in the cytoplasm (**Figure 4A**). Then we used LncBase Predicted v.2 in DIANA tools to analyze the predicted targets of lncRNA ANRIL, and we identified a lot of potential targets including miR-181a, miR-122-5p, miR-144, miR-125a, miR-7-5p, and so on. Among them, studies have shown that miR-7-5p plays a critical regulatory role in bone differentiation of MSCs. Thus, miR-7-5p was chosen for further study (Chen et al., 2020; Tang et al., 2020).

To investigate the relationship between miR-7-5p and lncRNA ANRIL, iPDLSCs were transfected with miR-7-5p mimics, miR-7-5p inhibitor, and correspond to miR-NC. Transfection efficacy results showed approximately 43-fold upregulation of miR-7-5p in the mimics group and 0.3-fold miR-7-5p expression in miR-7-5p inhibitor-transfected cells (**Figure 4B**). qRT-PCR results revealed that lncRNA ANRIL expression was reduced in the mimics group and elevated in the inhibitor group (**Figure 4C**) ($P < 0.05$). Moreover, dual-luciferase reporter assay uncovered that miR-7-5p mimics could specifically lessen only the luciferase activity of wild-type ANRIL but not

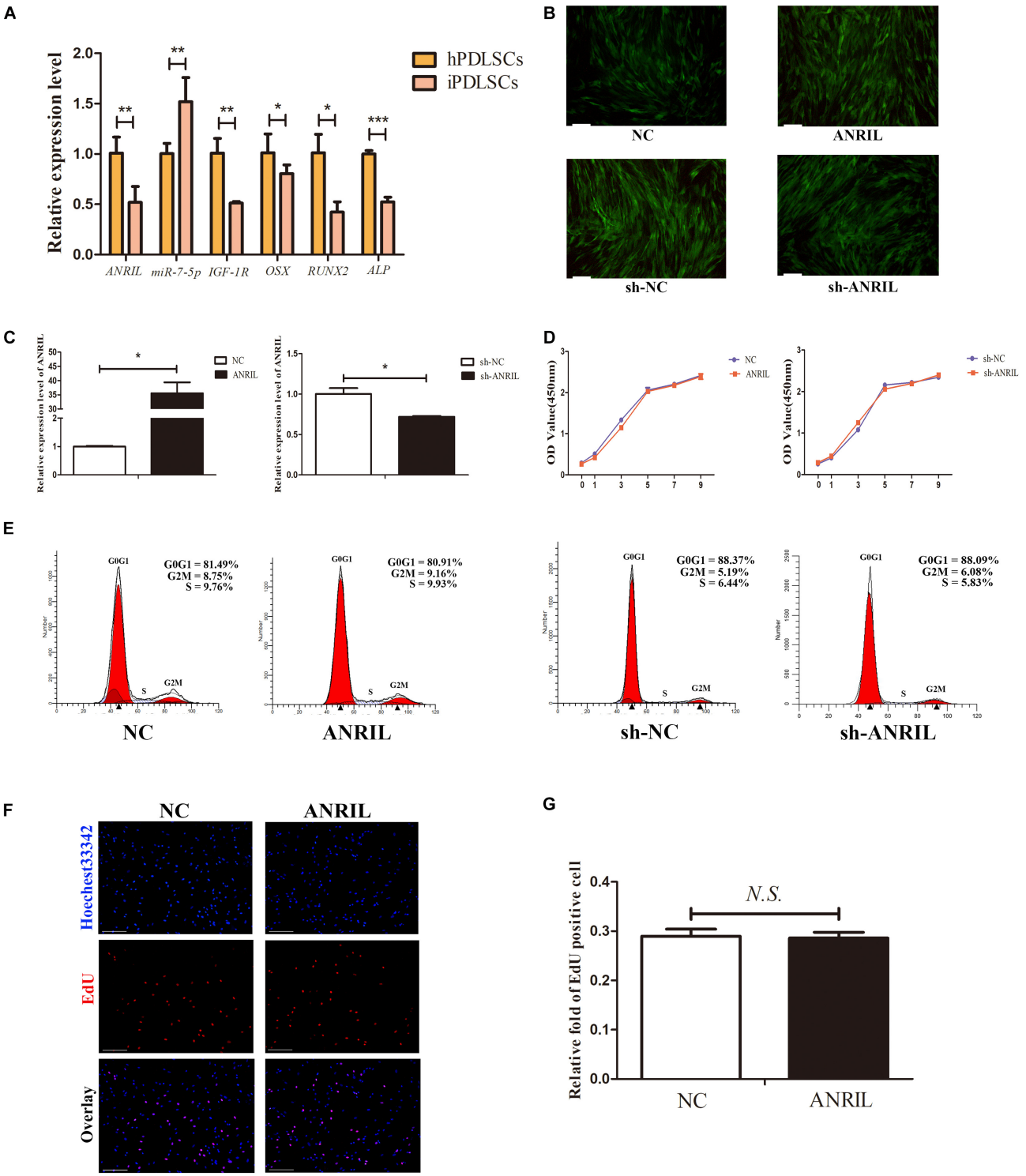

FIGURE 1 | (A) The expression levels of long non-coding RNA (*lncRNA) antisense non-coding RNA INK4 locus (ANRIL),miR-7-5p, insulin-like growth factor-1 receptor (IGF-1R), alkaline phosphatase (ALP), OSX,* and *RUNX2* were determined by quantitative real-time polymerase chain reaction (qRT-PCR) in healthy periodontal ligament stem cells (hPDLSCs) and inflamed periodontal ligament stem cells (iPDLSCs). **(B)** Fluorescent photomicrographs showed lentivirus transfection in NC, ANRIL, sh-NC, sh-ANRIL groups. (Scale bar = 100 μm). **(C)** The transfection efficacy of ANRIL was measured by qRT-PCR in ANRIL group and sh-ANRIL group. GAPDH was used for normalization. **(D)** Proliferative ability of iPDLSCs was detected by Cell Counting Kit-8 (CCK-8) assay. **(E–G)** Flow cytometry and EdU assay demonstrated that ANRIL had no significant difference in cell proliferation of iPDLSCs on day 7 (Scale Bar = 100 μm; N.S., $P > 0.05$) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

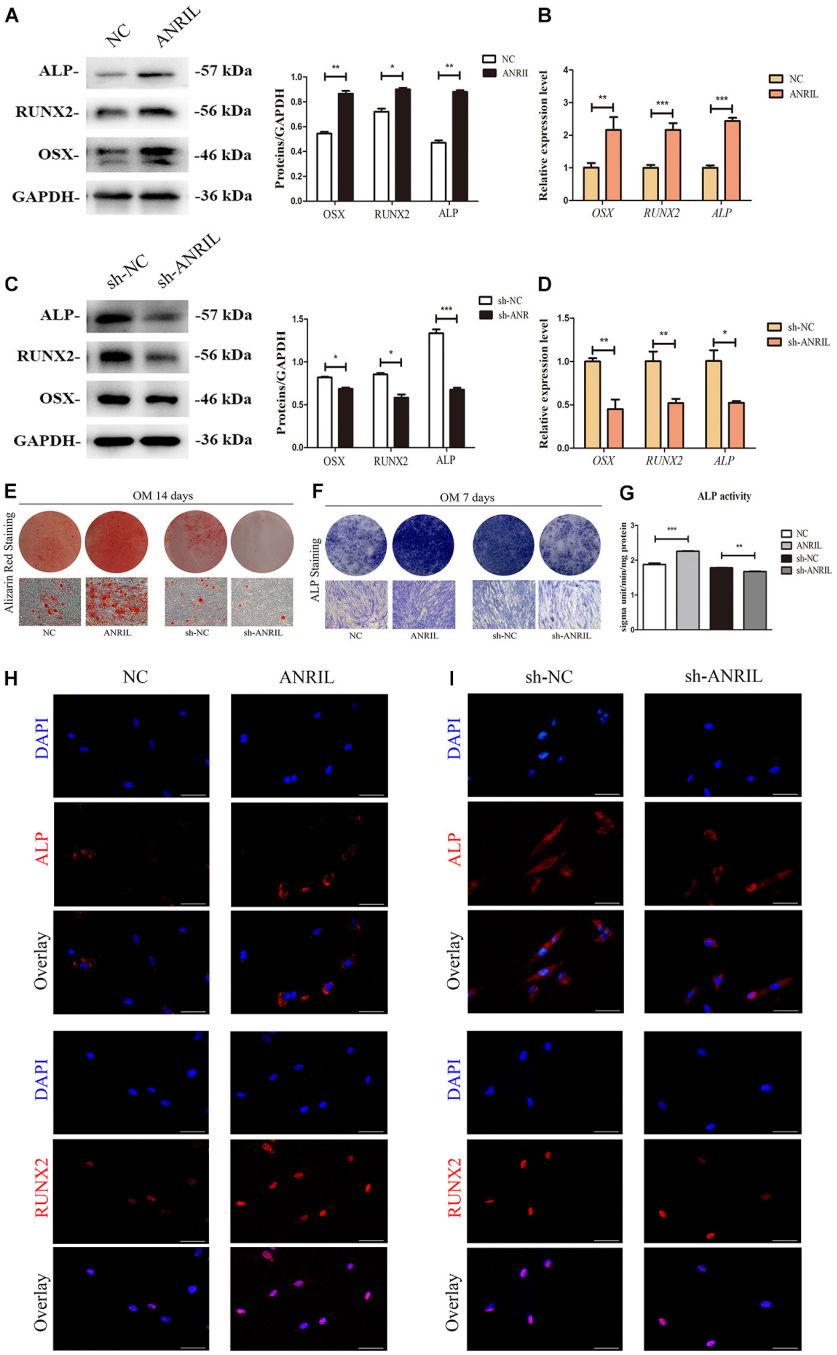

FIGURE 2 | LncRNA ANRIL regulates osteogenic differentiation of iPDLSCs. **(A–D)** ALP/*ALP*, RUNX2/*RUNX2*, and OSX/*OSX* expressions were measured by western blot and qRT-PCR on day 7 after osteogenic induction. GAPDH served as an internal control. **(E–G)** Osteogenic differentiation of iPDLSCs was determined by Alizarin Red S, ALP staining and ALP activity assay at 14 and 7 days after osteogenic induction. **(H,I)** The expressions of ALP and RUNX2 in transfected iPDLSCs were also determined by immunofluorescence assay (Scale Bar = 50 μm) (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

miR−NC, while there was no apparent difference between the mutant ANRIL + miR-7-5p mimics group which further identified the miR-7-5p was a binding target of lncRNA ANRIL (**Figures 4D,E**) ($P < 0.05$). In general, the results above demonstrated the direct binding effect between lncRNA ANRIL and miR-7-5p in iPDLSCs.

MiR-7-5p Inhibits the Osteogenic Differentiation of Inflamed Periodontal Ligament Stem Cells

Cells were transfected with miR-7-5p mimics, miR-7-5p inhibitor, and correspond miR-NC; and transfection efficacy was confirmed

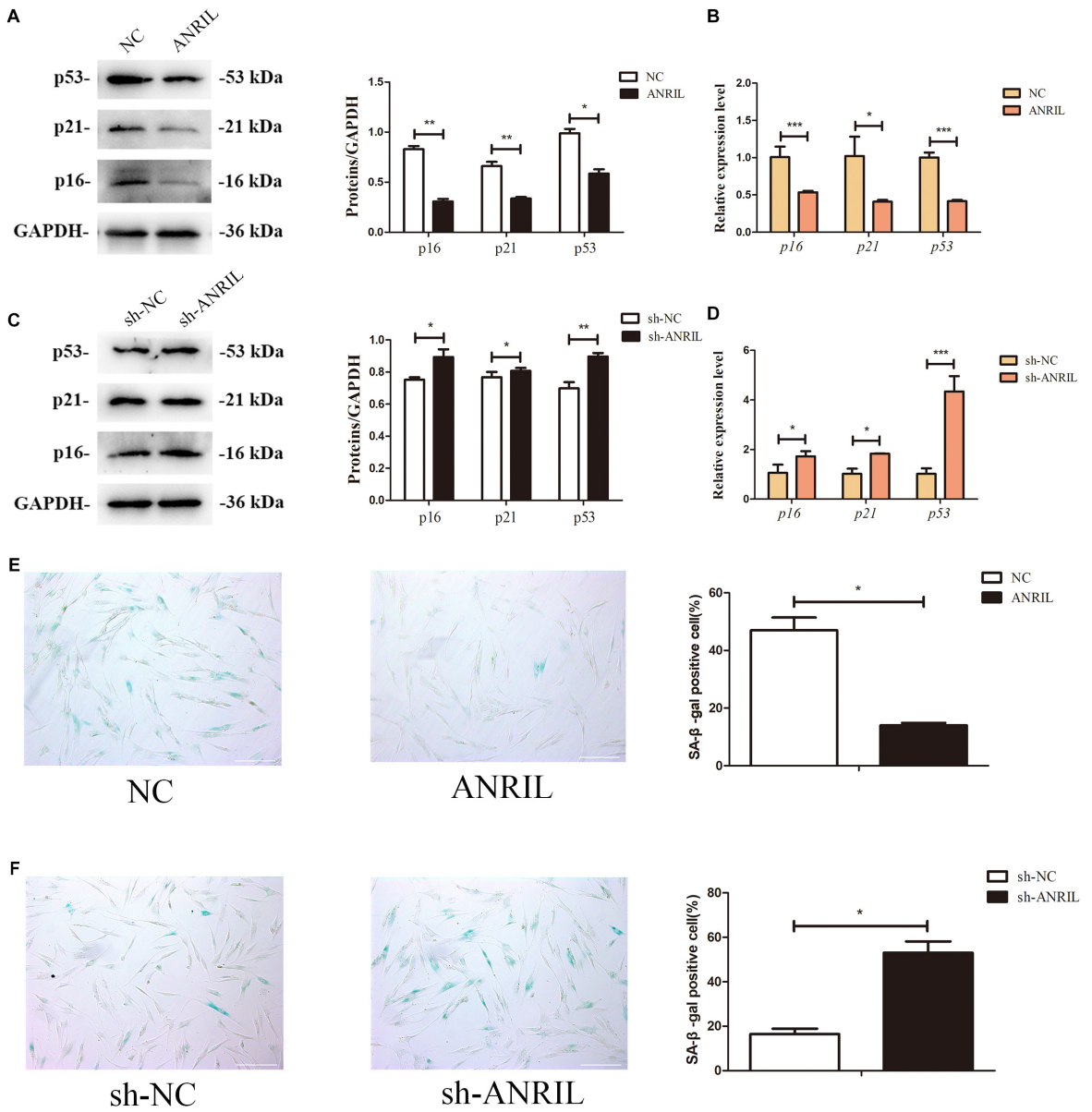

FIGURE 3 | LncRNA ANRIL affects the senescence of iPDLSCs. **(A)** Protein expression of p16, p21, and p53 in the ANRIL group and NC group. **(B)** mRNA levels of *p16*, *p21,* and *p53* in the ANRIL group and NC group. **(C)** Protein expression of p16, p21, and p53 in the sh-ANRIL group and sh-NC group. **(D)** mRNA levels of *p16*, *p21,* and *p53* in the sh-ANRIL group and sh-NC group. **(E,F)** SA-β-gal staining showed less senescent cells in the ANRIL group and less senescent cells in the sh-ANRIL group. Scale bar = 100 μm (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

by qRT-PCR (**Supplementary Figure 3B**) ($P < 0.05$). As shown in **Figures 5A,C**, expression of ALP, RUNX2, and OSX was significantly decreased in the mimics group, while increased in the inhibitor group on day 7. qRT-PCR also showed the same trend (**Figures 5B,D**) ($P < 0.05$). ARS, ALP activity, and ALP staining further verified that miR-7-5p inhibitor could promote the formation of mineralized nodules, whereas the miR-7-5p mimics impaired these processes (**Figures 5E–G**). Identically, expression of ALP and RUNX2 were downregulated by miR-7-5p mimics and upregulated by miR-7-5p inhibitor in iPDLSCs as immunofluorescence revealed (**Figures 5H,I**). These results indicated that miR-7-5p inhibited the process of osteogenic differentiation of iPDLSCs.

MiR-7-5p Downregulates Insulin-Like Growth Factor-1 Receptor Expression in Inflamed Periodontal Ligament Stem Cells

Potential downstream target genes (12,264) of miR-7-5p were predicted according to bioinformatic analyses including miRTarBase, miRWalk, miRDB, and TargetScan algorithms

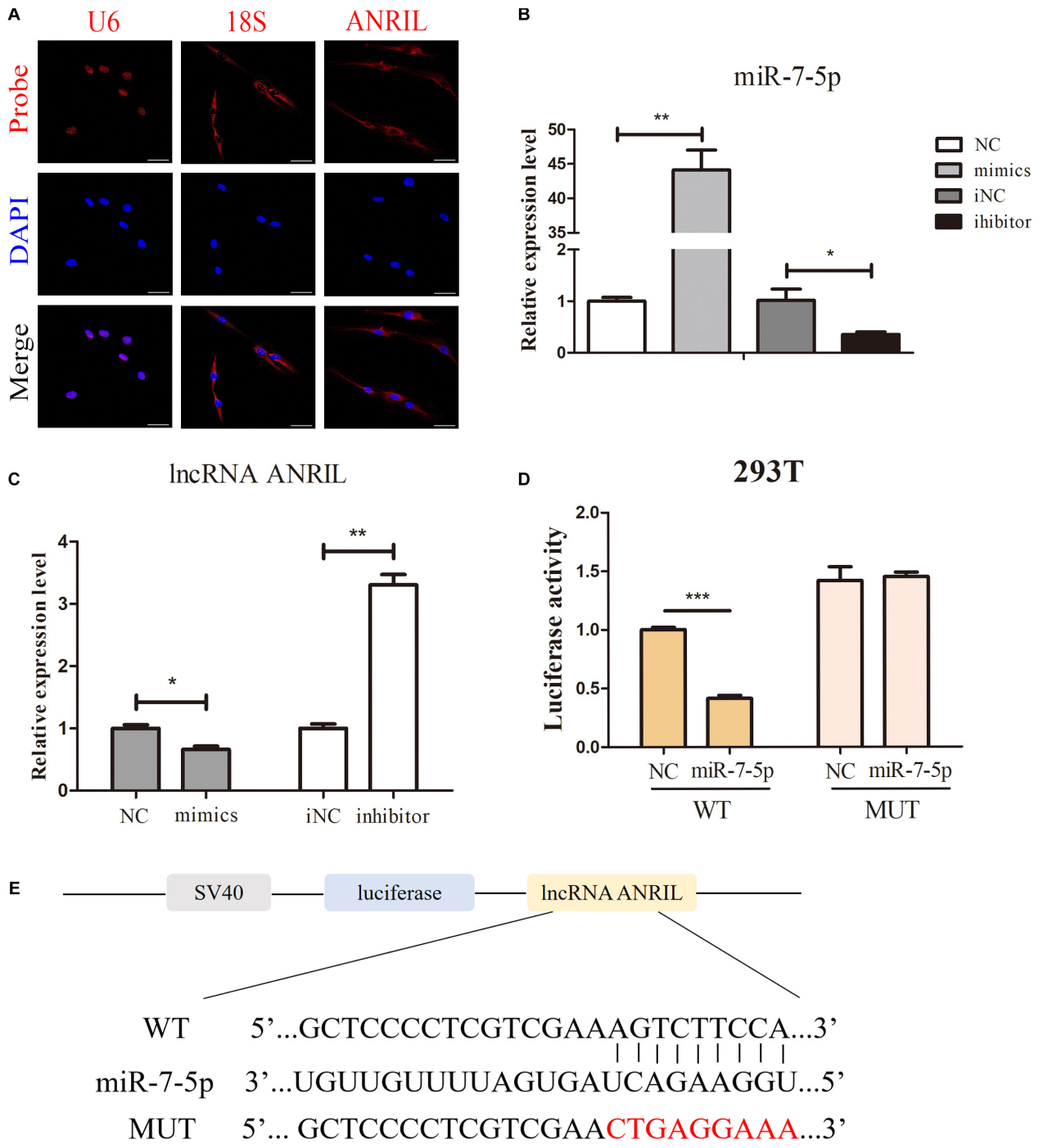

FIGURE 4 | LncRNA ANRIL acts as a sponge of miR-7-5p. **(A)** FISH assay showed the localization of lncRNA ANRIL in the cytoplasm. 18S and U6 were the internal control. **(B)** Transfection efficacy of miR-7-5p mimics and inhibitor was determined by qPCR. **(C)** Relative lncRNA ANRIL expression level was measured by qPCR in iPDLSCs after transfected with miR-7-5p mimics or miR-7-5p inhibitor. **(C)** FISH assay showed the localization of lncRNA ANRIL in the cytoplasm. 18S and U6 were the internal control. **(D,E)** The binding of miR-7-5p to ANRIL was validated by conducting the luciferase reporter assay. IPDLSCs were co-transfected with mimics-NC or miR-7-5p mimics and wild-type lncRNA ANRIL or mutant lncRNA ANRIL. Relative Renilla luciferase activity was normalized to that of firefly luciferase. Scale bar = 100 μm (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

(**Figure 6A**). GO annotation and KEGG pathway analysis suggested that these target genes participate in a variety of biological processes and cellular pathways, such as the PI3K-Akt signaling pathway (**Figures 6B,C**). However, most of these target genes which could play roles in iPDLSCs were not validated before. Among these genes, we found that

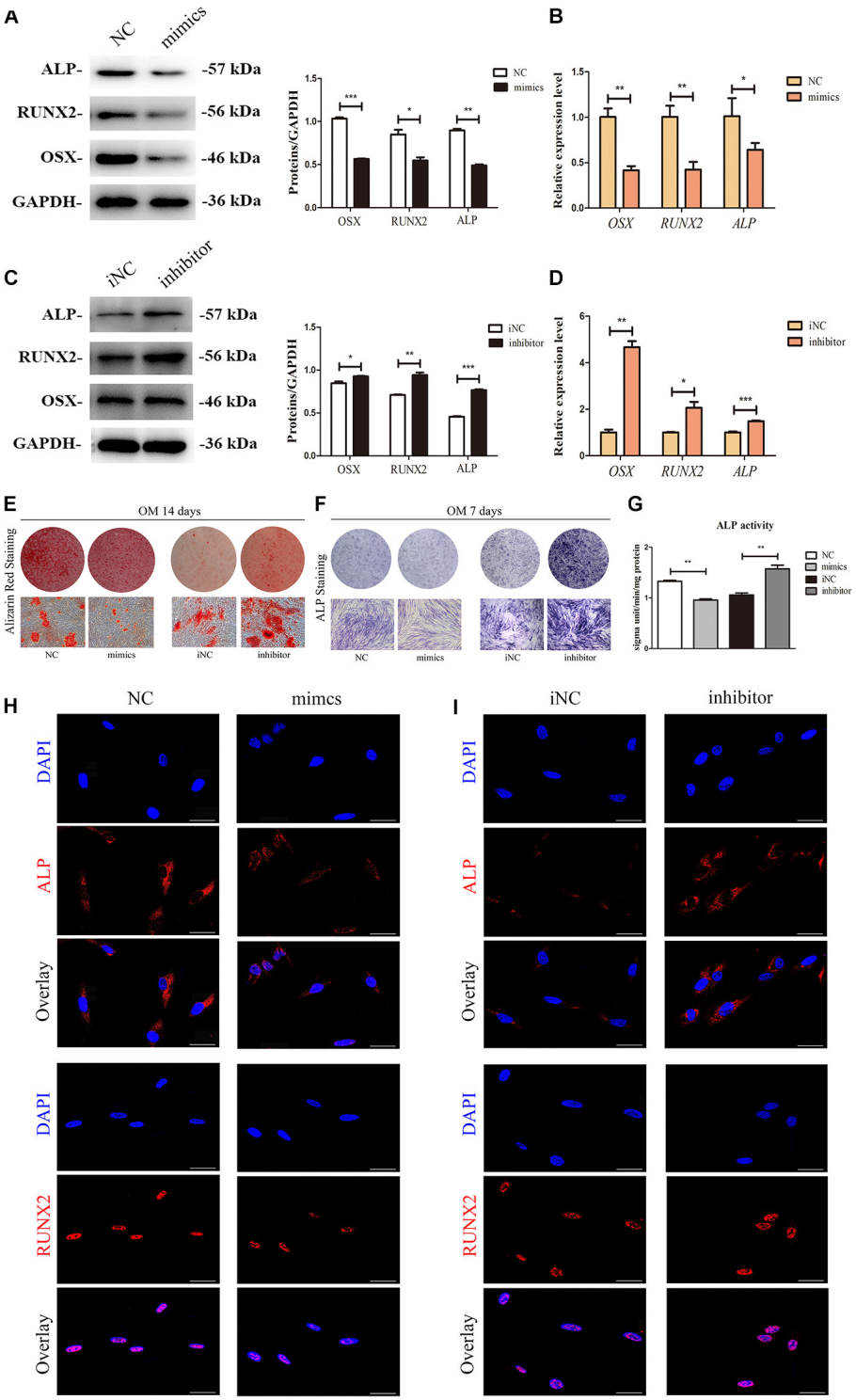

FIGURE 5 | MiR-7-5p participates in osteogenic differentiation of iPDLSCs. **(A)** Western blot assay showed higher protein levels of ALP, RUNX2, and OSX in mimics group than the control group. GAPDH was the internal control. **(B)** qRT-PCR analysis of *RUNX2, OSX,* and *ALP* levels of iPDLSCs in control group and mimics group. **(C)** Western blot assay showed lower protein levels of ALP, RUNX2, and OSX in inhibitor group than the control group. GAPDH was the internal control. **(D)** qRT-PCR analysis of *RUNX2, OSX,* and *ALP* levels of iPDLSCs in control group and inhibitor group. **(E)** After 14 days of culture, Alizarin red staining showed more calcified nodules in the mimics NC group than mimics group and less calcified nodules in the inhibitor NC group than inhibitor group. **(F,G)** The ALP staining assay and ALP activity at Day 7 in the NC group, mimics group, iNC group and inhibitor group. **(H,I)** Immunofluorescence assay revealed downregulated ALP and RUNX2 in mimics group compared with NC group and upregulated ALP and RUNX2 in inhibitor group than iNC group. Scale bar = 100 μm (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

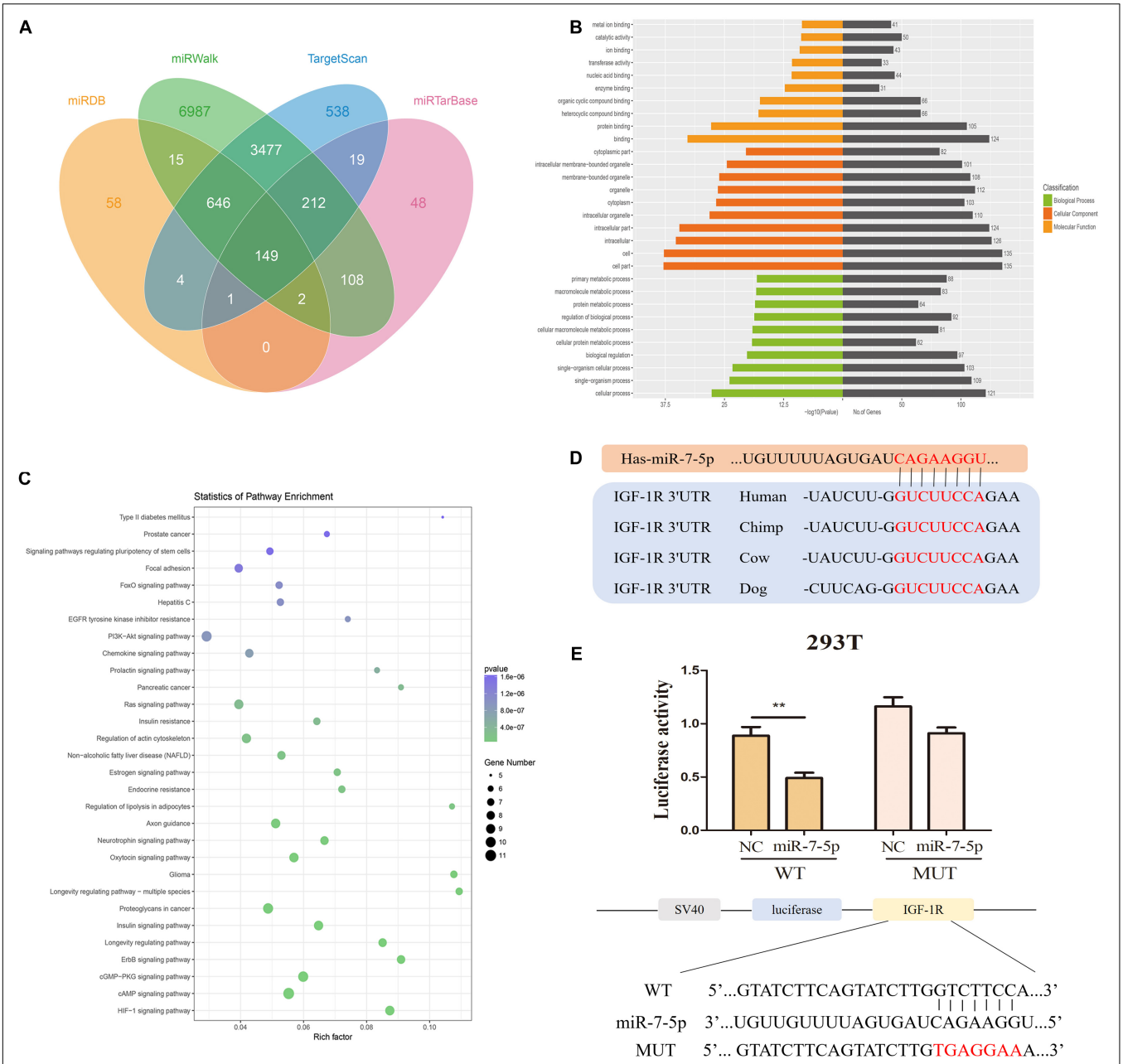

FIGURE 6 | MiR-7-5p directly targets IGF-1R. **(A)** Venn diagram of four datasets (miRDB, miRTarBase, miRWalk, and TargetScan) showed miR-7-5p predicted target genes. **(B)** Gene Ontology (GO) is a commonly used approach for annotating genes and gene products with functions including molecular function, biological process, and cellular component. GO analysis showed the top 10 GO terms of each function which sorted by *P*-value. **(C)** Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis showed that target genes were enriched in regulation of significant signaling pathways. **(D)** The binding sequences between miR-7-5p and IGF-1R were predicted online. The red letters delegate the binding sequence of miR-7-5p. **(E)** Luciferase activity in 293T further illustrated that miR-7-5p could bind with IGF-1R (**$P < 0.01$).

IGF-1R is the mutual target gene of miR-7-5p in miRWalk, TargetScan, and miRTarBase databases. Furthermore, TargetScan 2.0 was performed to obtain the binding sites of miR-7-5p with IGF-1R, and the complementary regions were also highly conserved among different species (**Figure 6D**). Importantly, the positive regulatory effect of IGF-1R on the osteogenesis of tooth-derived stem cells had been documented in our previous studies (Shu et al., 2016; Liu et al., 2018).

Furthermore, dual-luciferase reporter assay showed no significant change in mutant-type IGF-1R group, but it was notably reduced in wild-type group, validating that miR-7-5p could bind with IGF-1R (**Figure 6E**). The above results proved that IGF-1R is a direct target of miR-7-5p.

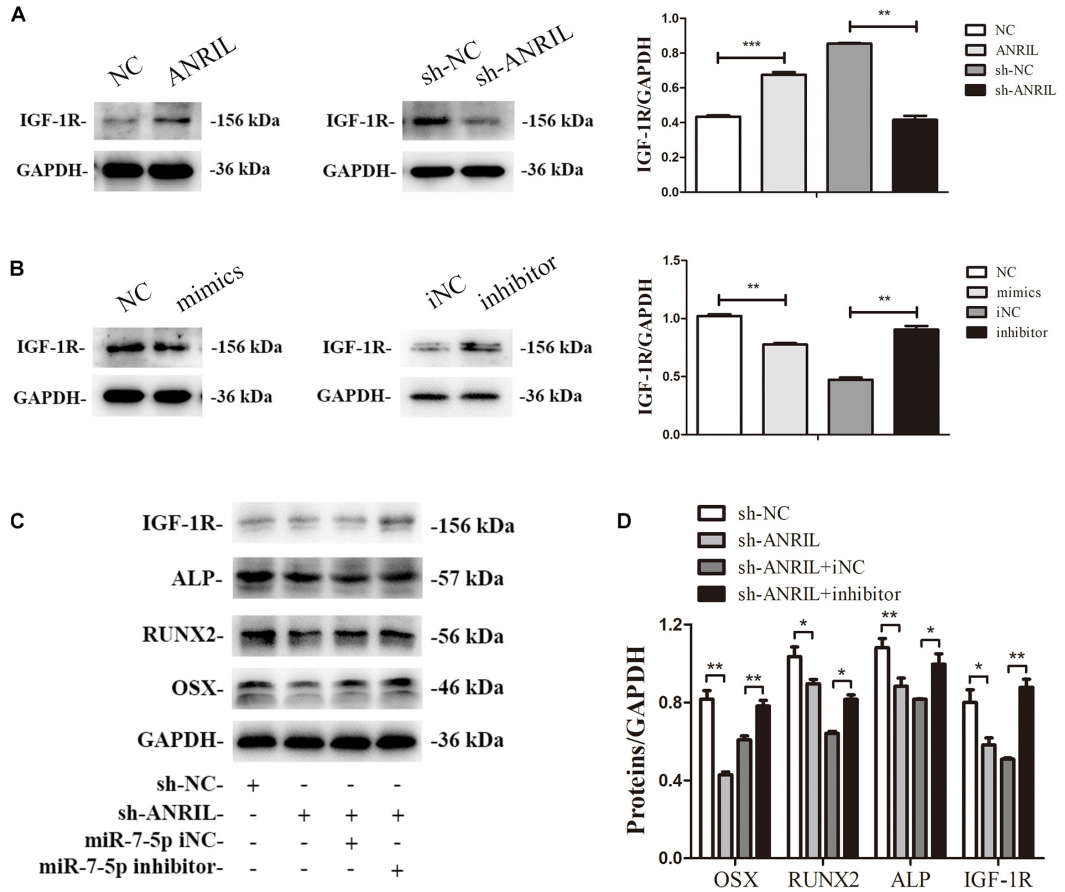

FIGURE 7 | MiR-7-5p inhibitor rescues the role of ANRIL knockdown on committed differentiation of iPDLSCs. **(A)** IGF-1R expression in iPDLSCs transfected with overexpression and low-expression ANRIL. **(B)** IGF-1R expression in iPDLSCs transfected with miR-7-5p mimics, mimics NC, miR-7-5p inhibitor and inhibitor NC. **(C,D)** Expression of IGF-1R/*IGF-1R*, ALP/*ALP*, RUNX2/*RUNX2*, and OSX/*OSX* in iPDLSCs co-transfected with lncRNA ANRIL and miR-7-5p (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

MiR-7-5p Inhibitor Rescues the Role of Long Non-coding RNA Antisense Non-coding RNA INK4 Locus Shortage on Inflamed Periodontal Ligament Stem Cells Osteogenesis

To identify the interaction between miR-7-5p and lncRNA ANRIL and how they influence the IGF-1R in the osteogenic differentiation of iPDLSCs, we performed a rescue experiment in iPDLSCs transfected with sh-ANRIL and miR-7-5p inhibitor. The transfection efficacy was confirmed in **Supplementary Figures 3C,D** ($P < 0.05$). As presented in **Figures 7A,B**, Western blot proved that lncRNA ANRIL increased protein expression of IGF-1R and miR-7-5p suppressed IGF-1R in iPDLSCs. The Western blot showed that the expression of ALP, RUNX2, OSX, and IGF-1R declined in the sh-ANRIL group, while miR-7-5p inhibitor reversed the suppression of ANRIL knockdown on osteogenic differentiation of iPDLSCs (**Figures 7C,D**). In a word, lncRNA ANRIL absorbs miR-7-5p as a ceRNA and stimulates the IGF-1R to promote the committed differentiation of iPDLSCs.

DISCUSSION

Inflammation of the pathological periodontium in the periodontal pocket can change the cell biology of PDLSCs. Low-intensity chronic inflammation breaks the dynamic balance between pro-inflammatory and anti-inflammatory responses in the body. The body is in a state of inflammation for a long time, which accelerates the aging process and reduces the osteogenic differentiation of iPDLSCs. At the same time, aging also aggravates the state of inflammation, affects the resistance of patients to bacteria, and causing more severe and rapid destruction of periodontal tissues (An et al., 2018; Ebersole et al., 2018). Once it is damaged, the periodontium has a limited regeneration capacity, which relies on the availability of stem cells (Liu J. et al., 2019). Studies have shown that there are many other methods to regulate periodontal bone regeneration, such as bone grafts, scaffolds, and growth factors (Mahajan and Kedige, 2015). Our study focuses on the periodontal ligament stem cells in the inflammatory state to enhance osteogenic differentiation.

Recently, many studies have found that lncRNAs are not only associated with the occurrence and development of a variety of

diseases but also with the committed differentiation of MSCs. For example, LncRNA H19 regulates the committed differentiation of SCAPs via miR-141/SPAG9 pathway (Li et al., 2019). Besides, LncRNA TUG1 facilitates osteogenic differentiation of PDLSCs by targeting Lin28A (He et al., 2018). LncRNA ANRIL was initially identified from patients with familial melanoma and encoded 3,834 nt RNA (Zhang J. J. et al., 2018). Jun-Jun et al. (2016). demonstrated that lncRNA ANRIL could inhibit the cell senescence of epithelial ovarian cancer. Moreover, lncRNA ANRIL markedly affects cell senescence of vascular smooth muscle cells directly sequesters miR-181a in the cytoplasm (Tan et al., 2019). However, the regulatory mechanisms of lncRNA ANRIL on osteogenic differentiation and senescence in periodontitis remain unclear.

In this study, we explored the expression of lncRNA ANRIL in iPDLSCs and hPDLSCs, and found that ANRIL was reduced in iPDLSCs. Studies have shown that periodontitis inhibits the formation of osteogenic, so we speculated that whether lncRNA ANRIL is associated with the osteogenesis of iPDLSCs (Peng et al., 2018). Here, we concluded that lncRNA ANRIL had no obvious effect on the proliferative ability of iPDLSCs. However, the influence of lncRNA ANRIL on cell proliferation was different in other experiments. For example, ANRIL could promote cell growth in head and neck squamous cell carcinoma (Matsunaga et al., 2019). Thus, we deduced that the effect of lncRNA ANRIL on cell proliferation might be different in various cell types. Herein, we checked the function of the lncRNA ANRIL in iPDLSCs osteogenic differentiation. The western blot and qRT-PCR showed that RUNX2/*RUNX2*, ALP/*ALP*, and OSX/*OSX* were increased, and aging indicators P53/*P53*, P21/*P21*, P16/*P16* were decreased when lncRNA ANRIL was overexpressed. It was confirmed that lncRNA ANRIL could promote the osteogenic differentiation and delay the senescence of iPDLSCs. Some studies have confirmed that osteogenic differentiation capacity decreases during the process of cell senescence. Therefore, we speculated the regulatory effects of lncRNA ANRIL on osteogenesis might be partly mediated by aging (Fan et al., 2018).

Many lncRNAs exert their miRNA sponge potential in multiple biological processes and compete for binding sites to affect the activity of targeted factors (Chen et al., 2017; Li et al., 2017; Tan et al., 2019). Increasing studies have shown that lncRNA ANRIL could be used as a ceRNA to regulate miRNAs expression and the activity of target genes. For example, lncRNA ANRIL promotes cell growth and represses apoptosis in retinoblastoma cells by targeting miR-99a (Wang X. et al., 2019).

MiR-7-5p has diverse roles in development and disease, and it may be an abnormal expression in different diseases. Emerging studies have confirmed that miR-7-5p acts as a tumor suppressor in various cancers including bladder cancer and pancreatic ductal adenocarcinoma (Li J. et al., 2018; Weihua et al., 2018). Meanwhile, miR-7-5p can play a regulatory role in MSCs osteogenesis (Chen et al., 2020; Tang et al., 2020). Nevertheless, the specific role of miR-7-5p in iPDLSCs has not been clarified yet. A previous study displayed that knockdown of lncRNA ANRIL could increase miR-7-5p expression in H9c2 cells (Shu et al., 2020). Given that, we assumed that lncRNA ANRIL also could function by binding with miR-7-5p in iPDLSCs. As expected, the sequence of lncRNA ANRIL binding with miR-7-5p was confirmed by bioinformatic analyses and dual-luciferase reporter gene assay. In this study, miR-7-5p knockdown silenced by a specific inhibitor could significantly promote osteogenesis, indicating the vital role of miR-7-5p in regulating the bone regeneration of periodontitis. We additionally found that the silence of miR-7-5p can attenuate the effects of ANRIL-knockdown on iPDLSCs, which further confirmed that ANRIL exerted its impacts on bone formation through sponging miR-7-5p. In this study, we indicated that miR-7-5p suppressed the osteogenic differentiation, which was the same as Li et al. confirmed, while Ta was different from cell types.

We studied the possible mechanism of how miR-7-5p regulates the osteogenesis of iPDLSCs. Bioinformatic analyses using miRDB, miRTarBase, miRWalk, and TargetScan algorithms have found that IGF-1R is a target gene of miR-7-5p. IGF-1R is a class of growth factor receptor proteins, which can play an essential role in the growth, proliferation, and differentiation of biological cells by promoting the protein synthesis process (Martin et al., 2019). Moreover, IGF-1R can mediate the biological actions of IGFs, which undergo autophosphorylation and thereby initiates cellular signaling cascades upon ligand binding. On the surface of periodontal ligament-derived fibroblasts, researchers discovered the presence of IGF-1R and an increase in IGF-1R expression during osteogenic differentiation of these cells (Reichenmiller et al., 2004; Gotz et al., 2006). Our previous studies indicated that the IGF-1/IGF-1R/*hsa-let-7c* axis affects the biological properties of dental stem cells by Tang et al. demonstrated that miR-7-5p might promote the osteoblastic differentiation of MSCs (Li X. B. et al., 2018; Tang et al., 2020). MiR-7-5p has an opposite effect on osteogenesis, which may be activating JNK and p38 MAPK pathways (Shu et al., 2016; Liu et al., 2018). Sergi et al. (2019) also found that following the ligand binding, the activated IGF-1 or insulin receptor can lead to the activation of two major signaling pathways, which are the MAPK pathway and the PI3K-PKB/AKT pathway. However, how IGF-1R plays a role in the senescence of iPDLSCs is still a question worthy of further study.

CONCLUSION

To conclude, in this research we found that lncRNA ANRIL promotes bone formation by regulating miR-7-5p/IGF-1R, which further confirms the role of lncRNA ANRIL in bone formation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethics Committee of the

Stomatological School of Nanjing Medical University. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MB, YY, and YL conducted the project design and the experiments, and wrote the manuscript. ZZ, XW, and XY performed the data analysis and reviewed the data. JY conceived and designed the study, and provided financial support and study materials. All authors read and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.604400/full#supplementary-material

Supplementary Figure 1 | Phenotype identification of hPDLSCs. **(A)** The morphology of second-generation hPDLSCs. **(B)** Immunofluorescence assay showed that cultured hPDLSCs were positive for STRO-1. **(C)** The expression of CD29, CD73, CD90, CD105, CD34, and CD45 was detected by flow cytometry analysis. **(D)** Tri−lineage differentiation of hPDLSCs was performed *in vitro*. Scale bar = 100 μm.

Supplementary Figure 2 | Phenotype identification of iPDLSCs. **(A)** The morphology of second-generation iPDLSCs. **(B)** Immunofluorescence assay revealed that cultured iPDLSCs were positive for STRO-1. **(C)** Flow cytometry analysis showed that iPDLSCs were positive for CD29, CD73, CD90, and CD105, and negative for CD34 and CD45. **(D)** Tri−lineage differentiation of IPDLSCs was performed *in vitro*. Scale bar = 100 μm.

Supplementary Figure 3 | (A) The transfection efficacy of ANRIL was measured by qRT-PCR. **(B)** The transfection efficacy of miR-7-5p mimics and inhibitor was determined by qPCR. **(C)** The transfection efficacy of ANRIL was measured by qRT-PCR. **(D)** The transfection efficacy of miR-7-5p mimics and inhibitor was determined by qPCR (*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$).

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Conflict 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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ORIGINAL RESEARCH
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N-Cadherin Regulates the Odontogenic Differentiation of Dental Pulp Stem Cells via β-Catenin Activity

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Dental pulp stem cell (DPSC) transplantation has shown new prospects in dental pulp regeneration, and is of great significance in the treatment of pulpitis and pulp necrosis. The fate and regenerative potential of stem cells are dependent, to a great extent, on their microenvironment, which is composed of various tissue components, cell populations, and soluble factors. N-cadherin-mediated cell–cell interaction has been implicated as an important factor in controlling the cell-fate commitment of mesenchymal stem cells. In this study, the effect of N-cadherin on odontogenic differentiation of DPSCs and the potential underlying mechanisms, both *in vitro* and *in vivo,* was investigated using a cell culture model and a subcutaneous transplantation mouse model. It was found that the expression of N-cadherin was reversely related to the expression of odontogenic markers (dentin sialophosphoprotein, DSPP, and runt-related transcription factor 2, Runx2) during the differentiation process of DPSCs. Specific shRNA-mediated knockdown of N-cadherin expression in DPSCs significantly increased the expression of DSPP and Runx2, alkaline phosphatase (ALP) activity, and the formation of mineralized nodules. Notably, N-cadherin silencing promoted nucleus translocation and accumulation of β-catenin. Inhibition of β-catenin by a specific inhibitor XAV939, reversed the facilitating effects of N-cadherin downregulation on odontogenic differentiation of DPSCs. In addition, knockdown of N-cadherin promoted the formation of odontoblast-like cells and collagenous matrix in β-tricalcium phosphate/DPSCs composites transplanted into mice. In conclusion, N-cadherin acted as a negative regulator via regulating β-catenin activity during odontogenic differentiation of DPSCs. These data may help to guide DPSC behavior by tuning the N-cadherin-mediated cell–cell interactions, with implications for pulp regeneration.

Keywords: dental pulp stem cells, pulp regeneration, odontogenic differentiation, N-cadherin, β-catenin

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INTRODUCTION

Dental caries is one of the most common bacterial infectious diseases worldwide, leading to destruction of the tooth structure and irreversible pulpitis or pulp necrosis. Root canal therapy is the common endodontic treatment for pulp diseases that involves the extirpation of diseased pulp and the subsequent obturation of root canal systems with bioinert synthetic materials,

resulting in a permanently inactivated tooth that is more prone to fractures and re-infection (Gong et al., 2016). In recent years, the advancement of tissue engineering and regenerative medicine have greatly spurred the development of regenerative endodontics, which seeks to replace the inflammatory/necrotic pulp tissue with regenerated pulp-like tissue to reestablish the protective functions, including sensibility, healing, innate and adaptive immunity (Cao et al., 2015). Remarkably, dental pulp stem cell (DPSC)-mediated approaches for regeneration have demonstrated promising results in terms of generating dental pulp-like tissues with morphologic characteristics that resemble those of normal dental pulp *in vivo* and *in situ* (Gronthos et al., 2000, 2002; Huang et al., 2010; Nakashima et al., 2017; Xuan and Li, 2018). Nevertheless, the effectiveness of stem cell-based regeneration is not always realized, because of the dynamic and complex microenvironment in which it is applied. The fates and regenerative potential of stem cells are dependent on their microenvironment, which is composed of various tissue components, cell populations, and soluble factors (Zheng et al., 2019; Sui et al., 2020). Therefore, the improvement of regenerative endodontic procedures requires a better understanding of how the microenvironment controls DPSC behavior.

Cell–cell and cell–extracellular matrix (ECM) interactions mediated by adhesion molecules (cadherins and integrins, respectively) are important mechanisms controlling tissue morphogenesis and cell fate (Marie et al., 2014b). Previously, we found that knockdown of Integrin α5 in DPSCs impairs proliferation and migration, while enhancing their odontogenic differentiation capacity (Cui et al., 2014; Xu et al., 2015). Further, the Integrin α5 priming synthetic cyclic peptide promotes the deposition of ECM, the activity of ECM-receptor, and hence the odontogenic differentiation of DPSCs (Wang et al., 2019).

However, how cadherins regulate odontogenic differentiation of DPSCs remains largely unknown. Cadherins are composed of an extracellular domain that mediates calcium-dependent homophilic interactions between cadherin molecules, a transmembrane domain, and an intracellular domain (Derycke and Bracke, 2004). The intracellular domain interact with cytoskeletal proteins, allowing cell anchorage, and with several signaling molecules, including vinculin, α-catenin, and β-catenin, indicating the involvement of cadherins in modulating cellular signaling processes in addition to cell–cell adhesion (Marie et al., 2014b). In mesenchymal cells, cell–cell interaction is mediated through N-cadherin (Cosgrove et al., 2016). Thus, in this study, the effect of N-cadherins on the odontogenic differentiation of DPSCs, and the potential underlying mechanism, was investigated. Further, the *in vivo* effect was examined using a subcutaneous transplantation mouse model.

MATERIALS AND METHODS

Cell Culture and Treatment

Healthy dental pulp tissues were collected from caries-free teeth of patients (age 18–25 years old) undergoing extraction of fully erupted third molars, according to the informed protocol approved by the Ethics Committee of Nanfang Hospital, Southern Medical University, Guangzhou, China. Primary human DPSCs were isolated, identified and cultured in Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, United States) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Gibco), as described previously (Chen et al., 2020). The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2 and 95% air. Cells cultured for 3–5 passages were used for the following experiments.

To induce odontogenic differentiation, the cells were incubated in odontogenic medium consisting of basal medium, 10 nM dexamethasone (Sigma-Aldrich, St. Louis, MO, United States), 50 μg/mL ascorbic acid 2-phosphate (Sigma-Aldrich), and 10 mM β-glycerophosphate (Sigma-Aldrich) for the indicated times. The medium was changed every 2–3 days. For the β-catenin pathway inhibition experiment, cells were cultured with 2 μM XAV939 (Selleck, Houston, TX, United States). Dimethyl sulfoxide (DMSO) (<0.2%) was used as for the vehicle-only control.

Quantitative Real-Time Polymerase Chain Reaction

Total RNA was extracted from DPSCs using the TRIzol reagent (Invitrogen, Carlsbad, CA, United States) following the manufacturer's instructions. Complementary DNA synthesis was performed using the PrimeScript™ RT Master Mix (TaKaRa, Kyoto, Japan). Quantitative PCR analyses were performed using the LightCycler 480 Real-time PCR System (Roche, Indianapolis, IN, United States) with SYBR® Premix Ex Taq™ (TaKaRa). The $2^{-\Delta\Delta Ct}$ value was used to calculate the relative fold change of gene expression normalized to an internal control (β-actin). The primer sequences used were as follows:

N-cadherin: forward 5′-TCTGGGTCTGTTTTATTACTCCTGG-3′, reverse 5′-GCGAGCTGATGACAAATAGCG-3′;

Runx2: forward 5′-CACTGGCGCTGCAACAAGA-3′, reverse 5′-CATTCCGGAGCTCAGCAGAATAA-3′;

DSPP: forward 5′-TCACAAGGGAGAAGGGAATG-3′, reverse 5′-TGCCATTTGCTGTGATGTTT-3′;

β-actin: forward 5′-CCATCGTCCACCGCAAAT-3′, reverse 5′-CCTGTAACAACGCATCTCATA-3′.

Western Blotting

Total protein was extracted from DPSCs using RIPA buffer in the presence of protease inhibitor and phosphatase inhibitor (Beyotime, Shanghai, China) following the manufacturer's instructions. The Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime) was used in cases where the extraction of separate cytoplasmic and nuclear protein fractions was necessary. The Enhanced BCA Protein Assay Kit (Beyotime) was used to measure the protein concentrations. Proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, transferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, United States), and then incubated with primary antibodies overnight at 4°C, followed by horseradish peroxidase-conjugated secondary antibodies for 1 h at room

temperature. The following primary antibodies were used: anti-N-cadherin (sc-59987, 1:1000; Santa Cruz, Dallas, TX, United States), anti-Runx2 (sc-390715, 1:1000; Santa Cruz), anti-DSPP (sc-73632, 1:1000; Santa Cruz), anti-β-catenin (ab32572, 1:5000; Abcam, Cambridge, United Kingdom), anti-Lamin B1 (ab16048, 1:1000; Abcam), and anti-β-actin (ab8226, 1:5000; Abcam). Proteins were visualized using enhanced chemiluminescence substrate. Protein levels were normalized to the β-actin or Lamin B1 signal.

Lentivirus Transfection

A lentivirus expressing a short hairpin RNA (shRNA) specific to the N-cadherin (oligo sequence: CCGGCCTAAG ATCATTCGCCAAGAACTCGAGTTCTTGGCGAATGATCTT AGGTTTTTG) and a negative control lentivirus (oligo sequence: CCGGTTCTCCGAACGTGTCACGTCTCGAGACGTGACACG TTCGGAGAATTTTTG) were provided by GeneChem (Shanghai, China). DPSCs were seeded into 6-well plates at a density of 6×10^4 cells/well and were infected with viral supernatants (multiplicity of infection = 50) supplemented with polybrene (5 μg/mL) for 10 h, and then incubated under normal growth conditions for a further 72 h. After screening with puromycin (1 μg/mL), DPSCs stably expressing the N-cadherin-specific shRNA were established. The knockdown efficiency was confirmed by quantitative real-time polymerase chain reaction (qPCR) and western blotting.

Alkaline Phosphatase Staining and Activity

After culture in odontogenic medium for 7 days, DPSCs were fixed with 4% paraformaldehyde for 30 min at room temperature and washed with distilled water. Alkaline phosphatase (ALP) staining was performed with the BCIP/NBT ALP Color Development Kit (Beyotime) according to the manufacturer's instructions. The total protein content was determined in the same sample by an ALP activity kit (Jiancheng, Nanjing, China). The absorbance of each well was determined by measurements at 520 nm. ALP activity relative to the control treatment was calculated after normalization to the total protein content.

Alizarin Red S Staining and Quantification

After culture in odontogenic medium for 14 days, DPSCs were fixed with 4% paraformaldehyde for 30 min at room temperature and washed with distilled water. Calcium deposition in the ECM was stained with 2% Alizarin Red S (ARS) (pH 4.2; Sigma-Aldrich) for 15 min. The mineralized nodules were observed and photographed using an inversed microscope (Olympus, Tokyo, Japan). To further quantify the mineralized nodules, the stain was dissolved in 10% cetylpyridinium chloride (Sigma-Aldrich) for 1 h, and the calcium concentration was determined by absorbance measurements at 562 nm.

Immunofluorescence Staining

Dental pulp stem cells cultured on coverslips were fixed with 4% paraformaldehyde at 4°C for 15 min, and then permeabilized with 0.3% Triton X-100 for 5 min. After blocking with 10% normal goat serum for 1 h, the cells were incubated at 4°C overnight with anti-β-catenin (ab32572, 1:250; Abcam) primary antibody. Subsequently, the cells were incubated at 37°C for 1 h in the dark with goat anti-rabbit conjugated with Alexa Fluor 647 (ab150079, 1:1000; Abcam) secondary antibody. 4′,6-diamidino-2-phenylindole (DAPI) (Roche) was applied to visualize the nuclei. Images were captured using a fluorescent microscope (Olympus).

DPSC Subcutaneous Transplantation

Animal experiments were performed in accordance with the regulations of the Ethics Committee of Nanfang Hospital, Southern Medical University, Guangzhou, China. Five male BALB/c immunocompromised nude mice (weight: 15–18 g; age: 6 weeks) were purchased from the Medical Laboratory Animal Center of Guangdong Province (China) and were maintained under pathogen-free conditions in the Experimental Animal Center at Nanfang Hospital, Southern Medical University.

Beta-tricalcium phosphate (β-TCP) blocks were provided by the Biological Materials Manufacturing Core, Sichuan University. DPSCs were transfected with either the control or N-cadherin-specific shRNA lentivirus, and a total of 1×10^6 cells were loaded onto the β-TCP scaffold per block. The composites of β-TCP blocks and cells were transplanted into the left and right dorsal subcutaneous region of mice as previously described (Li et al., 2018). Four weeks after transplantation, the mice were euthanized, and the composites were harvested, fixed in 4% paraformaldehyde at 4°C for 48 h, decalcified in 10% EDTA (pH 7.4) at room temperature for 10 days, and embedded in paraffin.

Hematoxylin and Eosin Staining and Masson's Trichrome Staining

The embedded samples were serially sectioned at 4-μm thickness. The sections were deparaffinized in xylene, rehydrated through a series of graded ethanol solutions, and stained with hematoxylin and eosin (H&E) or Masson's trichrome (Solarbio, Beijing, China) according to the manufacturer's instructions.

Immunohistochemistry Staining

The sections were dehydrated, subjected to antigen retrieval, and incubated with primary antibodies at 4°C overnight, followed by a horseradish peroxidase-conjugated secondary antibody for 40 min at room temperature. The immunostained proteins were visualized by the application of diaminobenzidine solution. The following primary antibodies were used: anti-DSPP (sc-73632, 1:500; Santa Cruz), anti-nestin (ab105389, 1:100; Abcam), and anti-β-catenin (ab32572, 1:500; Abcam). The sections were lightly counterstained with hematoxylin, mounted, and analyzed under a light microscope (Olympus) by a technician who was blinded to the samples. The number of positive cells exhibiting brown staining were counted at 400× the original magnification in five randomly picked fields per slide, and the mean value of these fields was calculated for each group.

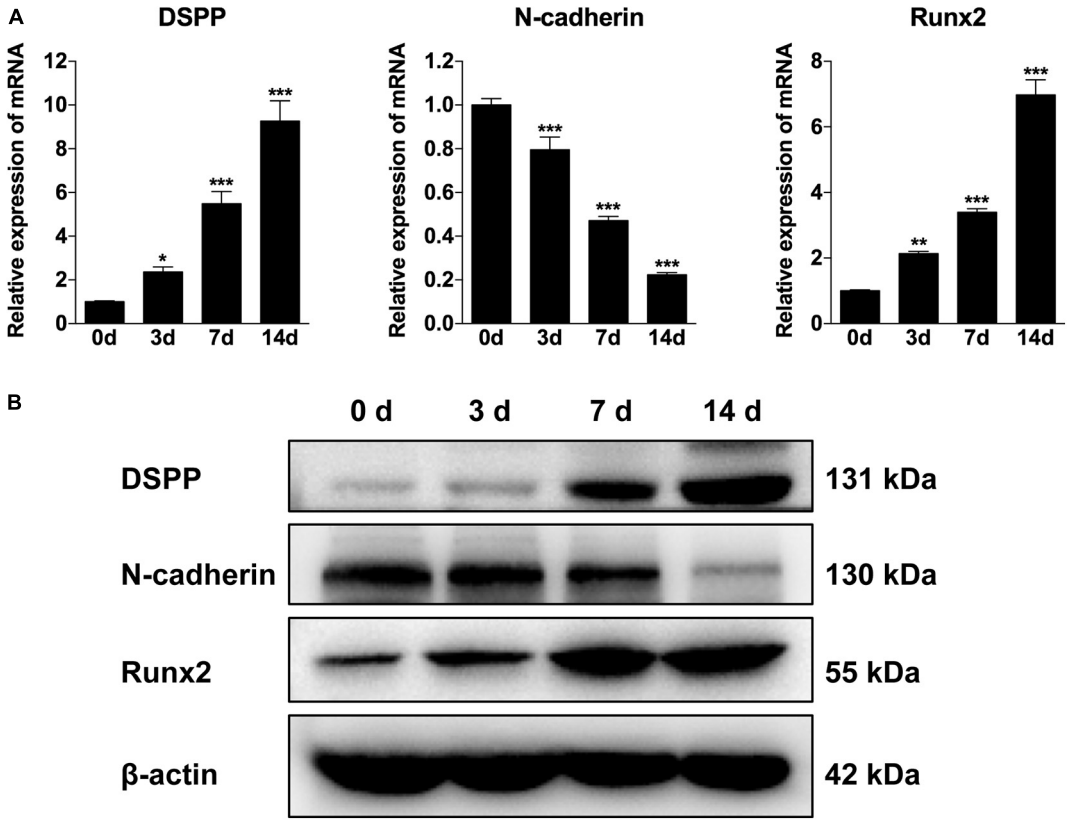

FIGURE 1 | The expression of N-cadherin was decreased during odontogenic differentiation of dental pulp stem cells (DPSCs). DPSCs were cultured in odontogenic medium for 3, 7, and 14 days, and then the expression pattern of N-cadherin and odontogenic markers (dentin sialophosphoprotein, DSPP, and runt-related transcription factor 2, Runx2) were examined by qPCR **(A)** and western blot **(B)** analysis. Data are presented as the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Statistical Analyses

All experiments were repeated at least thrice. Data are presented as the means ± SEM unless otherwise stated and were analyzed using SPSS software (version 21.0; IBM, Chicago, IL, United States). Data normality and homogeneity of variances were confirmed with Shapiro–Wilk test and *F*-test, respectively. Samples with normal distribution were analyzed with the independent sample *t*-test for two groups or one-way analysis of variance (ANOVA) followed by Bonferroni post-test for multiple groups. Samples without normal distribution were analyzed using the non-parametric Mann–Whitney *U* test for two groups or Kruskal–Wallis test for multiple groups. The statistical significance level was set at $P < 0.05$.

RESULTS

The Expression of N-Cadherin Was Decreased During Odontogenic Differentiation of DPSCs

To explore the involvement of N-cadherin in the odontogenic differentiation of DPSCs, DPSCs were cultured in odontogenic medium for 3, 7, and 14 days, and then the expression pattern of N-cadherin and odontogenic markers (DSPP and Runx2) were examined by qPCR and western blot analysis. The expression of DSPP and Runx2 gradually increased over time as expected, whereas the expression of N-cadherin gradually decreased both at the mRNA and protein level (**Figures 1A,B**). These results indicate that a functional decrease in N-cadherin was required during odontogenic differentiation of DPSCs.

N-Cadherin Knockdown Promoted Odontogenic Differentiation of DPSCs

Given that there is a negative correlation between N-cadherin and the odontogenic markers, it was investigated whether inhibition of N-cadherin could promote DPSC differentiation by knocking-down N-cadherin via lentiviral transfection (**Figure 2A**). qPCR and western blot analysis showed that N-cadherin was significantly downregulated both at the mRNA and protein level after N-cadherin shRNA lentivirus transfection in DPSCs (**Figures 2B,C**).

Next, lentivirus-transfected DPSCs were subjected to odontogenic differentiation. Following induction for 7 and 14 days, ALP staining, alizarin red staining, qPCR, and western blotting were performed to identify the differentiation of DPSCs. Compared with the control shRNA lentivirus-transduced

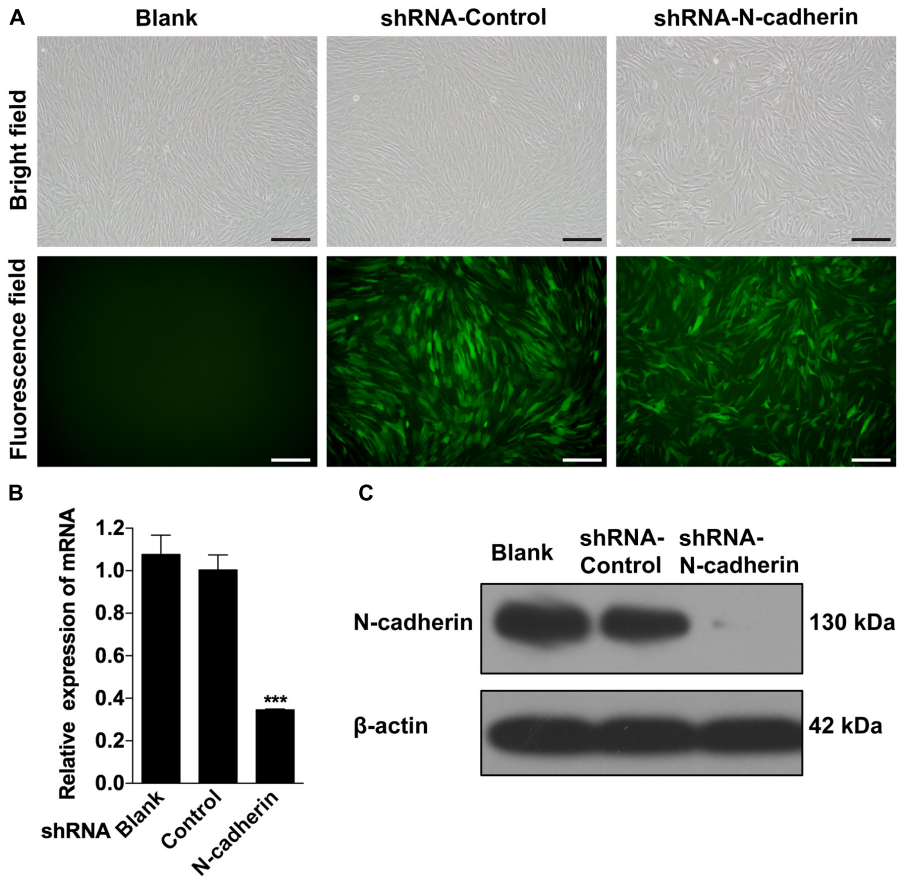

FIGURE 2 | Stable downregulation of N-cadherin in dental pulp stem cells (DPSCs). DPSCs were transfected with either the N-cadherin shRNA or control shRNA lentivirus. The transfection efficiency was evaluated using fluorescence microscopy **(A)**. The expression level of N-cadherin mRNA and protein were detected by qPCR and western blotting, respectively **(B,C)**. Scale bars = 200 μm. Data are presented as the mean ± SEM of three independent experiments. ***$P < 0.001$.

cells, the expression of odontogenic-related genes, DSPP and Runx2, were significantly upregulated in the N-cadherin shRNA lentivirus-transduced cells (**Figures 3A,B**). Meanwhile, ALP activity and the presence of mineralized nodules were increased in N-cadherin shRNA lentivirus-transduced cells (**Figures 3C,D**). These results suggest that N-cadherin acted as a negative regulator during odontogenic differentiation of DPSCs.

N-Cadherin Downregulation Enhanced the Odontogenic Differentiation of DPSCs via Increasing β-Catenin Activity

β-catenin is involved in both cadherin-mediated cell adhesion and the canonical Wnt/β-catenin pathway. To determine nucleus translocation and accumulation of β-catenin, a marker for β-catenin signaling activation was detected by western blotting and immunofluorescent staining. As shown in **Figure 4A**, shRNA-mediated knockdown of N-cadherin increased the expression of β-catenin in the nucleus and cytoplasm, as well as the expression of total β-catenin. In line with this, silencing of N-cadherin promoted the translocation of β-catenin to the nucleus (**Figure 4B**). These results indicate that the inhibition of N-cadherin increased β-catenin activity during the odontogenic differentiation of DPSCs.

Further, lentivirus-transfected DPSCs were treated with XAV939, a specific inhibitor of β-catenin. As expected, XAV939 decreased the expression of nuclear, cytoplasmic, and total β-catenin, as well as odontogenic-related genes (DSPP and Runx2) (**Figure 5A**). Consistently, ALP activity was also decreased by XAV939 (**Figure 5B**). These results suggest that inhibition of β-catenin by XAV939 could reverse the effects of N-cadherin downregulation on DPSC odontogenic differentiation.

N-Cadherin Downregulation Enhanced β-Catenin Activity and Odontogenic Differentiation *in vivo*

To confirm the impact of N-cadherin on odontogenic differentiation of DPSCs *in vivo*, we performed subcutaneous transplantation with β-TCP/DPSCs composites in BALB/c immunocompromised nude mice for 4 weeks (**Figure 6A**). H&E staining showed a pulp-like structure, comprised of a thick layer of cells lining along the surfaces of β-TCP scaffold's macropores and an interstitial tissue infiltrated with blood

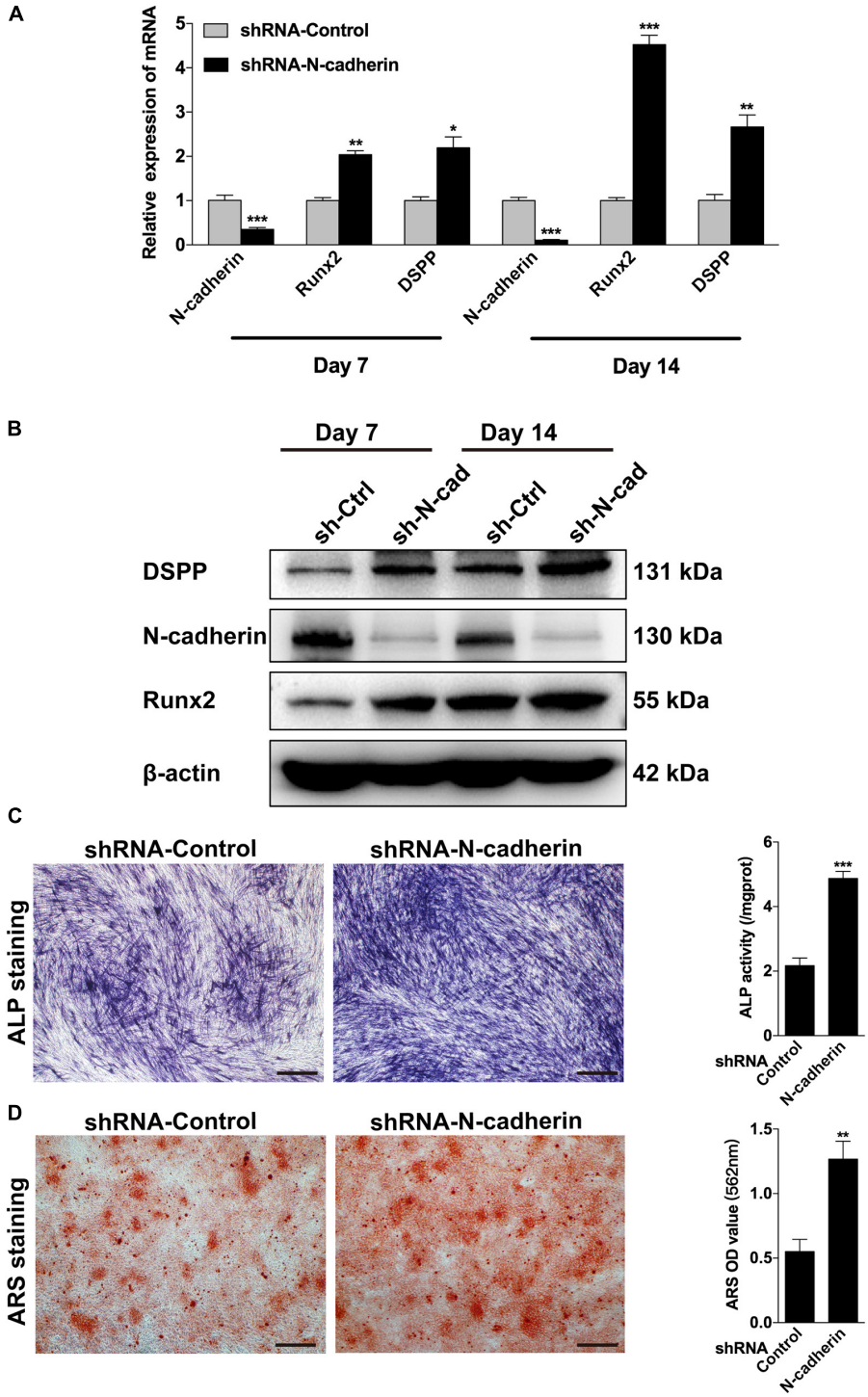

FIGURE 3 | N-cadherin knockdown promoted the odontogenic differentiation of dental pulp stem cells (DPSCs). DPSCs transfected with a control or N-cadherin-specific shRNA lentivirus were subjected to odontogenic induction for 7 or 14 days. The expression level of odontogenic-related genes (DSPP and Runx2) were detected by qPCR and western blotting on day 7 and 14 **(A,B)**. The alkaline phosphatase (ALP) activity was measured by ALP staining on day 7 **(C)**. The formation of mineralized nodules was detected by alizarin red staining (ARS) on day 14 **(D)**. Scale bars = 500 μm. Data are presented as the mean ± SEM of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

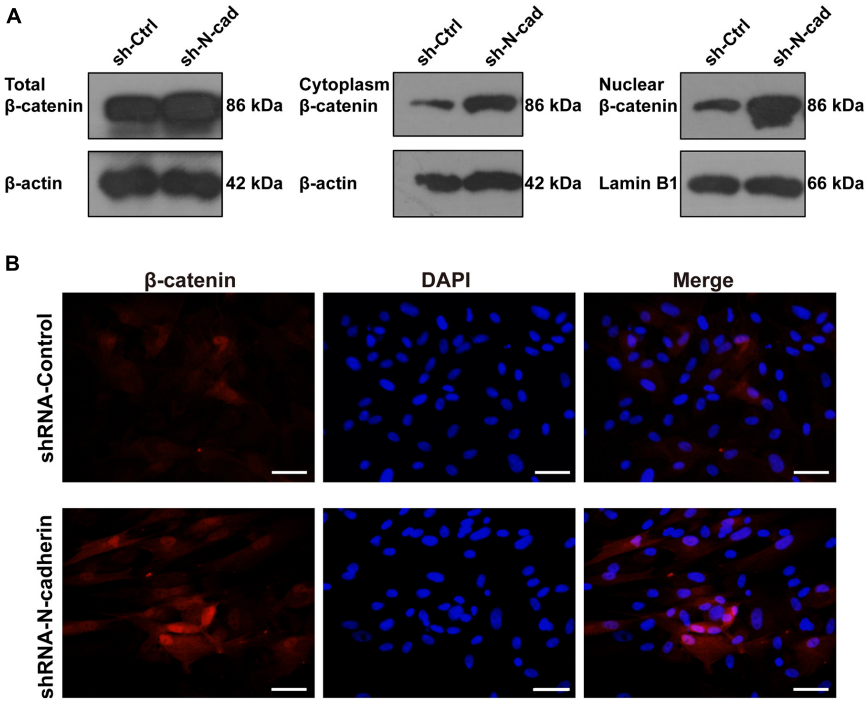

FIGURE 4 | N-cadherin downregulation enhanced β-catenin activity in dental pulp stem cells (DPSCs). DPSCs transfected with a control or N-cadherin-specific shRNA lentivirus were subjected to odontogenic induction for 14 days. The expression level of nuclear, cytoplasmic, and total β-catenin was detected by western blotting **(A)**. The nuclear translocation of β-catenin was detected by immunofluorescent staining. Nuclei were counterstained with DAPI **(B)**. Scale bars = 50 μm.

vessels in the shRNA-N-cadherin group. By contrast, the shRNA-control group showed a disordered structure with cells evenly distributed throughout the whole tissue (**Figure 6B**). Masson's trichrome staining revealed an increase of collagenous matrix within the scaffold's macropores of the shRNA-N-cadherin group (**Figure 6C**). The layer of cells formed in the shRNA-N-cadherin group were further identified to be odontoblast-like cells with positive DSPP and nestin staining (**Figures 6D,E**). Similarly, immunohistochemical analysis revealed the presence of β-catenin localized in the nuclei of the cells (**Figure 6F**). The number of cells that were positive for DSPP, nestin or β-catenin were significantly higher in the shRNA-N-cadherin group than in the shRNA-control group (**Figures 6G–I**). These results suggest that knockdown of N-cadherin promoted the formation of odontoblast-like cells and collagenous matrix in β-TCP/DPSCs composites transplanted into mice.

DISCUSSION

The fundamental goal of endodontic treatment is to retain the natural dentition. Regeneration of a functional pulp-dentin complex is promising for the overall prognosis of the tooth (He et al., 2017). To regenerate the pulp-dentin complex, an appropriate microenvironment is of great importance for stimulating the differentiation of DPSCs. The current study aimed to clarify the involvement of N-cadherin, mediator of cell–cell adhesion, in the odontogenic differentiation of DPSCs. We found that a functional decrease of N-cadherin level was required during the odontogenic differentiation of DPSCs. Artificial downregulation of N-cadherin expression in DPSCs significantly upregulated β-catenin signaling, and consequently enhanced the odontogenic differentiation *in vitro*, which was abrogated by pretreatment of the cells with XAV939, a specific inhibitor of β-catenin. In addition, knockdown of N-cadherin promoted the formation of odontoblast-like cells and collagenous matrix in β-TCP/DPSC composites transplanted into mice.

Mounting evidence shows that the mechanical and biochemical signals from cell–cell adhesion are key to stem cell fate decision (Alimperti and Andreadis, 2015). It was reported that during differentiation of odontoblastic MDPC-23 cells, E-cadherin expression gradually increased over time, whereas the expression of N-cadherin gradually decreased (Lee et al., 2014). Similarly, N-cadherin was expressed in undifferentiated dental bud stem cells, and its expression remained constant at the early stage, but decreased at the late stage of osteogenic differentiation (Di Benedetto et al., 2015). Overexpression of N-cadherin in bone marrow-derived mesenchymal stem cells inhibited osteogenesis and ectopic bone formation, while silencing N-cadherin could promote osteogenesis *in vitro* (Xu et al., 2013). In accordance with these findings, this study determined that the expression of N-cadherin was decreased over time and was reversely related to the expression of odontogenic markers during the differentiation process. Furthermore, knockdown of N-cadherin promoted the odontogenic differentiation of DPSCs *in vitro* and *in vivo*. Collectively, a functional decrease of

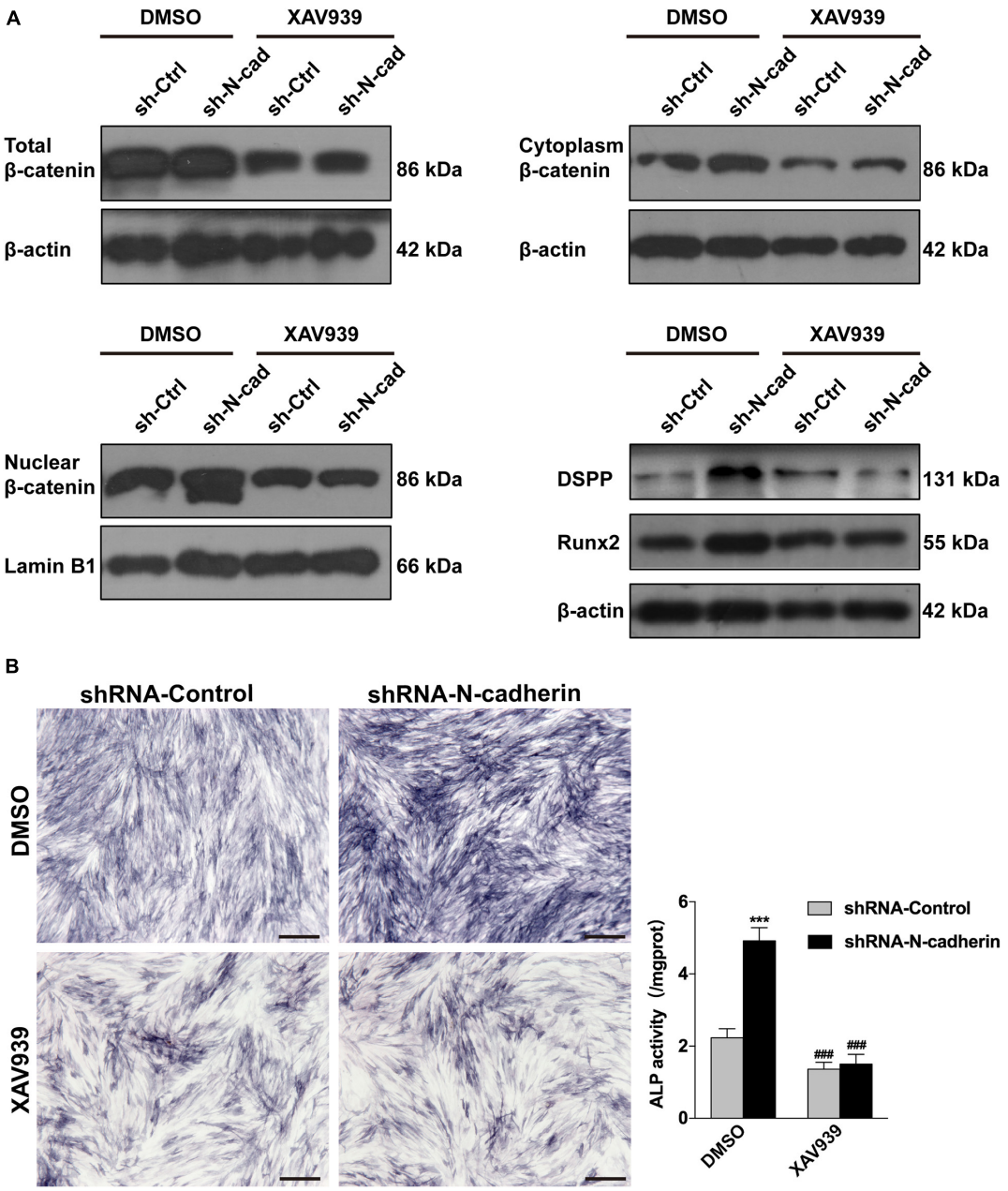

FIGURE 5 | Inhibition of β-catenin by XAV939 reversed the effects of N-cadherin downregulation on dental pulp stem cells (DPSCs) odontogenic differentiation. DPSCs transfected with a control or N-cadherin-specific shRNA lentivirus were subjected to odontogenic induction in the presence or absence of XAV939 (a β-catenin inhibitor). DMSO was used as the vehicle-only control. The expression of nuclear, cytoplasmic, and total β-catenin, and the odontogenic-related genes (DSPP and Runx2), were detected by western blotting on day 14 **(A)**. The alkaline phosphatase (ALP) activity was measured by ALP staining on day 7 **(B)**. Scale bars = 500 μm. Data are presented as the mean ± SEM of three independent experiments. $^{***}P < 0.001$ compared with the shRNA-Control group, $^{###}P < 0.001$ compared with the DMSO group.

N-cadherin expression appears to be a universal phenomenon during odontogenic/osteogenic differentiation. This may be explained by lessons learned from tooth development, since there are parallels between pulp development and regeneration (Schmalz and Smith, 2014). During early steps of odontogenesis, N-cadherin initiates dental mesenchymal condensation, which is a fundamental mechanism involved in morphogenesis by facilitating cell–cell interactions (Xiao and Tsutsui, 2012; Giffin et al., 2019). However, as mesenchymal development progresses, the ECM gradually deposits and the microenvironment changes from one that rich in cell–cell interactions to one that dominated by cell–ECM interactions (Cosgrove et al., 2016). In this situation, N-cadherin-mediated cell–cell interactions are increasingly restrained and then become less predominant

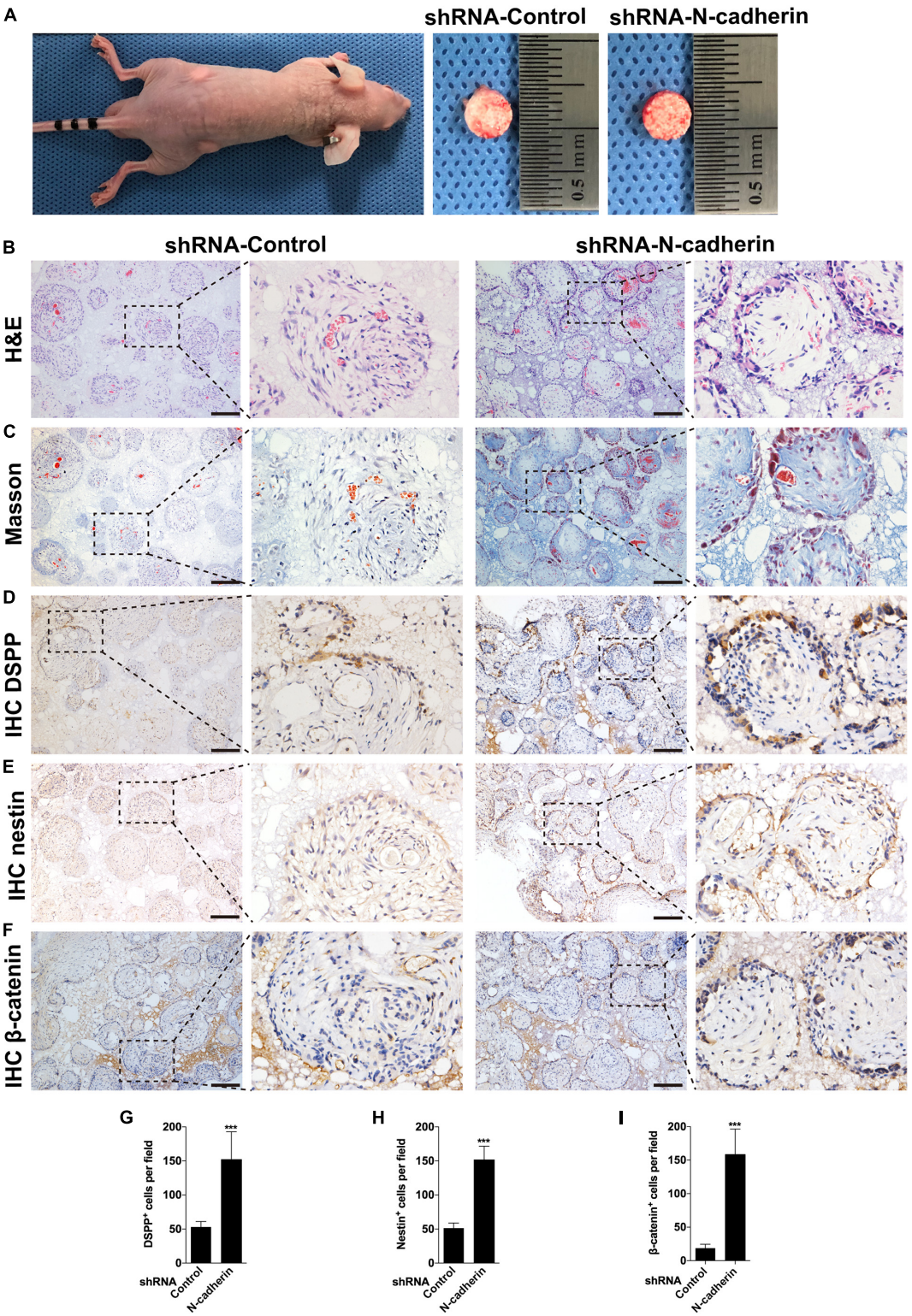

FIGURE 6 | N-cadherin downregulation enhanced β-catenin activity and odontogenic differentiation *in vivo*. Dental pulp stem cells transfected with a control or N-cadherin-specific shRNA lentivirus were loaded onto the β-tricalcium phosphate scaffold, and transplanted subcutaneously into immunocompromised nude mice for 4 weeks **(A)**. The samples were subjected to hematoxylin and eosin (H&E) staining **(B)**, Masson's trichrome staining **(C)**, DSPP, nestin and β-catenin immunohistochemistry (IHC; **D–F**, respectively). The numbers of DSPP, nestin and β-catenin positive cells were quantified (**G–I**, respectively). Scale bars = 200 μm. Data are presented as the mean ± SD (n = 5 per group). ***$P < 0.001$.

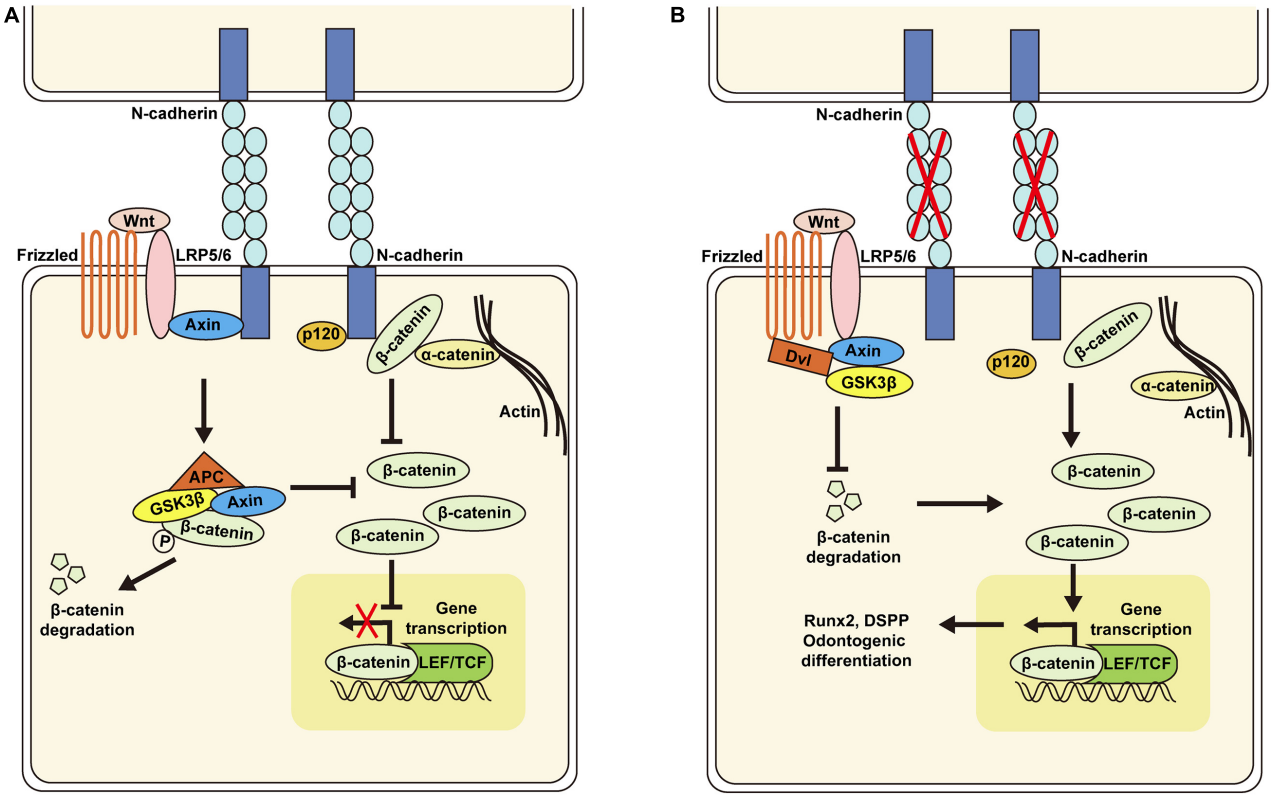

FIGURE 7 | Schematic of the proposed model for N-cadherin/β-catenin signaling in the regulation of odontogenic differentiation of dental pulp stem cells (DPSCs). N-cadherin can sequester β-catenin at the cell–cell adhesion site and associate with the Wnt co-receptor LRP5/6 and Axin to inhibit β-catenin signaling **(A)**. N-cadherin downregulation can promote β-catenin release at the cell membrane and decrease interaction with LRP5/6, resulting in β-catenin signaling activation, Runx2 and DSPP upregulation, and odontogenic differentiation of DPSCs **(B)**.

owing to the dense surrounding matrix. Hence, the fine-tuning of cell–cell and cell–matrix interactions in different phases of odontogenesis is crucial for allowing differentiation to proceed. In addition, integrins and cadherins are linked to each other through the intracellular actin–myosin network. And such linkage regulates the intracellular forces, which is a central mechanism underlying cell fate determination (Mui et al., 2016).

It is well-established that the role of N-cadherin involves both cell–cell interactions and interference with intracellular signaling, particularly the Wnt/β-catenin pathway. N-cadherin binds to β-catenin and modulates its cytoplasmic pools and transcriptional activity (Marie et al., 2014a). In addition, N-cadherin also interferes with low-density lipoprotein receptor-related protein 5 and 6 (LRP5/6) signaling by sequestering these receptors in inactive pools via Axin binding (Haÿ et al., 2009; Revollo et al., 2019). In this study, we found that knockdown of N-cadherin promoted the β-catenin nucleus translocation and accumulation, while inhibition of β-catenin by XAV939 impeded the odontogenic differentiation of DPSCs. This could be further supported by previous research reporting that the expression of β-catenin was significantly upregulated during odontogenic differentiation of DPSCs, and knockdown of β-catenin disrupted odontogenic differentiation, which could be reversed by the lithium chloride-induced accumulation of β-catenin (Han et al., 2014). Besides, mounting evidence shows that numerous regulators, including but not limited to enhancer of zeste homolog 2, Stathmin, vacuolar protein sorting 4B, R-Spondin 2, and lncRNA *DANCR*, regulate the odontogenic differentiation of DPSCs through the Wnt/β-catenin pathway (Chen et al., 2016; Li et al., 2018; Pan et al., 2019; Zhang et al., 2019; Gong et al., 2020).

Moreover, we found that knockdown of N-cadherin increased the expression of odontogenic markers Runx2 and DSPP. Studies have illustrated that β-catenin binds to the Runx2 promoter and controls its transcription (Gaur et al., 2005), and odontogenic differentiation increases such binding (Han et al., 2014). Runx2 upregulates DSPP expression through binding to the 5′-TACCTCA (−3950 to −3944 bp) and 5′-ACCACA (−3106 to −3101 bp) specific sites in the *DSPP* promoter (Guo et al., 2019). Hence, we propose that N-cadherin silencing induced β-catenin nucleus translocation and accumulation, which then promoted β-catenin binding to the Runx2 promoter and the expression of Runx2 and DSPP, thus stimulating odontogenic differentiation of DPSCs. However, it has been reported that Runx2 needs to be down-regulated to acquire full odontoblast differentiation for dentinogenesis (Miyazaki et al., 2008). The stage-specific roles of Runx2 in odontoblast differentiation

and the underlying mechanism warrants further exploration in future studies.

Pulp regeneration, in its most strict sense, must include functional re-innervation of the pulp–dentin complex (Diogenes, 2020). Interestingly, N-cadherin has been implicated in regulating the differentiation of neural progenitor cells during development (Miyamoto et al., 2015), as well as the neurogenic differentiation of menstrual blood-derived endometrial stem cells (Liu et al., 2018). Since DPSCs originate from neural crest cells and are able to differentiate into neural-like cells (Gronthos et al., 2002; Rafiee et al., 2020), how N-cadherin regulates the neurogenic differentiation of DPSCs warrants further investigation.

CONCLUSION

The results presented here demonstrate that knockdown of N-cadherin enhances β-catenin activity possibly through release of β-catenin at the cell membrane and decreased interaction with the Wnt coreceptor LRP5/6, resulting in Runx2 and DSPP upregulation, and odontogenic differentiation of DPSCs (**Figure 7**). These data may help to guide DPSC behavior by tuning the N-cadherin-mediated cell–cell interactions, with implications for pulp regeneration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Nanfang Hospital, Southern Medical University, Guangzhou, China. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Ethics Committee of Nanfang Hospital, Southern Medical University, Guangzhou, China.

AUTHOR CONTRIBUTIONS

ZD, WY performed the experiments, analyzed the data, and wrote the manuscript. XD, MC, and QQ participated in the experiments and revised the manuscript. WZ and BW designed the study and revised the manuscript. All authors have read and approved the final version of the manuscript.

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Fibrocartilage Stem Cells in the Temporomandibular Joint: Insights From Animal and Human Studies

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Temporomandibular disorders (TMD) are diseases involving the temporomandibular joint (TMJ), masticatory muscles, and osseous components. TMD has a high prevalence, with an estimated 4.8% of the U.S. population experiencing signs and symptoms, and represents a financial burden to both individuals and society. During TMD progression, the most frequently affected site is the condylar cartilage. Comprising both fibrous and cartilaginous tissues, condylar cartilage has restricted cell numbers but lacks a vascular supply and has limited regenerative properties. In 2016, a novel stem cell niche containing a reservoir of fibrocartilage stem cells (FCSCs) was discovered in the condylar cartilage of rats. Subsequently, FCSCs were identified in mouse, rabbit, and human condylar cartilage. Unlike mesenchymal stem cells or other tissue-specific stem/progenitor cells, FCSCs play a unique role in the development and regeneration of fibrocartilage. More importantly, engraftment treatment of FCSCs has been successfully applied in animal models of TMD. In this context, FCSCs play a major role in the regeneration of newly formed cartilage. Furthermore, FCSCs participate in the regeneration of intramembranous bone by interacting with endothelial cells in bone defects. This evidence highlights the potential of FCSCs as an ideal stem cell source for the regeneration of oral maxillofacial tissue. This review is intended to detail the current knowledge of the characteristics and function of FCSCs in the TMJ, as well as the potential therapeutic applications of FCSCs. A deep understanding of the properties of FCSCs can thus inform the development of promising, biologically based strategies for TMD in the future.

Keywords: temporomandibular disorders, osteoarthritis, regeneration, condylar cartilage, mesenchymal stem cells

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INTRODUCTION

The temporomandibular joint (TMJ) is a unique articulation between the mandible and the temporal bone that consists of the temporal bone fossa, mandibular condyle, and articular disc (Ottria et al., 2018). The articular disc lies bilaterally between the glenoid fossa and condyle, separating the TMJ into upper and lower joint cavities. Characterized as a distinct hinge structure,

the TMJ exhibits a complex range of movements, including sliding and rotation (Singh and Detamore, 2009; Bordoni and Varacallo, 2020). Among them, the lower joint compartment plays an essential role in rotational movement. The lubrication of synovial fluid and the glazed surface of condylar cartilage ensure smooth rotation and minimal abrasion of the TMJ (Vazquez et al., 2019). However, this condition is disrupted in the development of temporomandibular disorders (TMD) (Gauer and Semidey, 2015). The etiology of TMD is complex and multifactorial, including biological, environmental, emotional, and social triggers (Gauer and Semidey, 2015). However, the etiology of TMD progression is not fully delineated, and the primary pathology involves degeneration of the TMJ, known as osteoarthritis (OA) (Scrivani et al., 2008). The degenerative condition in the lower compartment of the TMJ directly affects the biomechanical properties of the cartilage and bone (Scrivani et al., 2008). Therefore, mandibular condylar cartilage is one of the most frequently affected sites (Iwasaki et al., 2017; Nickel et al., 2018). The current treatment strategies include non-surgical and surgical methods, which mainly relieve pain and improve the range of motion (Dimitroulis, 2018). These traditional therapies fail to recover the integrated structure of the TMJ. More importantly, due to the deficiency in nerves, blood vessels, and lymphatic cycling and the effect of persistent weight-bearing, there is a paucity of options to restore impaired condylar cartilage (Gauer and Semidey, 2015; Stoustrup and Twilt, 2015). Furthermore, unlike the hyaline cartilage covering the joint head in other synovial articulations, mandibular condylar cartilage is composed of fibrocartilage containing both fibrous and cartilaginous tissues, making regeneration more challenging (Huey et al., 2012). With advances in regenerative medicine, stem cell-based therapies have attracted much attention as an alternative way to repair diseased tissue in TMD (Cui et al., 2017; Jiang et al., 2020). Considering immune rejection, pathogen transmission, potential tumorigenesis, and host tissue engraftment, resident stem cells have profound advantages compared to exogenic stem cells (Huey et al., 2012; Centeno, 2014; Waskow, 2015). In this context, scientists have recently discovered a novel stem cell niche in the superficial zone of condylar cartilage, termed fibrocartilage stem cells (FCSCs) (Embree et al., 2016; Bi et al., 2020). FCSCs conform to the criteria of mesenchymal stem cells (MSCs) and have potential in cartilage and bone regeneration. This review outlines recent discoveries related to FCSCs, with a particular focus on their distinct characteristics and regulatory networks among species. An in-depth and comprehensive understanding of the properties of FCSCs can thus inform the development of biologically based strategies for TMD and other maxillofacial defects.

ISOLATION OF FCSCS FROM ANIMALS AND HUMAN

Of mesodermal origin, cartilage is a special connective tissue found in various sites throughout the body. Based on its composition and function, it comprises three types: hyaline cartilage, fibrocartilage, and elastic cartilage (Benjamin and Evans, 1990). Fibrocartilage contains a large number of collagen fibers and shows both the elasticity of cartilage tissue and the flexibility and toughness of fibrous tissue (Benjamin and Ralphs, 2004). Fibrocartilage has been discovered in the tendon, pubic symphysis, intervertebral discs, menisci, and TMJ (Benjamin and Ralphs, 2004). Histologically, TMJ fibrocartilage is divided into four layers: a fibrous superficial zone (SZ), a polymorphic zone, a zone of chondrocytes, and a zone of hypertrophic chondrocytes (Shibukawa et al., 2007) (**Figure 1**). A stem cell population, FCSCs, has been recently discovered in the SZ. FCSCs are mesenchymal-derived cells originating from condylar primordium blastema. The niche of FCSCs probably forms during the late period of the embryonic stage, participating in condyle development (Ruscitto et al., 2020). It is speculated that FCSCs exist over the span of a lifetime to maintain the homeostasis of mandibular condylar cartilage (Liang et al., 2016). To date, FCSCs in rats, mice, rabbits, and *Homo sapiens* have been identified by mesenchymal cell markers and location (Embree et al., 2016; Nathan et al., 2018; Bi et al., 2020; Ruscitto et al., 2020; **Table 1**).

Fibrocartilage stem cells were first isolated by Embree et al. (2016) from the rat TMJ. In this study, condyles were dissected from Sprague–Dawley rats at 6–8 weeks of age, followed by a 15-min digestion process containing 4 mg/ml dispase II at 37°C to separate the SZ. Then, the SZ was continuously digested in 4 mg/ml dispase II and 3 mg/ml collagenase I to harvest all nucleated cells. Cellular suspensions were cultured in basal medium containing 55 mM 2-mercaptoethanol. Rat FCSCs were confirmed by surface markers of MSCs. They were positive for CD90, CD44, CD29, CD105, and CD146 but negative for CD45, CD79a, and CD11b (Embree et al., 2016).

Subsequently, mouse FCSCs in the TMJ condyle were identified by using the stem cell label α-SMA (Embree et al., 2016). α-SMA is commonly known as a myofibroblast marker and has been implicated in organ fibrosis (Bhowmick et al., 2004; Darby and Hewitson, 2007). Lee et al. proved that human MSCs have a stepwise process of fibroblast differentiation *in vitro*. By analyzing α-SMA expression, they found that human MSCs express α-SMA under connective tissue growth factor (CTGF) and transforming growth factor (TGF)-β1 stimulation. In this study, cells expressing α-SMA were deemed to present a myofibroblast phenotype (Lee et al., 2010). In addition, α-SMA expressed in hypertrophic chondrocytes was one of the indicators of fibrosis of condylar cartilage in TMJOA progression (Wang et al., 2012). Furthermore, Zhang et al. (2019) found that the percentage of α-SMA$^+$ cells was positively correlated with Mankin scores of OA. These studies focused on the expression of α-SMA in the layer of chondrocytes and hypertrophic chondrocytes. The role of α-SMA as a typical skeletal stem/progenitor cell marker has been recently discovered. α-SMA was originally found in smooth muscle cells and vascular pericytes as a cell marker. As the osteogenic potential of pericytes was proven (Doherty et al., 1998), scientists assumed that pericytes have similar characteristics to MSCs. After that, Shi and Gronthos (2003) labeled α-SMA in MSCs derived from bone marrow (BMMSCs) and dental pulp stem cells (DPSCs) and found

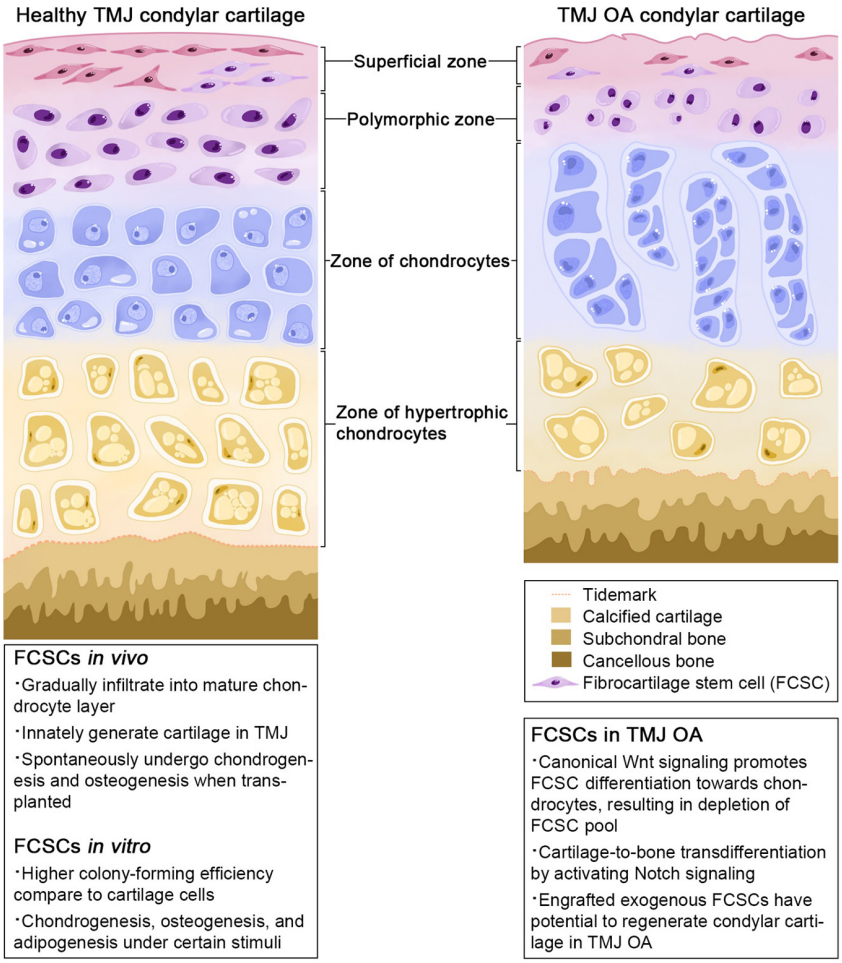

FIGURE 1 | Zonal structure of TMJ condylar cartilage.

that α-SMA$^+$ cells have characteristics similar to those of smooth muscle cells and pericytes. Furthermore, Grcevic et al. (2012) identified α-SMA$^+$ cells as typical skeletal progenitor cells responsible for new bone formation and fracture healing. Hence, Embree et al. performed a lineage-tracing experiment using α-SMACreERT2/Ai9 transgenic mice as an indicator of progenitor cells in condylar cartilage. α-SMACreERT2/Ai9 mice were injected with tamoxifen at postnatal day 16 (P16) and sacrificed after 2 and 15 days. The results suggested that α-SMA$^+$ cells were concentrated in the SZ 2 days after tamoxifen administration and increased in condylar cartilage after 15 days. Moreover, it has been verified that the extracellular matrix of FCSCs in the SZ is different from that of mature chondrocytes in condylar cartilage. FCSCs do not express aggrecan and collagen II (Col II), proteins secreted from mature chondrocytes, but are surrounded by lubricin and collagen I (Col I) (Embree et al., 2016). Therefore, the SZ may provide a niche for undifferentiated cells, and these α-SMA$^+$ cells in the SZ are able to differentiate into chondrocytes. In addition, Ruscitto et al. found that Notch signaling in FCSCs participated in TMJ morphogenesis, indicating that Notch-Venus reporter mice are an alternative choice to label FCSCs in mouse condylar cartilage during embryonic development (Nathan et al., 2018). However, mouse FCSCs have not been successfully isolated and cultured *in vitro* due to their small number in the mouse TMJ. Therefore, further research is required to optimize the isolation method to harvest mouse FCSCs.

More recently, we cultivated human FCSCs from condylar pieces harvested from patients with condylar comminuted fractures (Bi et al., 2020). Owing to the large volume of human tissue, the superficial zone of the condylar cartilage was cut into 1 mm × 1 mm pieces before digestion. The following enzymatic digestion method was similar to that used for rat FCSC isolation. Then, the surface markers of the cultured cells were identified by flow cytometry. The results showed that human FCSCs were positive for CD44, CD73, CD90, and CD105 but negative for CD29, CD34, and CD45, consistent with the cellular surface markers of rodent FCSCs (Embree et al., 2016; Bi et al., 2020). According to the report from Dominici et al. (2006), MCSs express CD73, CD90, and CD105 but are negative for CD34, CD45, CD14, CD11b, CD19, and CD79α. Both human FCSCs and rat FCSCs expressed classical Dominici MSC markers, such

TABLE 1 | Characteristics and regulatory networks of FCSCs in animals and humans.

Species	Markers	Characteristics	Regulatory networks	Author and year
Rat	Positive: CD90, CD44, CD29, CD105, CD146 Negative: CD45, CD79a, CD11b	Reside in the SZ; Chondrogenic, osteogenic, and adipogenic differentiation capacity *in vitro*; High colony formation ability compared to that of cartilage cells; Spontaneous osteogenesis and chondrogenesis when transplanted *in vivo*	Wnt: deplete FCSCs pool and enhance chondrogenesis Notch: promote differentiation of FCSCs into chondrocytes and osteoblasts SOX9: induce chondrogenesis of FCSCs	Embree et al., 2016 Nathan et al., 2018 Ruscitto et al., 2020 Bi et al., 2020
Mouse	α-SMA+ cells in the SZ Notch+ cells during embryonic development	Reside in the SZ and gradually infiltrate into the mature chondrocyte layer	Notch: participate in TMJ morphogenesis and cartilage-to-bone transdifferentiation in TMJ OA	Embree et al., 2016 Ruscitto et al., 2020
Human	Positive: CD44, CD73, CD90, CD105 Negative: CD29, CD34, CD45	Spindle-shaped cells; Comparable propagation ability, apoptosis rate, and colony forming efficiency to hOMSCs; Weak migration capability; Chondrogenesis under chondrogenic induction *in vivo*	SOX9: induce chondrogenesis of FCSCs	Bi et al., 2020
Rabbit	N/A	N/A	SOST: maintain the FCSC pool	Embree et al., 2016

FCSCs, fibrocartilage stem cells; SZ, superficial zone; hOMSCs, human orofacial bone marrow-derived mesenchymal stem cells; SOX9, Sry-related HMG box-9; TMJ, temporomandibular joint; OA, osteoarthritis; SOST, sclerostin.

as CD44, CD90, and CD105. Comparatively, human FCSCs were negative for CD34 and CD45 (Bi et al., 2020), while rat FCSCs lacked expression of CD45, CD11b, and CD79α (Embree et al., 2016). Notably, CD29 was found to be positive in rat FCSCs but negative in humans. Moreover, human FCSCs are positive for CD73, but this was not verified in rat FCSCs. Whether there are other diverse surface markers among species remains to be determined. Further analysis, such as using single-cell technology, may unravel the specific markers of FCSCs and help to dissect the desired cell population and generate novel mouse models for directly targeting FCSCs.

CHARACTERISTICS OF FCSCS UNDER PHYSIOLOGICAL CONDITIONS

Proliferation

A colony-forming assay was performed to evaluate the proliferation rates of rat FCSCs *in vitro*, showing that rat FCSCs formed sixfold more colonies than donor-matched cartilage cells (Embree et al., 2010, 2016). Canonical Wnt signaling was closely tied to the proliferative activity of rat FCSCs. By adding sclerostin (SOST), an inhibitor of Wnt signaling, the proliferation rates of FCSCs were significantly reduced (Embree et al., 2016). In *H. sapiens*, FCSCs show self-renewal ability and are able to maintain their spindle-shaped morphometry, proliferation, apoptosis, and senescence abilities after expansion. They have comparable propagation ability, apoptosis rates, and colony-forming efficiency to orofacial bone marrow-derived mesenchymal stem cells (OMSCs) (Bi et al., 2020).

Differentiation

Fibrocartilage stem cells possess many *in vitro* features of MSCs, including clonogenicity and multipotential differentiation capacity. Under stimulation, FCSCs can differentiate into osteogenic, chondrogenic, and adipogenic cells. When rat FCSCs were cultured in chemically defined media, over 87% of individual colonies exhibited heterogeneous differentiation potential (22.5% trilineage, 64.5% bilineage) (Embree et al., 2016). Human FCSCs, comparing to human OMSCs, had a comparable adipogenic potential but a reduced osteogenesis potential during multi-lineage differentiation *in vitro*. Increasing evidence has revealed that multiple signaling pathways control FCSC differentiation *in vitro*, such as the canonical Wnt and Notch signaling pathways. After transfection of β-catenin, FCSCs exhibited decreased expression of cartilage-related transcription factors, such as *sox5, sox6, and sox9* (Embree et al., 2016). Another study found that SOX9 was more highly expressed in human FCSCs than in cartilage cells (Embree et al., 2010, 2016) and other mesenchymal stem cells (Bi et al., 2020; Jiang et al., 2020). When SOX9 expression was interfered with, FCSCs were unable to form well-organized cartilaginous tissue under chondrogenic induction (Bi et al., 2020). Furthermore, Ruscitto et al. (2020) revealed that Notch signaling plays a key role in promoting FCSC differentiation into chondrocytes and osteoblasts but not adipogenic cells *in vitro*.

Fibrocartilage stem cells also present multipotential capacity *in vivo*. FCSCs traced by the skeletal stem/progenitor cell marker α-SMA in adult mice showed that the α-SMA$^+$ cells in the SZ gradually infiltrated into the mature chondrocyte layer and expressed the chondrocyte marker Col II. This result indicated that FCSCs have the potential to differentiate toward chondrocytes to maintain the homeostasis of condylar cartilage under physiological conditions (Embree et al., 2016). Wnt signaling is also involved in the chondrogenesis of FCSCs. The downstream Wnt mediator β-catenin is expressed in mature chondrocytes but not in the SZ, implicating that Wnt activity is restrained in the SZ. Notably, SOST suppressed FCSC proliferation as previously noted, yet SOST knockout mice showed depletion of the FCSC pool (Embree et al., 2016). It has been speculated that in SOST knockout mice, Wnt signaling was enhanced significantly in the SZ and thus induced the differentiation of FCSCs toward chondrocytes, emphasizing the function of Wnt signaling in directing FCSC fate.

Moreover, recent research has confirmed the strong osteogenic and chondrogenic capability of exogenic FCSCs. Subcutaneously

transplanted rat FCSCs with collagen sponges could form cartilaginous-like tissue, which then gradually transformed into transitional tissue (bone, cartilage, and osteoclast-mediated tissue) and resulted in well-organized trabecular bone-like tissue (Embree et al., 2016). Collectively, these results indicate that FCSCs are able to differentiate into multiple cell lineages and spontaneously recapitulate endochondral ossification when transplanted *in vivo* (Yang et al., 2014). Human FCSCs have different fates than rat FCSCs in xenograft models. Human FCSCs need chondrogenic induction before transplantation; otherwise, they are not able to undergo a similar process to rat FCSCs (Bi et al., 2020).

The regulation of stem cell populations is tightly controlled by the local microenvironment according to the requirements of the host tissue (Fuchs and Segre, 2000; Bianco and Robey, 2001). When injected into mandibular condylar cartilage defects, FCSCs spontaneously formed cartilage, and no bone-forming process was observed during follow-up (Bi et al., 2020). FCSCs undergo a chondrogenic differentiation fate in the microenvironment of the lower joint compartment. However, ectopic xenografts of rat FCSCs begin to form bone-like tissue after 4 weeks of observation, indicating that FCSCs have the tendency to undergo hypertrophy to form bone in a subcutaneous environment (Embree et al., 2016). These distinct differentiation patterns highlight the importance of the microenvironment in FCSC fate decisions. To date, the detailed modulatory mechanisms by which the microenvironment affects FCSC fate have not been fully characterized, but this could be a target for researchers in future studies of FCSCs.

Migration

The analysis of FSCS migration is somewhat limited. By performing the scratch wound healing assay, we found that human FCSCs presented a weaker migration capability than OMSCs (Bi et al., 2020). The migratory ability of FCSCs *in vivo* and whether these endogenous stem cells can be recruited to defect sites remain to be determined.

Trophic and Immunomodulatory Functions

Increasingly, the mechanisms underlying the therapeutic effects of MSCs are attributed to the secretion of trophic factors, particularly extracellular vesicles (EVs) (Meirelles Lda et al., 2009). EVs are cell-derived membrane-bound nanoparticles that play an important role in the maintenance of biophysiological homeostasis as well as cellular, physiological, and pathological processes (Yáñez-Mó et al., 2015). EVs have significant diagnostic and therapeutic potential. MSCs, as prolific producers of EVs, have recently attracted much attention (Baglio et al., 2012; Liang et al., 2014). Of note, exosomes, one type of EV with a 40–100 nm diameter (Raposo and Stoorvogel, 2013), secreted by MSCs are found to have a great effect on the treatment of OA (Zhu et al., 2017). In TMJOA treatment, Zhang et al. (2016, 2019) administered exosomes isolated from human embryonic stem cell-derived MSCs to treat TMJOA and found that they could promote TMJ repair. However, the trophic function of FCSCs is still not well characterized. FCSCs have the potential to secrete trophic factors, particularly exosomes, which are crucial for therapeutic function. Moreover, a previous report compared exosomes secreted by synovial membrane MSCs and induced pluripotent stem cell-derived MSCs in the treatment of OA in the knee joint. Both exosomes could attenuate OA, but the latter had a better therapeutic effect (Zhu et al., 2017). Because exosomes secreted by different types of MSCs show distinct regenerative capacities, it is crucial to investigate the trophic function of FCSCs as well as their cell-specific properties of trophic factors in TMJOA treatment.

Moreover, increasing evidence indicates that MSCs play an immunomodulatory role primarily through the release of EVs and paracrine factors (Spees et al., 2016; Li and Hua, 2017). Previous reports have found that MSC-derived exosomes and microparticles play an anti-inflammatory role independently to modulate T and B lymphocytes in inflammatory arthritis (Cosenza et al., 2018). Whether FCSCs have immunomodulatory capacity during condylar cartilage regeneration under pathological conditions remains to be determined. Scholars have found that FCSCs can secrete VEGF-A in a paracrine manner *in vitro* (Nathan et al., 2018). This may help to explain how FCSCs organize the hematopoietic microenvironment *in vivo* (Embree et al., 2016), highlighting the possibility of interactions of FCSCs and surrounding cells in a paracrine manner. Further study of the trophic and immunomodulatory functions of FCSCs is needed.

Differences Between FCSCs and BMMSCs

Compared with BMMSCs, FCSCs express similar cell surface markers, including CD90, CD44, CD29, CD105, and CD146, but lack leukocyte markers, such as CD45, CD79a, and CD11b (Soleimani and Nadri, 2009; Robey et al., 2021). As noted above, they show heterogeneous differentiation potential similar to that of BMMSCs *in vitro* (Embree et al., 2016). It is important to note that FCSCs show distinct progress of osteogenesis when transplanted onto the dorsum of athymic nude mice. FCSCs formed cartilaginous-like tissue first and then transformed into bone-like tissue, while BMMSCs directly formed bony tissue without cartilaginous tissue transition (Embree et al., 2016). Notably, chondrogenically precultured BMMSCs could form unstable cartilage with hypertrophy, vascular invasion, and terminal matrix calcification (Pelttari et al., 2006). In general, compared to BMMSCs, FCSCs have innate chondrogenic capacity in the context of transplantation.

Comparison Among FCSCs and Other Fibrocartilage Tissue-Derived Stem Cells

While FCSCs are stem cells in the fibrocartilage of the TMJ, there are various stem cells that can be isolated from fibrocartilage in other organs, including meniscus-derived mesenchymal stem cells (MMSCs), annulus fibrosus-derived stem cells (AFSCs), and tendon-derived stem cells (TDSCs). MMSCs are isolated from avascular zone of meniscus, which express MSCs surface markers, such as CD44 and CD90 (Gui et al., 2015; Huang et al., 2016).

When compared to BMMSCs, MMSCs showed a stronger chondrogenesis *in vitro* and a better repair of damaged meniscus *in vivo* (Ding and Huang, 2015). Similar to FCSCs, MMSCs preferentially differentiate into chondrocytes (Huang et al., 2016). Annulus fibrosus is a fibrocartilaginous tissue in intervertebral disc (Liu et al., 2014). AFSCs express common MSCs surface markers, including CD29, CD44, and CD166 (Liu et al., 2014; Guo et al., 2018). They could form a hierarchical structure approximating native AF tissue (Chu et al., 2018; Zhou et al., 2021). TDSCs express a similar surface marker with MSCs, including CD44 and CD90 (Bi et al., 2007; Liu et al., 2018). Unlike FCSCs and MMSCs, TDSCs preferentially differentiated into tenocyte-like cells but not chondrocytes (Guo et al., 2016), emphasizing the potential of TDSCs in repairing bone-tendon junction, a fibrocartilaginous structure in tendon (Benjamin and Ralphs, 1998; Qin et al., 2020). These data suggest that stem cells originated from certain fibrocartilage tissue may have their unique differentiation signature, possibly reflecting their site of origin.

Interactions Between FCSCs and Human Umbilical Vein Endothelial Cells

Angiogenesis is a tightly regulated process involved in the growth and repair of bone tissue. Several studies have verified that human umbilical vein endothelial cells (HUVECs) can indirectly regulate BMMSCs *via* angiocrine factors (Villars et al., 2000; Zhu et al., 2020). However, Nathan et al. proved that the secreted factors of HUVECs were not sufficient to stimulate FCSCs *in vitro*. Only when in direct contact with HUVECs were the osteogenic transcription factors of FCSCs markedly upregulated (Nathan et al., 2018). Furthermore, FCSCs in turn support angiogenesis. Vascular endothelial growth factor A (VEGF-A), a cytokine promoting HUVEC proliferation, was highly expressed in FCSCs when cultured *in vitro*. The number of HUVECs significantly increased when cultured in FCSC-conditioned medium (Nathan et al., 2018). However, some studies reported different results. The fibrinogen gel bead angiogenesis assay (FIBA) suggested that direct interactions between FCSCs and HUVECs impeded angiogenesis (Nathan et al., 2018). Therefore, more research is needed to explore whether other FCSC-derived factors affect HUVECs in addition to their paracrine function through VEGF-A.

THERAPEUTIC APPLICATION

Treatment of Temporomandibular Joint Osteoarthritis

Temporomandibular joint OA is one of the most severe subtypes of TMD due to degeneration of various hard and soft tissues, including cartilage degeneration, viscous synovial fluid accumulation, and osteophyte formation (Rando and Waldron, 2012; Bechtold et al., 2016; Ibi, 2019). Existing treatments for TMJ OA mainly focus on pain relief and functional rehabilitation. There is difficulty in recovering the physiological morphology and function of condylar cartilage. Therefore, clinical therapy is urgently needed to restore the TMJ structure and regenerate defects. Residing in the SZ of cartilage, FCSCs harbor multilineage differentiation potential and participate in cartilage formation, implying their potential in repairing defects in TMJ OA. It was discovered that the application of an exogenous Wnt inhibitor could repair and regenerate injured fibrocartilage by maintaining the FCSC pool and regulating FCSC differentiation. Embree et al. arranged SOST injection into a rabbit TMJ OA model and found that the condyles had mild surface irregularities after SOST administration. The contralateral PBS-treated condyles displayed severe surface irregularities and had significantly higher Osteoarthritis Research Society International (OARSI) recommended macroscopic scores. Moreover, SOST treatment led to a significantly greater number of cells surviving in the SZ, indicating that Wnt inhibitors could protect FCSCs from depletion and improve the morphology of condylar cartilage in the progression of TMJ OA (Embree et al., 2016).

Accelerated cartilage-to-bone transformation is one of the main causes of condylar bone reconstruction in TMJ OA (Liu et al., 2015). Ruscitto et al. discovered that Col II/Runx2 double-positive cells located at the cartilage/bone interphase did not express Notch1 in the normal mandibular condyle. However, after local delivery of TNF-α to induce TMJ OA, Col II/Runx2^{+} cells appeared in the SZ and were positive for Notch1, implying that Notch1 mediated FCSCs cartilage-to-bone transformation in the setting of TMJ OA (Ruscitto et al., 2020). Therefore, Notch inhibitors offer promising therapeutic potential in the treatment of TMJ OA by maintaining the morphology of the condyle. The Notch inhibitors γ-secretase inhibitor IX and N-[N-(3,5-difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT) significantly reduced the expression of *Notch 1, Runx2,* and *Ocn* in FCSCs and suppressed osteogenesis of FCSCs *in vitro* (Ruscitto et al., 2020). Although the effect of Notch inhibitors on FCSCs has not been evaluated *in vivo*, increasing evidence suggests the potential role of Notch inhibitors in the treatment of TMJ OA by targeting FCSCs (Luo et al., 2018).

More recently, we transplanted exogenous FCSCs into a TMJ defect rat model to assess their function in cartilage repair (Bi et al., 2020). After 4 weeks, engrafted FCSC lineages could be observed in the SZ, polymorphic zone, and zone of chondrocytes. Under gross observation, the condylar surface of the defect sites was smoother in the FCSC-treated group than in the vehicle-treated group. The International Cartilage Regeneration and Joint Preservation Society (ICRS) score and modified Mankin score were utilized to evaluate the effectiveness of FCSC treatment, showing that FCSC treatment improved the arrangement of cartilage structures. These results indicate that FCSCs are an optimal stem cell source facilitating TMJ cartilage repair *in vivo*.

Regeneration of Maxillofacial Bone

Previous studies have demonstrated that the interactions of FCSCs and HUVECs could promote osteogenic differentiation of FCSCs *in vitro*. Researchers further generated a mouse model with critical-size defects in the calvaria to mimic the vascularized bone niche and found that FCSC transplantation directly formed bone-like tissue in the defect region (Nathan et al., 2018).

FCSCs were able to differentiate and form *de novo* bony tissue that expressed OCN. In addition, the neovasculature localized at the periphery of the FCSC engraftment area was $CD31^+$, suggesting that FCSC integration was coupled with endothelial cell recruitment (Embree et al., 2016; Nathan et al., 2018). Notably, dorsum-transplanted FCSCs regenerated cartilage before calcification, which differed from the direct formation of bone-like tissue in calvarial defects. Scholars speculated that the microenvironment may contribute to FCSC fate decisions. In the ectopic xenograft model, the innate chondrogenic capacity of FCSCs dominated the regeneration process, while the osteogenesis of FCSCs observed in the vascularized bone defect may rely on FCSC–HUVEC interactions. At present, the mechanisms modulating FCSC differentiation toward chondrogenesis and osteogenesis *in vivo* remain uncertain, and further investigation is warranted.

CONCLUSION

Fibrocartilage stem cells, a novel stem cell population, have been recently identified in the condylar cartilage of animals and humans. Under physiological conditions, FCSCs play an indispensable role in the development and homeostasis of condylar cartilage. They present clonogenicity and multipotency, sharing similar *in vitro* properties with MSCs. Recent attention has been focused on the regulatory mechanisms of FCSCs, implying their distinct characteristics during development. However, whether FCSCs have a unique signature compared to other resident dental MSC populations remains to be determined. More importantly, endogenous and exogenous FCSCs hold enormous promise in cartilage and bone repair and regeneration in pathologic states. The mechanism may involve the Wnt and Notch signaling pathways, but the precise regulatory networks have not been fully clarified. There is still controversy regarding the differentiation process of FCSCs when transplanted in different sites; thus, it is of crucial importance to perform a more comprehensive analysis of *in vivo* changes as well as the interaction between FCSCs and their microenvironment. In summary, understanding the functions and regulatory mechanisms of FCSCs will aid the establishment of FCSC-based strategies for cartilage and bone regeneration.

AUTHOR CONTRIBUTIONS

YF, CC, PLi, PLy, YL, and RB collected the literature and drafted the manuscript. YF, RB, and SZ supervised the procedures and approved the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

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Unraveling the Role of the Apical Papilla During Dental Root Maturation

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The apical papilla is a stem cell rich tissue located at the base of the developing dental root and is responsible for the progressive elongation and maturation of the root. The multipotent stem cells of the apical papilla (SCAP) are extensively studied in cell culture since they demonstrate a high capacity for osteogenic, adipogenic, and chondrogenic differentiation and are thus an attractive stem cell source for stem cell-based therapies. Currently, only few studies are dedicated to determining the role of the apical papilla in dental root development. In this review, we will focus on the architecture of the apical papilla and describe the specific SCAP signaling pathways involved in root maturation. Furthermore, we will explore the heterogeneity of the SCAP phenotype within the tissue and determine their micro-environmental interaction. Understanding the mechanism of postnatal dental root growth could further aid in developing novel strategies in dental root regeneration.

Keywords: apical papilla, SCAP, dental, root, development

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INTRODUCTION

Dental tooth development can be subdivided into different steps and starts with the formation of the crown during the bud, cap and bell stages. Once the crown has taken shape, the dental root starts to grow under coordination of the Hertwig's epithelial root sheath (HERS) (Luan et al., 2006). This double layer of the epithelial sheath grows apically and positions itself between the dental papilla and dental follicle. During root elongation and formation of dentin, the HERS will be fragmented into the epithelial rests of Malassez allowing dental follicle cells to establish contact with the newly formed dentin and to differentiate into cementoblasts (Huang X. et al., 2009). Collagen fibers secreted by dental follicle cells are deposited against the cementum to enable a firm connection to the alveolar bone (Zhou et al., 2019). Consequently, tooth root formation and elongation is associated with eruption and positioning of the newly formed tooth. Complete elongation and maturation of the dental root is guided by the apical papilla (**Figure 1**) which originates from the ectomesenchyme (Huang et al., 2008). From a developmental point of view, the architectural composition of the apical papilla and its role in dental root maturation are poorly studied. Here, we will provide an in depth overview on the current knowledge of the apical papilla tissue and how the stem cell rich content drives dental root development through specific signaling pathways and micro-environmental changes.

From a macroscopic point of view, the apical papilla is located apical to the epithelial diaphragm and is partitioned from the dental pulp by a cell-rich zone (Sonoyama et al., 2008). Within the collagenous-rich matrix of the apical papilla, a high concentration of mesenchymal

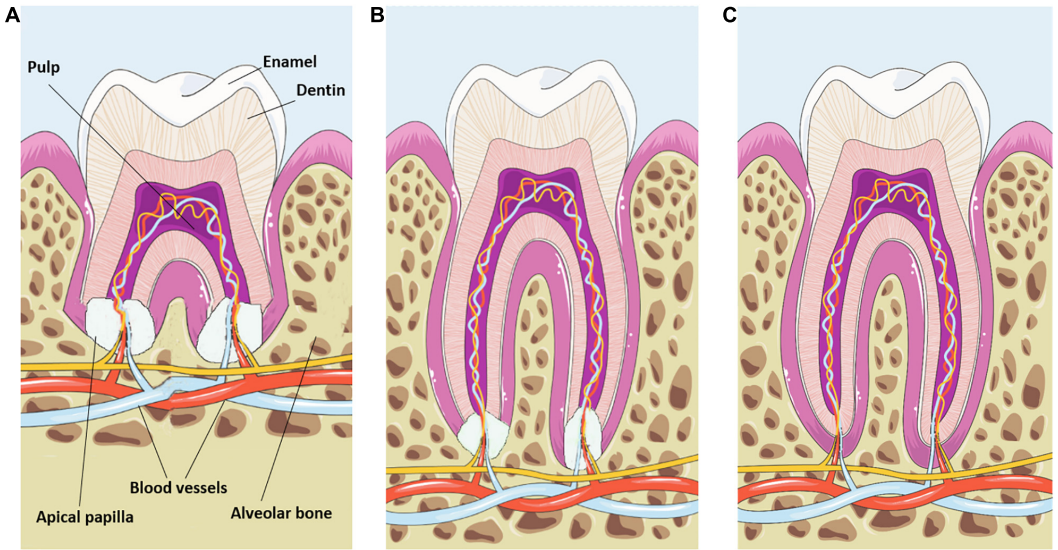

FIGURE 1 | Schematic representation of apical papilla-induced root maturation. **(A)** The apical papilla is located at the growing part of the maturing dental root. Note the different components of the tooth and the dental vasculature entering the apical papilla and pulp tissue. **(B)** During root elongation the apical papilla progressively decreases in size. **(C)** Fully mature roots are associated with a complete loss of the apical papilla tissue. This image was created using Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License, available online at https://smart.servier.com/.

stem cells reside which are defined as stem cells of the apical papilla (SCAP). These highly proliferative SCAP will contribute to the formation of dentin by differentiating into odontoblasts and/or are recruited to the connected pulp tissue. Since the discovery of the SCAP within the apical papilla, it became clear that multipotent SCAP are highly suitable for osteogenic, adipogenic and chondrogenic differentiation (Huang G. T. J. et al., 2009). Together with their immunomodulatory capacity (Gaudin et al., 2018; Liu et al., 2019; Fehrmann et al., 2020), SCAP have a high potential for implementation in tissue regeneration. This offered the research community an easily accessible mesenchymal stem cell source with low ethical considerations. Recently, our group identified novel histological regions (Driesen et al., 2020) based on the collagen distribution and organization. At the surface layer, the connective tissue is organized as a dense collagen matrix with perpendicular aligned collagen fibers and which is defined as cortex fibrosa. The inner part of the apical papilla which is indicated as medulla consists of a loosely disorganized collagen matrix with a high concentration of SCAP. The whole apical papilla is furthermore encapsulated by a single layered cuboid epithelium and can be considered as an independent tissue structure with its own vascular network and innervation branching toward the central dental pulp vasculature and nerve. Therefore, the apical papilla is less susceptible to pathologic events leading to pulp necrosis and to apical periodontitis (Chepra et al., 2017). In addition SCAP have been demonstrated to own a high immune stability profile (Lei et al., 2021). Furthermore, resection of the dental pulp via pulpectomy (Jung et al., 2011) or periapical lesion after trauma (Jiang and Liu, 2020) with preservation of the apical papilla revealed that root maturation is continuous and is independent from the dental pulp.

MOLECULAR MECHANISM OF ROOT FORMATION

Recent studies have procured more information on the signaling pathways specifically involved in tooth root development. An important prerequisite for the start of dental root growth after crown formation is the de-activation of fibroblast growth factor-10 signaling in the dental papilla (Yokohama-Tamaki et al., 2006). At the apical site of root development, a dynamic interaction of SCAP in close vicinity with the HERS is observed which influences apical root morphogenesis and controls root number, length and dentin formation (Luder, 2015; **Figure 2**). HERS stimulates root formation in the mesenchyme of the apical papilla through secretion of Wnt3a and 4 and sonic Hedgehog (SHH) which upregulate the expression of the Nfic transcription factor, the central regulator for root formation. HERS triggers the Nfic pathway in SCAP by switching on the canonical Wnt pathway which has been shown to be essential for postnatal root maturation (Kim et al., 2013; Wang et al., 2020). HERS-secreted Wnt3a and Wnt4 proteins will couple to the seven-pass transmembrane Frizzled receptor and the low-density lipoprotein receptor-related protein 5 or 6 (LRP5/6) (Wen et al., 2020). Recruitment of Disheveled (DVL) promotes phosphorylation of the LRP6 receptor and binding of the Axin complex composed of tumor suppressor adenomatous polyposis coli gene product (APC), glycogen synthase kinase 3 (GSK3) and casein kinase 1 (CK1) (MacDonald et al., 2009). This will lead to a reduction of β-catenin phosphorylation and degradation allowing β-catenin to upregulate the expression of runt-related transcription factor 2 (Runx2) followed by direct activation of the Nfic pathway. The importance of β-catenin in root development has previously been shown in

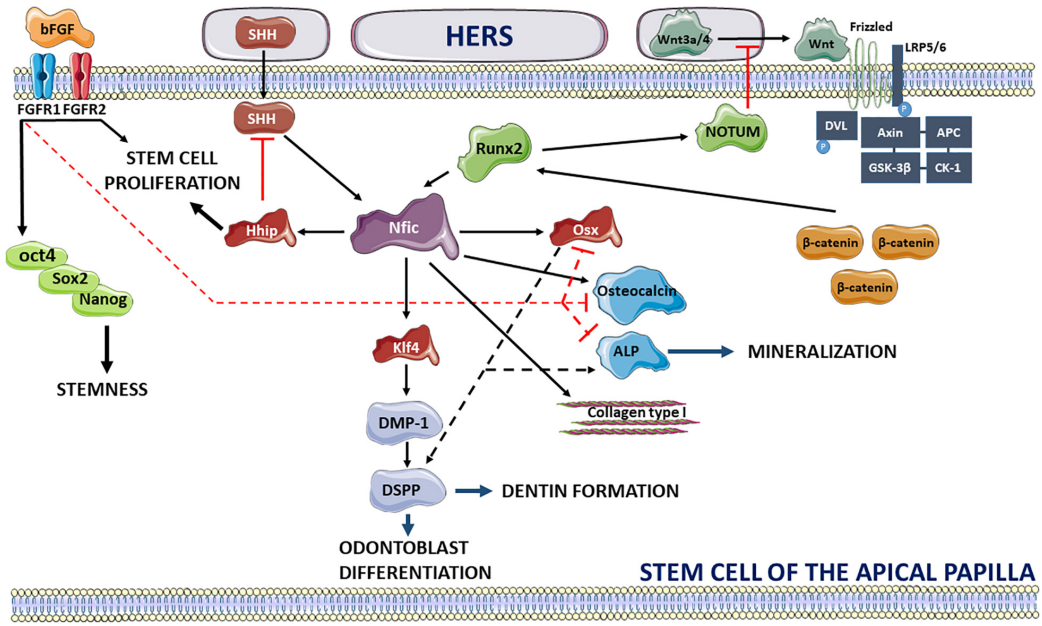

FIGURE 2 | Overview of signaling pathways regulating the stemness of SCAP and promoting dental root development via extrinsic signals of HERS. bFGF maintains the stemness of SCAP via activation of FGFR1 and 2 promoting expression of stemness factors oct4, Sox2, and Nanog, inhibits mineralization through interaction with Osx, Osteocalcin, and ALP and promotes cell proliferation. HERS stimulates dental root maturation by secretion of Wnt3a and Wnt4 interacting with the Frizzled and LRP5/6 receptors. Activation of Wnt signaling acts via binding and phosphorylation of DVL, phosphorylation of LRP5/6 and de-activation of the Axin, APC, GSK-3β, and CK-1 complex leading to inhibition of β-catenin degradation. Consequently, β-catenin interacts with Runx2 activating the Nfic pathway. Nfic triggers odontoblast differentiation and dentin formation by acting with Klf4 and promoting DMP-1 and DSPP expression. Secondly, Nfic induces mineralization by upregulating Osteocalcin, ALP and collagen type I and through interaction with Osx which promotes ALP production and DSPP expression. Alternatively, HERS activates Nfic signaling independent of Runx2 via secretion of SHH. Wnt and hedgehog signaling pathways are, respectively, antagonized by Runx2-driven secretion of NOTUM and Nfic-promoted expression of Hhip which promotes stem cell proliferation. Activators of signaling pathways are shown in black lines (full and dotted) whereas inhibition is depicted in red. This image was created using Servier Medical Art, licensed under a Creative Common Attribution 3.0 Generic License, available online at https://smart.servier.com/.

conditional knockout mice for β-catenin revealing impaired root elongation (Yang et al., 2020) and loss of HERS structural integrity. Consequently, activation of the Nfic signaling pathway will promote odontoblast differentiation and dentin formation via Krüppel-like factor 4 (Klf4) expression resulting in the production of dentin matrix acidic phosphoprotein-1 (DMP-1) and dentin sialophosphoprotein (DSPP) (Lee et al., 2014). Nfic-induced mineralization is stimulated via increased expression of collagen type I and osteocalcin (Zhang et al., 2015) and through upregulation of osterix (Osx) which activates ALP production and induces DSPP expression (He et al., 2016). Loss of Osx expression has been shown to result in failure of complete root maturation and a decrease in odontogenic differentiation (Zhang et al., 2015). The interplay between Runx2 and Wnt signaling, however, is considered complex and requires a delicate regulation for achieving optimal root elongation (Wen et al., 2020). Overexpression of Wnt signaling results in shorter roots whereas de-activation completely arrests root development. This balance is guided by the Runx2-induced secretion of NOTUM, an inhibitor of the Wnt signaling pathway by de-acetylating Wnt3a (Wen et al., 2020). It should be noted that the Ror2 mediated non-canonical Wnt signaling and its downstream Cdc42 mediator has revealed an impact on the mesenchyme cell proliferation and a contribution to root development size in mouse molars (Ma et al., 2021). Secondly, HERS triggers tooth root development by secretion of SHH which is regulated in the epithelial compartment by SMAD4-mediated transforming growth factor-beta (TGF-β)/bone morphogenetic protein (BMP) signaling (Huang and Chai, 2012). SHH contributes to the activation of the Nfic signaling pathway independent of Runx2 resulting in elevated expression levels of hedgehog interacting protein (Hhip) and promotion of SCAP proliferation (Liu Y. et al., 2015). It has been shown that hedgehog signaling activity is the highest in SCAP at the periphery of the apical papilla and diminishes toward the center of the tissue creating a concentration gradient (Li et al., 2015). In addition, the level of hedgehog activity is negatively regulated by the Nfic-activated Hhip ensuring proper root formation (Liu Y. et al., 2015).

Once root growth is initiated, apical odontoblasts emerge at the base of the developing roots displaying the expression of the aforementioned transcription factors i.e., Nfic, Osx, β-catenin and alkaline phosphatase (Bae et al., 2013). The differentiation level of this phenotype is more advanced compared to the pre-odontoblasts originating from the dental papilla which only express β-catenin. Differential regulation of odontogenic differentiation could be the result of a dynamic interplay between Nfic and TGF-β1 which is highly dependent on the

expression levels of both factors (He et al., 2014). TGF-β1 signaling promotes odontoblast differentiation via stimulation of the BMP/SMAD pathway and expression of DSPP (Iwamoto et al., 2017). On the other hand, TGF-β1 inhibits Nfic expression directly via SMAD3 upregulation and activation of the mitogen-activated protein kinase pathway which initiates Nfic degradation by SMURF1 and 2 (Lee et al., 2011). Nfic itself can counteract the inhibitory effect of TGF-β1 signaling by dephosphorylating the SMAD2/3 pathway. Comparing the expression levels of both signaling pathways during the progression of root elongation will shed more light on their close interaction.

HETEROGENEITY IN SCAP PHENOTYPE—LESSONS AND PITFALLS

A vast amount of differentiation protocols have been successfully optimized in primary cell cultures of SCAP to demonstrate the high capacity of SCAP for osteogenic and adipogenic differentiation. SCAP are easily isolated from the apical papilla by tissue explant culture or by enzymatic digestion as demonstrated previously for dental pulp stem cells (Hilkens et al., 2013). However, to study the contribution of SCAP to dental root development, one should be cautious in interpreting the function of SCAP in culture. In tissue, SCAP are part of a micro-environmental niche which determines cell cycle, functional behavior and differentiation capacity (Diao et al., 2017). Orchestration of progressive root maturation is coordinated in conjunction with changes in the extracellular matrix and expression of growth factors (Huang et al., 2020). Gene expression analysis has proven that disruption of the micro-environmental niche leads to high number of differentially expressed genes when compared between tissue and SCAP in culture (Diao et al., 2017). SCAP from early passage have been shown to retain a more original phenotype as demonstrated by their intact mesodermal differentiation capacity (Rémy et al., 2019). A similar observation was made when studying Wnt inhibitory factor 1 (WIF1) expression, a Wnt/β-catenin modulator which maintains stem cell commitment (Wang and Cao, 2019). High expression levels of WIF1 are encountered within the native tissue of the apical papilla but decrease rapidly in primary cultures of SCAP. When overexpressing WIF1 in SCAP, it became apparent that the Wnt pathway promotes dentinogenic differentiation as indicated by the increased expression of odontogenic genes i.e., DSPP, DMP1, Runx2, and Osx (Wang and Cao, 2019). Prevention of cell culture induced adaptations in SCAP can be limited through either recreating tissue atmospheric conditions by lowering the oxygen concentration (Rémy et al., 2019) or by re-integration in a 3-D scaffold system (Somoza et al., 2017).

SCAP are identified as mesenchymal stem cells based on the expression of a standard panel of markers including Stro-1, CD24, CD29, CD73, CD90, CD105, CD106, CD146, ALP, and absence of expression of CD34, CD45, CD18, and CD150. Recent studies have underscored the presence of a heterogeneous pool of SCAP phenotypes residing in the apical papilla. When evaluating these phenotypes throughout literature, a distinction can be made based on the localization within the tissue. The majority of cells within the apical papilla express CD90 and a high concentration of CD105 and CD73 positive cells is located near the blood vessels (Ruparel et al., 2013). A further characterization within the $CD146^+$ stem cell pool can be made based on STRO-1 expression (Bakopoulou et al., 2013). $STRO1^+$ SCAP were shown to retain a high expression of embryonic and mesenchymal stem cell markers and enhanced odontogenic differentiation via activation of SMAD and Erk signaling. These characteristics were completely absent in the $STRO1^-$ subpopulation. Importantly, the level of STRO-1 expression decreases progressively during increase in cell passages masking the original phenotype from the tissue. Treatment of SCAP using basic Fibroblast-Growth Factor (bFGF) maintains STRO-1 expression up to 10 passages, increases their proliferation, and preserves the undifferentiated state of the isolated stem cells (Wu et al., 2012). This process is regulated through binding of bFGF to FGF-receptors 1 or 2 expressed in SCAP (Chang et al., 2020) which initiate upregulation of oct4, Nanog and SOX2. These genes are highly involved in maintaining stem cell pluripotency and are part of a common stemness gene expression program observed in different types of dental stem cells originating from the bud and pulp tissue (Ballini et al., 2019). Furthermore, activation of FGF receptors enhances cell proliferation but negatively regulates mineralization by inhibiting Osx, osteocalcin, and ALP (Wu et al., 2012). Immunohistochemistry revealed the location of $NOTCH3^+$ and $CD146^+$ stem cells in the vicinity of the blood vessel walls (Jamal et al., 2015). The role of NOTCH3 has also been associated with the preservation of the undifferentiated state of stem cells and its expression is markedly present during tooth development (Zhang et al., 2008; Sun et al., 2014). FACS analysis identified 4 different SCAP subpopulations based on NOTCH3 and STRO-1 expression underscoring the heterogeneity of SCAP phenotypes in tissue (Jamal et al., 2015). Furthermore, CXCR4 is highly expressed in perivascular SCAP and is activated via SDF-1, indicating chemo-attraction (Liu J. Y. et al., 2015). SDF-1 activates phosphatidylinositol 3-kinase and protein kinase C signaling pathways in SCAP and contributes to the transmigration capacity (Chen et al., 2016). The latter is reflected by the formation of focal adhesions and the expression of focal adhesion molecules, i.e., p-focal adhesion kinase, p-paxillin, and vinculin, linking the adhesion complexes to a stress fiber network (Chen et al., 2016). In addition, the SDF-1/CXCR4 pathway contributes to induction of odontogenic differentiation via BMP-2 (Xiao et al., 2019).

INTERACTION OF SCAP WITH THE MICRO-ENVIRONMENT

From the aforementioned studies we can conclude that SCAP are a heterogeneous stem cell pool which have the capacity to migrate to the growing dental root and to differentiate into odontoblasts. However, the impact of SCAP is not only confined to mere differentiation but they also affect their surrounding

micro-environment by high secretion of a plethora of factors. Analysis of the SCAP secretome in conditioned medium revealed a diverse collection of proteins consisting of chemokines along with angiogenic, immunomodulatory, anti-apoptotic, neuroprotective factors, and extracellular matrix (ECM) proteins (Yu et al., 2020). Moreover, SCAP produce components of the renin-angiotensin system (RAS) (Macedo et al., 2021) which could increase SCAP proliferation rate via angiotensin 2 receptor activation (Pizzatto et al., 2020). Several studies indicate the capacity of the apical papilla to maintain cells in a non-differentiated state intrinsically and even in neighboring tissues via currently unknown secretory signaling molecules. Our group discovered that the central peripheral nerve within the apical papilla is surrounded by a resident vimentin negative cell population in contrast to the abundant vimentin positive SCAP in the medulla (Driesen et al., 2020). Therefore, it is hypothesized that the vimentin negative cell fraction maintains the opening of the root canal preventing odontoblast differentiation and dentin deposition. Indeed, in a co-culture system, SCAP were able to negatively regulate osteogenic differentiation of dental follicle stem cells (Wu et al., 2018). The apical papilla would thus exert an inhibitory effect on dental follicle stem cells during the formation of the dental root.

Since progression of dental root growth is associated with a reduction in apical papilla size and eventually disappears after final root maturation (**Figure 1**), one can hypothesize that the extracellular matrix is prone to continuous remodeling and degradation. Thus, an intrinsic balance between collagen digestion and cross-linking could serve as a mechanism for SCAP migration and recruitment to the growing root. Collagen digestion requires the secretion of enzymes with a collagenase activity such as membrane-type matrix metalloproteinase 1 (MT1-MMP) which is a zinc-endopeptidase and exclusively expressed during dental formation within the dental mesenchyme (Xu et al., 2016). Reduced activity of MT1-MMP has led to impaired root formation and as a result incomplete tooth eruption. In addition, we identified abundant expression of fibroblast-activation protein-α in dental mesenchymal stem cells and in particular within SCAP (Driesen et al., 2020). FAP-α is a member of the family of cell surface serine proteases and is highly expressed during embryonic development and contributes to extracellular matrix remodeling via collagenase type I activity. The presence of FAP-α in SCAP could point to a capacity to re-organize their collagen-based micro-environment creating a suitable substrate for enhanced migration toward the developing root.

FUTURE PERSPECTIVES AND CONCLUSION

Studies involved in comprehending the molecular mechanism of root formation has already provided novel insights in dental disorders. The pathology of short root anomaly is recognized by the development of abnormal short roots with a blunt appearance (Yu et al., 2021) and is a consequence of a dysregulation of nuclear factor 1 C-type, Osx, bone morphogenetic protein, TGF-β, Wnt-β catenin, and DKK1. Pulp necrosis and apical periodontitis in immature teeth negatively affects proper root development posing difficulties for proper dental treatment. Strategies are currently being investigated to restore or regenerate defective root formation. Berberine has been shown to induce root repair via activation of the Wnt/β-catenin pathway in SCAP leading to longer roots and thicker root walls (Cui et al., 2020). Mixture of SCAP and PDLSCs has proven to initiate regeneration of the root and periodontal structure in swine (Sonoyama et al., 2006). An interesting novel approach is the use of biomimetic scaffolds with flexible modeling of the scaffold's geometry. Integration of SCAP into these scaffolds with a stem-cell matching microarchitecture could procure new therapeutic strategies for bio-artificial root replacement (Ballini et al., 2017). However, many processes in dental root maturation are not resolved at the moment. Studying the interaction between the apical papilla, HERS and dental follicle will shed more light on the micro-environmental changes and extracellular matrix remodeling associated with apical papilla remodeling during root maturation. Identification and mechanistic understanding of the cellular heterogeneity within the apical papilla will be an important first step toward the development of tissue engineered apical papillae for future dental root regeneration therapy.

AUTHOR CONTRIBUTIONS

RD conceived and wrote the manuscript. TV, PG, and IL reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Roles of Dental Mesenchymal Stem Cells in the Management of Immature Necrotic Permanent Teeth

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Dental caries and trauma always lead to pulp necrosis and subsequent root development arrest of young permanent teeth. The traditional treatment, apexification, with the absence of further root formation, results in abnormal root morphology and compromises long-term prognosis. Regeneration endodontics procedures (REPs) have been developed and considered as an alternative strategy for management of immature permanent teeth with pulpal necrosis, including cell-free and cell-based REPs. Cell-free REPs, including revascularization and cell homing with molecules recruiting endogenous mesenchymal stem cells (MSCs), have been widely applied in clinical treatment, showing optimistic periapical lesion healing and continued root development. However, the regenerated pulp–dentin complex is still absent in these cases. Dental MSCs, as one of the essentials of tissue engineering, are vital seed cells in regenerative medicine. Dental MSC–based REPs have presented promising potential with pulp–dentin regeneration in large animal studies and clinical trials via cell transplantation. In the present review, we summarize current understanding of the biological basis of clinical treatments for immature necrotic permanent teeth and the roles of dental MSCs during this process and update the progress of MSC-based REPs in the administration of immature necrotic permanent teeth.

Keywords: dental mesenchymal stem cells, regenerative endodontics, pulp-dentin regeneration, immature permanent teeth, cell transplantation

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INTRODUCTION

Immature permanent teeth are prone to pulpal necrosis due to caries, trauma, or developmental malformation. These cases always lead to arrest of root formation, accompanied by thin root dentinal walls and open apices, which has been a challenge in endodontics (Shabahang, 2012). With apexification, the traditional treatment, either calcium hydroxide or mineral trioxide aggregate (MTA) is applied to achieve apical sealing (Andreasen and Bakland, 2011; Nicoloso et al., 2017). Apexification has been reported to resolve apical periodontitis with a success rate of 74–100% (Al Ansary et al., 2009). However, absence of further root formation with apexification still results in abnormal root morphology, such as thin dentinal walls with an increased risk of root fracture, consequently compromising long-term prognosis (Rafter, 2004).

Regeneration endodontics procedures (REPs) have been developed and considered as an alternative strategy for treatment of immature permanent teeth with pulp necrosis (Murray et al., 2006). The notion of tissue regeneration in the root canal was first proposed in the 1960s (Nygaard-Ostby, 1961). Banchs and Trope (2004) introduced a case report describing an alternative treatment for the management of necrotic immature permanent teeth called revascularization, in which a blood clot was induced inside the root canal after control of inflammation. Later, autologous platelet-rich plasma (PRP) and platelet-rich fibrin (PRF) took the place of the blood clot as alternative scaffolds because of their potential to induce tissue regeneration (Lovelace et al., 2010). A standard protocol for clinical REPs was proposed by the American Association of Endodontists [AAE], 2016b) in 2016 These REPs without exogenous cells, including revascularization and cell homing, have been successful in resolving apical periodontitis and arrest of root formation (Iwaya et al., 2001; Torabinejad and Turman, 2010; Shimizu et al., 2013). However, histological studies show that the pulp–dentin complex is absent in these cases although some of them have shown vital pulp (Shimizu et al., 2013; Ulusoy et al., 2019). Desired REPs are supposed to eliminate apical periodontitis; increase root length, dentinal wall thickness, and apical closure; and restore homeostatic function of the pulp–dentin complex, including inherent immunity, tertiary dentin formation with stimulus, and pulp sensibility. In particular, the reinstitution of pulp–dentin structure functions prolongs the life of the tooth. Hence, scientists and endodontists are keen to develop a novel regenerative strategy to achieve pulp vitality and organized pulp–dentin structure with homeostatic functions.

Three major elements have been recommended by Diogenes for further studies of pulp–dentin regeneration, including (i) reliable cell resources responsible for formation of root dentin, pulp tissue, and supporting tissue; (ii) an applicable scaffold to facilitate cellular proliferation and differentiation; and (iii) signaling molecules to motivate and direct tissue development, maturation, and neovascularization (Diogenes et al., 2016). Mesenchymal stem cells (MSCs) responsible for pulp–dentin regeneration might be indispensable for ideal REPs. Several preclinical studies reveal the regenerative potential of pulp–dentin tissue via cultured cell transplantation (Nakashima et al., 2017; El Ashiry et al., 2018; Xuan et al., 2018). With its accessibility and unique potential in dental tissue regeneration, including the pulp–dentin complex, dental MSCs play a decisive role of seed cells in REPs. In this context, the applications of dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHED), stem cells from apical papilla (SCAP), periodontal ligament stem cells (PDLSCs), and dental follicle stem cells (DFSCs) have been explored. In the latest clinical study (Xuan et al., 2018), implantation of autologous SHED aggregates generated pulp–dentin complex in immature necrotic permanent incisors of pediatric patients, including functional dental pulp tissue regeneration with vasculature, innervation, and the lining odontoblast layer. The regenerated dental pulp tissue promotes root elongation and apical foramen closure. Therefore, dental MSCs exert therapeutic applications and are of great importance in treating immature necrotic permanent teeth.

In this review, we briefly summarize the current understanding of the biological basis of clinical treatments for immature permanent teeth with pulpal necrosis and the roles of dental MSCs during this process and update the progress of MSC-based REPs in the treatment of immature necrotic permanent teeth.

BIOLOGICAL BASIS FOR REPs

Root development relies on temporospatial reciprocal action between dental epithelium (Hertwig's epithelial root sheath, HERS) and mesenchyme from the cranial neural crest (dental papilla and follicle) (Thesleff and Sharpe, 1997). When the tooth crown is formed, HERS is formed by the inner and outer enamel epithelium of the enamel organ, which lies between the dental papilla and follicle. Then, HERS extends apically with the dental papilla and follicle and eventually regulates root formation. The inner epithelial cells of HERS induce MSCs at the periphery of the pulp to form odontoblasts, which produce the root dentin (Huang et al., 2009). As SHED fragments, the dental follicle penetrates into the epithelial fenestrations, contacts the root dentin, and differentiates into cementoblasts, which form the cementum covering the root dentin (Zeichner-David et al., 2003; Sonoyama et al., 2007b; Huang et al., 2009). The dental follicle is also responsible for the formation of periodontal ligament and fiber bundles. Hence, HERS plays a vital role in the interaction between the dental epithelial and dental mesenchymal compartment during root formation (**Figure 1**).

Arrested root formation in immature necrotic permanent teeth is always related to severe HERS damage due to dental trauma (Andreasen et al., 1988). Inflammatory cytokines and chemokines induced by severe and chronic inflammation impair the stem cells during tissue repair (Cooper et al., 2014). Additionally, function of SCAP could be disrupted by proinflammatory cytokines (Johnson, 1997; Liu et al., 2016; Wang et al., 2017). Hence, root formation is halted in immature permanent teeth with pulpal necrosis. Once inflammation is controlled, proinflammatory cytokine and chemokines are reduced, which leads to resumption of the regulatory effect of HERS and, consequently, induces the continued formation of the incomplete root (Cooper et al., 2010; Diogenes and Hargeraves, 2017). MSCs are vulnerable to the inflammatory microenvironment, and their immunomodulatory capacities can vary unexpectedly with the exposure to different inflammatory conditions (Noronha et al., 2019). It is demonstrated that the TNFα/TNFR2 signaling pathway is involved in regulating the immunomodulatory properties of MSCs (Beldi et al., 2020a,b). The TNFα–TNFR2 axis mediates MSCs' anti-inflammatory effects and cell survival, indicated by the inhibition of T cell proliferation, the production of proinflammatory cytokines, and the inductive activation of regulatory T cells. The presence of the TNFR2 molecule is also involved in the regulatory effect of MSCs, such as the colony-forming unit, proliferation, and MSC-specific surface markers. TNFR2 is expressed predominantly in endothelial progenitor cells. The TNFα/TNFR2 signaling pathway is also critical in the regulation of endothelial progenitor

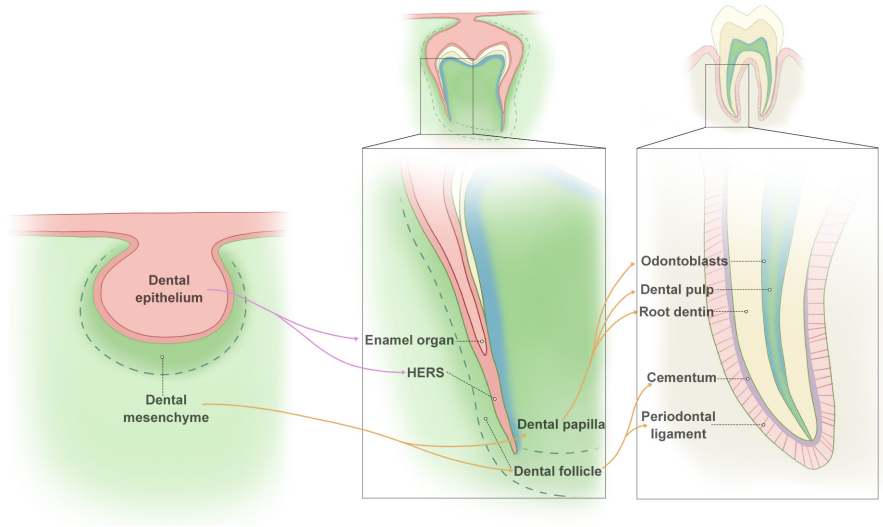

FIGURE 1 | Summary of root formation.

cell immunosuppression and the angiogenic effect to form new immunosuppressive vessels (Naserian et al., 2020). Whether the inflammatory environment caused by pulpal necrosis/apical periodontitis plays protective and essential roles in the biological functions of SCAPs needs further study. A previous study suggests the long-term viability of apical papilla under prolonged root canal infection and apical periodontitis (Diogenes and Hargeraves, 2017). On the contrary, MSCs responsible for the pulp–dentin complex in the root canal rarely survive during chronic endodontic infection, which explains the absence of pulp–dentin regeneration with revascularization or cell homing.

DENTAL MESENCHYMAL STEM CELLS

Human MSCs are multipotent cells from various tissues, such as skeletal muscle, adipose tissue, placenta, bone, and dental tissue (Pittenger et al., 1999). Based on minimal criteria proposed by the International Society for Cellular Therapy (ISCT), MSCs are plastic-adherent; possess multilineage differentiation potential *in vitro*; express at least CD105, CD73, and CD90; and negatively express CD11b, CD14, CD19, CD34, CD45, CD79α, and HLA-DR cell surface markers (Dominici et al., 2006; Han et al., 2019). According to MSC minimal criteria, dental MSCs derived from dental tissues, including impacted teeth and their supporting tissues, have been identified and characterized with typical MSC properties (Sharpe, 2016; **Table 1**). In addition to easy access, dental MSCs are genomically stable after multiple passages *in vitro*. Despite their multilineage differentiation capacity, dental MSCs are distinct from other MSCs because of the unique potential in dental tissue regeneration and have aroused much interest in regenerative medicine, especially the applications of REPs (Huang et al., 2009a). To date, dentin, dental pulp, or even pulp–dentin complex-like structure regenerations with the application of dental MSCs have been widely investigated, including DPSCs, SHED, SCAP, PDLSCs, and DFSCs.

DPSCs

Dental pulp tissue, formed by neural crest–derived dental papilla, is the soft tissue surrounded by the dentin. Responding to external stimuli, odontoblasts form the tertiary dentin. These odontoblasts are supposed to be derived from the progenitor cell populations within dental pulp. DPSCs, first isolated from adult third molar pulp tissues by Gronthos et al., possess definitive MSC characteristics, self-renewal capacity, and multilineage differentiation potential (Gronthos et al., 2000). DPSCs can differentiate into osteoblast-like cells with specific markers, forming new bone *in vivo* (Mortada and Mortada, 2018). A series of case reports indicate the potential application of DPSCs in treating intraosseous defects. In these patients with such defects caused by periodontitis, minimally invasive flap and collagen sponge integrated with autologous/allogeneic DPSCs have been applied. Results showed decreased probing depth reduction, achievement of clinical attachment, and formation of new bone with rare adverse effects, indicating the periodontal tissue regeneration potential of DPSCs (Aimetti et al., 2018; Ferrarotti et al., 2018; Hernández-Monjaraz et al., 2018). DPSCs are known to differentiate into odontoblasts that are indispensable for dentinogenesis. Dentin-like tissue is formed *in vivo* with DPSCs and hydroxyapatite/tricalcium phosphate (HA/TCP) scaffold, presenting a lining odontoblast-like cell layer of a specific odontoblastic-related marker expression, dentin sialophosphoprotein (DSPP) (Anitua et al., 2017; da Silva et al., 2019). Compared with human bone marrow MSCs, DPSCs exhibit notable neurogenic potential due to their origin of the neural crest and could differentiate into neurons upon specific differentiation induction (Pagella et al., 2019). The neurogenic potential was also confirmed with higher expression levels of neurotrophins when DPSCs were cocultured with trigeminal

TABLE 1 | Comparison of root formation-related dental MSCs in immature permanent teeth.

	Source	Surface marker positive	Surface marker negative	Multi-lineage differentiation potential	Immunomodulatory properties
DPSCs	Dental pulp tissue of permanent teeth	CD9, CD10, CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, NANOG, SOX2, OCT4, TRA1-60, TRA-1-80-1, and Nestin	CD14, CD19, CD24, CD31, CD34, CD45, and CD117	odontoblasts, osteoblasts, chondrocytes, adipocytes, neurons, cardiomyocyte, and hepatocytes	Immunosuppressive properties increased HGF, TGF-β, PGE-2, IL-6, and IDO; decreased IL-4 and IFN-γ; suppressed proliferation of T cells and PBMCs; increased number of regulatory T cells
SHED	Dental pulp tissue of exfoliated deciduous teeth	CD13, CD29, CD44, CD73, CD90, CD105, CD146, STRO-1, NANOG, adn Nestin	CD14, CD15, CD19, CD34, and CD45	odontoblasts, osteocytes, chondrocytes, adipocytes, neurons, and hepatocytes	Immunosuppressive properties increased IL-10; decreased IL-4 and IFN-γ; inhibited Th17 cell differentiation; increased number of regulatory T cells
SCAP	Apical papilla	CD13, CD24, CD29, CD44, CD49, CD51, CD56, CD61, CD73, CD90, CD106, CD146, CD166, STRO-1, NANOG, and Nestin	CD14, CD18, CD34, and CD45	odontoblasts, osteocytes, adipocytes, neurons, and hepatocytes	Low immunogenicity inhibited proliferation of T cells
PDLSCs	Periodontal ligament	CD9, CD10, CD13, CD29, CD44, CD59, CD73, CD90, CD105 CD106, CD146, CD166, and STRO-1	CD14, CD19, CD34, CD45, and HLA-DR	cementoblasts, osteoblasts, chondrocytes, adipocytes, and neurons	Immunosuppressive properties expressing TLR2 and TLR4; released HGF, TGF-β, and IDO; suppressed proliferation of PBMCs
DFSCs	Dental follicle	CD9, CD10, CD13, CD29, CD44, CD59, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1, NANOG, SOX2, OCT4, and Nestin	CD31, CD34, CD45, and CD133	odontoblasts, cementoblasts, osteoblasts, chondrocytes, adipocytes, neurons, and cardiomyocytes	Immunosuppressive properties expressing TLR2, TLR3, and TLR4; increased IL-6, TGF-β, and IDO-1; decreased IFN-γ, IL-4, and IL-8; suppressed proliferation and apoptosis of PBMCs; increased number of regulatory T cells

DPSCs, dental pulp stem cells; SHED, stem cells of human exfoliated deciduous teeth; SCAP, stem cells from apical papilla; PDLSCs, periodontal ligament stem cells; DFSCs, dental follicle stem cells; HFG, hepatocyte growth factor; IDO, indole amine 2,3-dioxygenase; IFN, interferon; IL, interleukin; PGE2, prostaglandin E2; TGF-β, transforming growth factor beta; Th17, T-helper 17; TLR, Toll-like receptor; PBMCs, peripheral blood mononuclear cells.

neurons (Jung et al., 2016; Kawase-Koga et al., 2019). DPSCs also display angiogenic potential for differentiation potential of endothelial cells and the formation of blood vessels after *in vivo* transplantation with HA scaffold (Jeong et al., 2020). A clinical study shows that implanted DPSCs achieve pulp-like tissue regeneration with vasculature and innervation in the root canal of traumatized incisors (Nakashima et al., 2017). The potential application of DPSCs in dental pulp tissue regeneration has also been indicated by another case report. The affected mature permanent tooth with symptomatic irreversible pulpitis shows a positive response in pulp vitality test, following the administration of autologous DPSCs and leukocyte PRF in the root canal of the affected tooth (Meza et al., 2018). The promising neurogenic, angiogenic, and odontoblastic differentiation potential makes DPSCs a major contributor to dentin regeneration and even whole pulp regeneration.

SHED

SHED were collected from children's exfoliated deciduous teeth with a similar methodology as that for DPSCs (Miura et al., 2003). SHED possess multilineage differentiation potential and can differentiate into various cell types, such as odontoblasts, adipocytes, and neurons (Miura et al., 2003). However, SHED show capacities of higher proliferation, more cell population doublings, and remarkable osteoinduction compared with DPSCs based on developmental differences between deciduous and permanent teeth. Regenerated new bone with larger osteoids and more collagen fibers by SHED with a polylactic-coglycolic acid membrane suggests that SHED exhibit outstanding potential for bone regeneration compared with DPSCs and bone marrow MSCs (Miura et al., 2003; Kunimatsu et al., 2018). As for the potential of neural regeneration, SHED show more intensive expression of neural differentiation markers than DPSCs under neural induction culture, such as β-III-tubulin and nestin (Wang et al., 2009) and can also promote neural functional recovery (Nicola et al., 2018). The odontoblastic differentiation capacity of SHED has been confirmed by *in vivo* transplantation that the composites of SHED and HA/TCP form a dentin-like structure containing DPSS-positive odontoblasts (Miura et al., 2003). SHED are also capable of forming functional dental pulp tissue, containing odontoblasts to regenerate tubular dentin in full-length root canals combined with collagen type I (Cordeiro et al., 2008). The abovementioned odontoblastic differentiation capacity renders SHED a promising cell source for dentin or pulp regeneration; and whole dental pulp regeneration has been achieved by SHED (Xuan et al., 2018).

SCAP

In the process of tooth development, dental papilla forms dental pulp and migrates apically (Sonoyama et al., 2007b). Several clinical case reports show that root formation continues in some necrotic immature permanent teeth, indicating that

TABLE 2 | Current preclinical and clinical studies of cell-free REPs.

Study/year	Sample size (teeth)	Animal model	Intracanal medication	Scaffold	Capping material	Observation period	Results or outcomes
Preclinical studies							
da Silva et al., 2009	40	Dogs	TAP	Empty scaffold	MTA	90 days	Hard tissue barrier, and increase of apical periodontal ligament thickness
Yamauchi et al., 2010	64	Dogs	TAP	Cross-linked collagen scaffold + blood clot, blood clot	MTA	3.5 months	Periapical healing and root wall thickening
Tawfik et al., 2013	108	Dogs	TAP	bFGF injectable scaffold + blood clot, blood clot	MTA	3 months	Negative results in this study: no change of root length and root thickness.
Khademi et al., 2014	36	Dogs	TAP	Blood clot	MTA	3-6 months	periapical healing, apical closure, and dentinal walls thickening
Yoo et al., 2014	30	Dogs	TAP	a collagen scaffold sponge (soaked with conditioned media from mouse preameloblasts) + blood clot	MTA	12 weeks	Continuous growth of root dentin, and hard tissue formation
Zhang et al., 2014	27	Dogs	TAP	PRP, blood blot	MTA	3 months	Root canal walls thickening, and apical closure
Londero Cde et al., 2015	20	Dogs	TAP	Gelatin-based scaffold (Gelfoam) + blood clot, blood clot	MTA	7 months	Increase in root length
Rodríguez-Benítez et al., 2015	40	Dogs	modified triple-antibiotics paste (mTAP)	PRP, blood blot	MTA	6 months	Root dentinal walls thickening, hard tissue deposition on dentinal walls, and apical closure
Saoud et al., 2015	17	Dogs	TAP	Blood clot	MTA	3 months	Not reported about root development and apical closure; but significant dentinal walls thickening, and periapical healing
Torabinejad et al., 2015	24	Dogs	TAP	Blood clot/Gelfoam, PRP	MTA	3 months	Apical narrowing, and hard tissue deposition in the apical third of the root
Altaii et al., 2017	4	Sheep	TAP	Blood clot	MTA	6 months	Significant increases in root length, root wall thickness and narrowing of root canal apical diameter
Study/year	**Sample size (teeth)**	**Age of patients (mean ± SD)**	**Intracanal medication**	**Scaffold**	**Capping material**	**Observation period (mean ± SD)**	**Results or outcomes**
Clinical studies							
Reynolds et al., 2008	2	11 years old	TAP	Blood clot	MTA	18 months	Significant root development with maturation of the dentin
Bose et al., 2009	88	-	TAP, Ca(OH)2, and formocresol	Blood clot	MTA	6 months-36 months	Continued root development: increased percentage of root length and dentinal wall thickness
Ding et al., 2009	12	8–11 years old	TAP	Blood clot	MTA	15 months	3 teeth of 12 exhibit complete root development with closed apex and positive response to electric pulp testing
Petrino et al., 2009	6	6, 11, and 13 years old	TAP	Blood clot	MTA	6-12 months	3 of 6 teeth showed continued root development, and 2 teeth displayed positive response to vitality testing
Iwaya et al., 2010	1	7 years old	$Ca(OH)_2$ paste (Vitapex)	Empty scaffold	Gutta-percha	30 months	Continued root development, root apex closure, and root canal thickness increase
Thomson and Kahler, 2010	1	12 years old	TAP	Blood clot	MTA	18 months	Continued root maturation and apical closure
Torabinejad and Turman, 2010	1	11 years old	TAP	PRP	MTA	5.5 months	Periapical lesion resolution, further root development, and continued apical closure of the root apex

(Continued)

TABLE 2 | Continued

Study/year	Sample size (teeth)	Age of patients (mean ± SD)	Intracanal medication	Scaffold	Capping material	Observation period (mean ± SD)	Results or outcomes
Chen et al., 2011	20	8-13 years old	$Ca(OH)_2$	Blood clot	MTA	6-26 months	periapical wound healing, and Increased thickening of root canal walls; 15 of 20 teeth continued root development ; 4 of 20 teeth exhibited severe hard tissue calcification in pulp canal; 2 of 20 teeth formed a hard tissue barrier in root canal space
Nosrat et al., 2011	2	8, and 9 years old	TAP	Blood clot	Calcium enriched mixture (CEM)	15-18 months	Periapical radiolucent lesions healing, and continued roots development
Jeeruphan et al., 2012	20	8-24 years old	TAP	Blood clot	MTA	21.15 ± 11.70 months	Increased percentage of root width and root length
Kim et al., 2012	3	10 and 12 years old	TAP	Blood clot	MTA	24, 42, and 48 months	Periapical radiolucency disappeared, and root wall thickness increased
Martin et al., 2012	1	9 years old	TAP	PRP + Blood clot	MTA	2 years and 1 months	Resolution of apical periodontitis; hard tissue of obliteration in distal canal, reduction in size of mesial canal space
Jadhav et al., 2013	6	10, 13, and 23 years old	TAP	PRP + blood clot, blood clot	Resin modified glass ionomer cement	12 months	Periapical healing, apical closure, and dentinal wall thickening
Kahler et al., 2013	16	7-12 years old	TAP	Blood clot	MTA	18 months	Patterns of continued root maturogenesis were variable: 90.3% resolution of the periapical radiolucency, 47.2% incomplete apical closure, 19.4% complete apical closure, 2.7% to 25.3% change of root length, and 1.9% to 72.6% change of root dentin thickness
Nagy et al., 2013	36	9-13 years old	TAP	FGF + blood clot, blood clot	MTA	18 months – 3 years	Periapical healing, increase in root length and width, and a decrease in apical diameter
Shimizu et al., 2013	1	9 years old	$Ca(OH)_2$	Blood clot	MTA	26 months	Resolution of periapical lesion, continued root development, thickening of the canal walls
Sönmez et al., 2013	3	9 years old	TAP	Blood clot	MTA	24 months	Continued thickening of the dentinal walls with apical closure; complete resolution of periapical radiolucencies
Alobaid et al., 2014	31	6-16 years old	TAP, BAP, $Ca(OH)_2$	Blood clot	MTA	14.5 ± 8.5 months	Apical closure and hard tissue barrier; but a greater incidence of adverse events in revascularization group
Bezgin et al., 2014	22	7–13 years old	TAP	PRP, blood clot	MTA	18 months	Complete apical closure, periapical tissue pathology resolution
Nagata et al., 2014	23	7-17 years old	TAP, $Ca(OH)_2$, and chlorhexidine	Blood clot	MTA	9-19 months	Periapical repair, apical closure, root length increase, dentinal walls thickening; but crown discoloration in teeth of TAP group
Saoud et al., 2014	20	11.3 ± 1.9 years old	TAP	Blood clot	MTA	1 year	Increase in radiographic root width and length and decrease in apical diameter
Narang et al., 2015	20	Below 20 years old	TAP	RPF + blood clot, PRP + collagen, blood clot	Resin-modified glass ionomer cement	6-18 months	PRF shows significant periapical healing, apical closure, root lengthening, and dentinal wall thickening in revascularization treatment
Nosrat et al., 2015	2	9, 10 years old	TAP	Blood clot	MTA	4 months	Progression of root development and maturation of the roots
Timmerman and Parashos, 2016	1	16 years old	$Ca(OH)_2$	Blood clot	MTA	3 years	Complete periapical healing, thickening of the dentinal root walls, and completed apex formation
Austah et al., 2018	2	8, and 10 years old	BAP, $Ca(OH)_2$	Blood clot + CollaPlug	MTA	43-54 months	Complete healing of periapical tissues, continued root development, root length increase, and dentin thickness increase

(Continued)

TABLE 2 | Continued

Study/year	Sample size (teeth)	Age of patients (mean ± SD)	Intracanal medication	Scaffold	Capping material	Observation period (mean ± SD)	Results or outcomes
Ajram et al., 2019	1	7 years old	$Ca(OH)_2$	Blood clot	Micro Mega-MTA (MM-MTA)	2 years	Complete apical healing, continued root growth, and apical closure
Rizk et al., 2019	26	8-14 years old	TAP	PRP, blood clot	MTA	12 months	Significant increase in root length, root width, and decrease in apical diameter of PRP-treated teeth compared with blood clot group; but higher amount of crown discoloration in blood clot-treated teeth
Alasqah et al., 2020	1	8 years old	$Ca(OH)_2$, TAP	Blood clot	MTA	24 months	Periapical healing with increased root thickness and length, and complete apical closure

BAP, bi-antibiotics paste; bFGF, basic fibroblast growth factor; Ca(OH)2, calcium hydroxide; MTA, mineral trioxide aggregate; PRF, platelet-rich fibrin; PRP, platelet-rich plasma; TAP, triple-antibiotics paste.

MSCs in apical papilla contribute to root development. SCAP are obtained from apical papilla of immature tooth roots and exhibit MSC properties, including expression of MSC surface markers and differentiation potential to a wide variety of cell types (Sonoyama et al., 2006, 2007a). SCAP possess neural differentiation potential similar to DPSCs and SHED, partially attributed to their common origin from the neural crest, and could be an alternative future therapy for spinal cord injury (De Berdt et al., 2015). Interestingly, SCAP have higher proliferation and greater odontoblastic differentiation potential than DPSCs, suggesting their potential applications for dentin regeneration (Sonoyama et al., 2006). *In vivo* studies show that SCAP are able to differentiate into odontoblast-like cells and generate dentin-like tissue with DSP expression (Sonoyama et al., 2006, 2007a). The dentin regeneration capacity of SCAP via cell homing strategy is enhanced by their greater migration ability following a scratch assay. SCAP can also form ectopic vascularized pulp-like tissue with DSPP and dentin matrix protein 1 (DMP1)-positive odontoblasts in mouse molars without exogenous growth factor application (Pelissari et al., 2018). Owing to their critical role in root development, SCAP are supposed to make a major contribution to root regeneration. After transplantation of SCAP and PDLSCs into a minipig model with a lower incisor extracted, a functional bioroot with root/periodontal–like complex was formed. Mineralized root-like tissue is able to support a porcelain crown and perform normal tooth function (Sonoyama et al., 2006).

PDLSCs

A population of MSCs exists in the periodontal ligament (PDL), and it is responsible for periodontal tissue homeostasis and regeneration (McCulloch and Melcher, 1983; Seo et al., 2004). These cells were first isolated from the PDL of third molars and named PDLSCs. The cementogenic/osteogenic differentiation potential is indicated by the formation of mineralization nodules with the expression of bone-specific markers after *in vivo* transplantation (Seo et al., 2004). The cementogenic/osteogenic differentiation potential and PDL tissue regeneration potential of PDLSCs are shown in a rat model of periodontal lesions, confirmed by newly formed cementum/PDL-like structures at the lesion area, such as Sharpey's fiber-like tissue (Seo et al., 2004; Iwata et al., 2010). A recent preclinical study using a novel cell transfer technology demonstrates the potential of PDLSCs in periodontal regeneration. In a rat model of surgical periodontal defects, the transplantation of PDLSC-amniotic membrane composite enhanced the periodontal defect recovery, manifested as newly formed PDL, bone, and cementum at surgically defective sites (Iwasaki et al., 2019).

DFSCs

Dental follicle contributes to alveolar bone formation during tooth development, and contains an MSC population to form supporting tissues, named DFSCs. DFSCs were separated from the dental follicle of developing teeth (Morsczeck et al., 2004; Han et al., 2009; Zhou et al., 2019). Compared with DPSCs, SHED, and PDLSCs, DFSCs show a higher proliferation and colony-forming capacity, indicating their application potential in regenerative medicine (Tian et al., 2015; Yildirim et al., 2016). DFSCs also exhibit superior osteogenic properties compared with DPSCs and SHED as shown by the higher expression levels of osteogenic genes (Yildirim et al., 2016). Under the administration of differentiation induction culture medium, DFSCs form osteoblasts and produce mineralized nodules with osteogenic differentiation marker expression, bone sialoprotein, and osteocalcin (Morsczeck et al., 2004; Han et al., 2009). DFSCs are capable of periodontal differentiation, indicated by the formation of PDL-like tissues or mineralized structures with bone- or cementum-like tissues (Morsczeck et al., 2004; Han et al., 2009). DFSCs generate complex tissues similar to cementum-PDL complex *in vivo*, in which PDL-like collagen fibers are inserted into newly formed cementum-like tissue (Han et al., 2009). The potential of odontoblastic differentiation has also been suggested in DFSCs because they have been shown to express higher level of DSPP compared with PDLSCs. The formation of dentin, including dentin, predentin, and calcospherites, is observed with treated dentin matrix induction

(Trubiani et al., 2019). All these findings suggest DFSCs as promising seed cells for both dentin and root regeneration.

CELL-FREE REPs

Roles of Dental MSCs in Cell-Free REPs

The first attempt at dental pulp tissue regeneration was proposed by Nygaard-Otsby et al. (Nygaard-Ostby, 1961; Nygaard-Ostby and Hjortdal, 1971). Over-instrumentation was applied to introduce blood from the periapical tissues into the root canal, followed by tissue growth. Later, Banchs and Trope (2004) proposed a protocol termed revascularization based on the experiments of Kling et al. (1986) on implanted teeth, Hoshino et al. (1996) on root canal disinfection, and (Nygaard-Ostby and Hjortdal, 1971) on blood clots in the canal space.

The standard REP protocol proposed by the American Association of Endodontists [AAE] (2016b; 2018) involves a multistep procedure. The first visit focuses on infection control of the affected tooth with the administration of a proper access cavity, canal irrigation, and disinfection. The common root canal dressing is calcium hydroxide or triple antibiotic paste (TAP), which is a mixture of ciprofloxacin, metronidazole, and minocycline. The second appointment aims to form the suitable scaffold formation for fresh tissue ingrowth and permanent coronal restoration following the absence of clinical signs and symptoms. During this appointment, the root canal is thoroughly irrigated with ethylenediaminetetraacetic acid to release the growth factor from the dentin. Apical bleeding is then stimulated by gentle irritation with a precurved K-file at 2 mm past the apical foramen to form a blood clot in the root canal. Finally, capping material, usually MTA, is placed over the blood clot, followed by the permanent coronal seal to prevent bacterial reinfection. At the follow-up, eliminating clinical signs and symptoms and healing periapical lesion are considered as primary goal of REPs. It is desirable, but not essential, that REPs increase the thickness of the root wall and/or length of the roots, which is the secondary goal. Some cases report that the teeth showed a positive response to pulp vitality testing, suggesting organized pulp tissue in the root canal, which achieves the tertiary goal.

As an amelioration to revascularization with blood clots, the cell homing strategy has been proposed to regenerate dental tissue via a cell-free strategy in which molecules encourage recruitment of the patient's endogenous MSCs to the root-canal space (He et al., 2016; Yin et al., 2017). Several endodontists believe that cell homing is conducive to achieving a more effective strategy of pulp–dentin regeneration than simple revascularization without exogenous cell transplantation (**Table 2**). Several molecules, including basic fibroblast growth factors, vascular endothelial growth factors, platelet-derived growth factors, nerve growth factors, and bone morphogenetic protein 7, have been applied as homing factors, showing promising outcomes in preclinical studies (Kim et al., 2010). These REPs without exogenous cell transplantation, including revascularization and cell homing, are considered cell-free REPs. Survival rates of cell-free REPs are reported close to 100% in some studies. Therefore, these studies suggest that cell-free REPs have an obvious therapeutic effect on necrotic immature teeth (**Figure 2A**).

Stem cells, homing to the injury site, have an essential role in wound healing (Rustad and Gurtner, 2011). The cells in the sites of injury and inflammation release chemokines, stem cell factors, and growth factors, which motivate the cell homing (Eramo et al., 2018). $CXCR4^{+}$ SCAP are demonstrated to be chemoattracted by stromal derived factor 1, a chemokine, and migrate into a scaffold made of collagen gel (Liu et al., 2015). In cell-free REPs, stem cells from the periapical tissues get into the root canal space in various ways, mainly by periapical bleeding and molecules in the scaffolds. It is demonstrated that a large number of MSCs with expression of CD105, CD73, and STRO1 were induced into the empty root canal by importing periapical bleeding (Lovelace et al., 2010). These cells were supposed to be MSCs from the adjacent apical papilla rather than systemic circulation although no direct evidence is shown in that study. Additionally, histological and immunohistochemical analysis presented the formation of cementum- and bone-like structures in necrotic immature permanent teeth with cell-free REPs. It suggests that stem/progenitor cells in periapical tissue, responsible for production of cementum and bone, also entered the root canal and participated in the formation of mineralized tissue during continued root formation (Martin et al., 2012; Shimizu et al., 2012, 2013; Torabinejad and Faras, 2012; Becerra et al., 2013; Nosrat et al., 2015). Therefore, undifferentiated MSCs originated from apical papilla, and periapical tissues are considered to be major cell sources for continued root formation and pulp–dentin regeneration. Cells from distant site, such as systemic circulation, are considered to be cell sources for cell-free REPs. However, these cells make little contribution to pulp regeneration, considering their small numbers.

Limitations of Cell-Free REPs

Although cell-free REPs are suggested to be effective in eliminating apical periodontitis and even revitalization of non-vital immature teeth in some case reports, their outcomes are still unpredictable. Elimination of apical periodontitis associated with necrotic immature permanent teeth, the primary goal of REPs, can be easily achieved once the infection in the root canal is controlled with disinfection. However, the vitality of cells in apical papilla, dental follicle, and HERS is determined by severity, origin, and duration of inflammation from immature permanent teeth with pulpal necrosis, which is beyond the control of the endodontists. Once severe damage happens to the apical papilla or follicle, there are no dental MSCs supporting odontoblast differentiation or dentin formation, which results in a lack of continued root formation (**Figure 3**). It is impossible to clearly define the status of MSCs in the apical papilla and dental follicle; thus, endodontists in the clinic always fail to predict the outcomes of cell-free REPs in the necrotic immature permanent teeth.

American Association of Endodontists [AAE] (2016a) has defined REPs as "biologically based procedures designed to physiologically replace damaged tooth structures, including dentine and root structures, as well as cells of the pulp – dentin complex." This suggests that endogenous stem cells introduced by periapical bleeding might achieve pulp–dentin

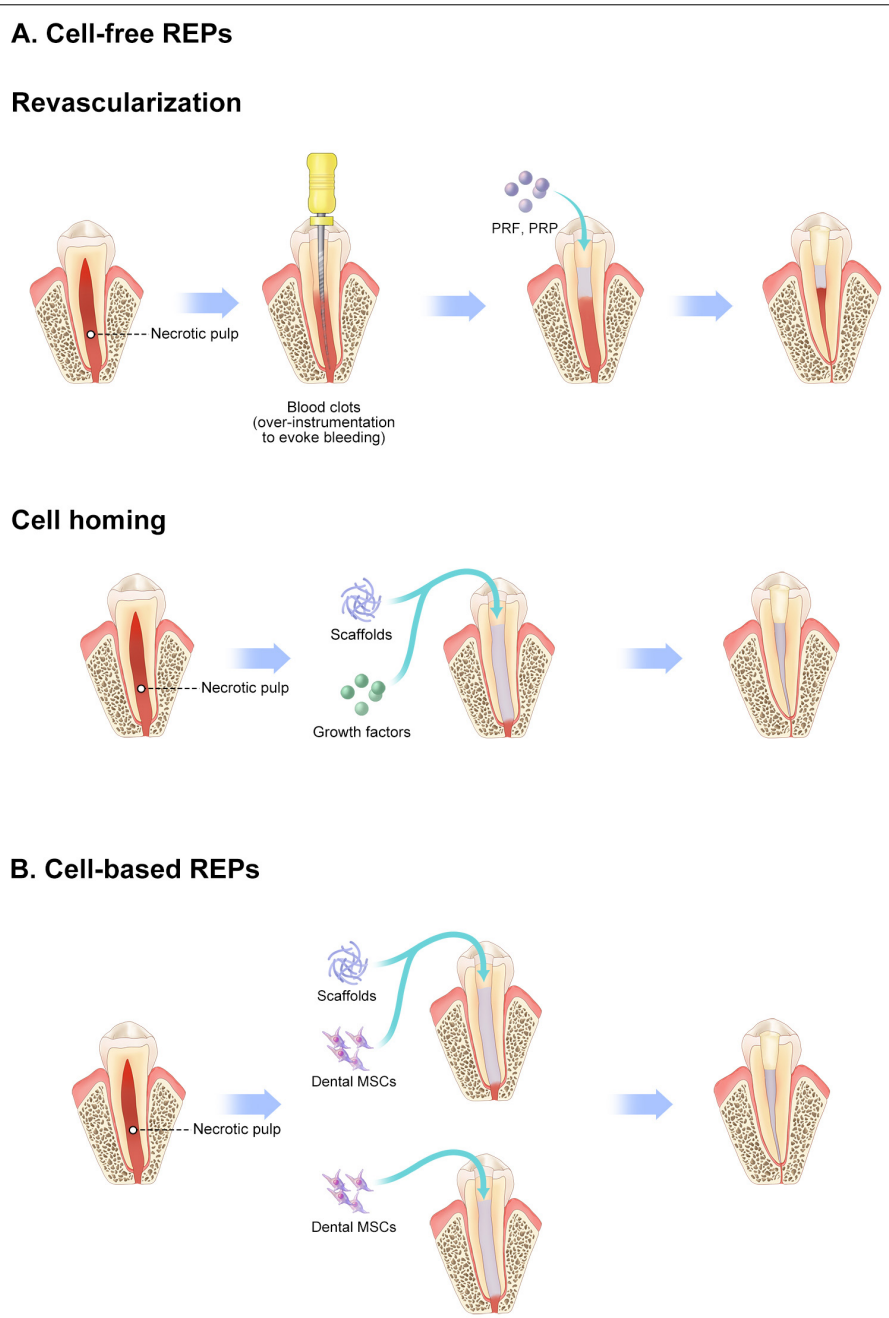

FIGURE 2 | Schematic diagram of REPs. **(A)** Cell-free REPs. **(B)** Cell-based REPs. REPs, regeneration endodontics procedures.

regeneration, which produce odontoblast-like cells and form dentin. However, both animal and preclinical studies fail to show such results. Formation of bone, cementum, and fibrous tissue is observed with revascularization in dogs. The regeneration of the pulp–dentin complex is rarely detected in the root canal. Additionally, histological studies of human teeth present similar cementum apposition, ectopic bone, and fibrous tissues in human mandibular molars treated with revascularization (Torabinejad and Faras, 2012; Nosrat et al., 2015). Only one human study shows regenerated pulp–dentin complex with odontoblast-like cells and dentin-like tissue in necrotic immature permanent teeth with cell-free REPs, which is assigned to survival of odontoblasts in the root canal (Austah et al., 2018). These studies suggest that the cell-free REPs of necrotic immature permanent teeth are "repair" rather than "regeneration" procedures (Diogenes et al., 2016). Unlike immature teeth with pulpal necrosis, teeth with reversible or irreversible pulpitis can regenerate pulp–dentin complex after cell-free REPs. This might result from the remaining pulp tissue, which means the presence of MSCs

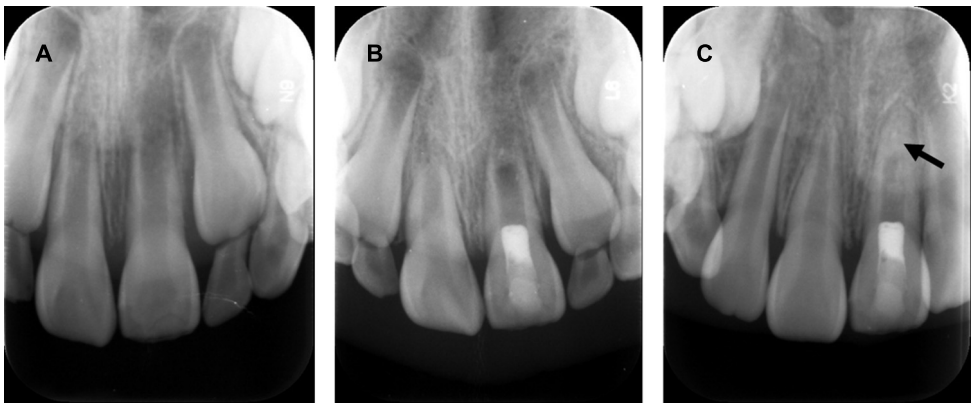

FIGURE 3 | Revascularization promotes continued root development and resolution of periapical lesion, but disorganized radio-opaque changes occur within the apical root canal. **(A)** Radiograph of a maxillary left central incisor: immature root formation with a wide-open apex and periapical lesion. **(B)** Postoperative radiograph of revascularization and coronal restoration. **(C)** Radiograph of one-year follow-up: resolution of periapical lesion and root apex closure. Arrow: non-specific radio-opaque calcific deposit within apical root canal. Courtesy of Dr. Xin Zhou.

responsible for pulp–dentin complex is indispensable for true regeneration in endodontics.

CELL-BASED REPs

Roles of Dental MSCs in Cell-Based REPs

Mooney et al. (1996) achieved cell-based pulp regeneration by applying pulp cells and polyglycolic acid *in vitro* as early as 1996. In 2005, stem cells were introduced as one of the essential elements of pulp–dentin regeneration in endodontics by Nakashima and Akamine (2005). Soon afterward, Murray et al. (2006) proposed regenerative endodontics as biologically based procedures, in which stem cells play a vital role. Since then, some studies demonstrate the effectiveness of cultured stem cell transplantation in pulp–dentin regeneration (**Table 3**). Huang et al. (2010) reported that MSC transplantation regenerated pulp–dentin complex in human root fragments compared with formation of fibrous tissue with scaffold alone, which was the first *in vivo* study of pulp–dentin regeneration. Pulp–dentin complex, a layer of odontoblast-like cells on nascent mineralized tissue, is observed in dental MSC–transplanted human dentin with polylactic acid, suggesting a requirement for cell transplantation in pulp–dentin regeneration (Sakai et al., 2010). Later, the necessity of cell transplantation was confirmed with animal studies. Pulp–dentin complex regeneration in large animals was first reported by Iohara et al. (2009) in a pulpotomy model in dogs, in which fractionated side-population cells enriched with $CD31^{-}/CD146^{-}$ were transplanted. They also indicate that pulp tissue is regenerated in the root canal with a combination of $CD105^{+}$ DPSCs and SDF-1 (Iohara et al., 2010). SDF-1 is considered an important homing signal by recruiting MSCs to injury sites and facilitating regeneration in various tissues (Suzuki et al., 2011). However, pulp tissue is hardly detected in the root canal with SDF-1 alone. These studies further demonstrate that homing signaling alone is insufficient for pulp–dentin regeneration. The transplantation of pulpal MSCs into the root canal is necessary for pulp–dentin regeneration (**Figure 2B**).

In recent years, cell-based REPs have aroused growing concern for pulpless teeth. Several clinical studies demonstrate whole dental pulp regeneration. In a preclinical trial, a composite containing human mobilized DPSCs (MDPSCs) and a collagen scaffold was utilized (Nakashima and Iohara, 2014). Upon autologous transplantation with the composite into the root canals of canine mature teeth after pulpectomy, vasculature and innervation- regenerated pulp-like tissue was formed with odontoblast-like cells on the surface of the root dentinal wall and newly formed dentin along the dentinal wall. It suggests that complete dental pulp regeneration similar to healthy dental pulp is achieved along with restoration of tooth function. The rarity of adverse events has confirmed the safety of MDPSC-based REPs. The biological characteristics of MDPSCs do not vary with age, including their stability and regenerative potential. Thus, MDPSCs have been applied to a clinical study to further explore the therapeutic potential and clinical safety of autologous MDPSC transplantation in pulpectomized human teeth (Nakashima et al., 2017). The results show mineralized structure formation of cone beam computed tomography (CBCT), similar signal intensity of magnetic resonance imaging to that of normal dental pulp in untreated controls, robust positive response of a pulp vitality test, and minor adverse events or toxicity. Therefore, human MDPSCs are suggested as safe and efficacious dental MSCs candidates in cell-based REPs.

The therapeutic potential of DPSCs in pulp–dentin regeneration via cell-based REPs is also explored in a minipig pulpectomy model with empty root canals (Xuan et al., 2018). The whole functional pulp tissue regenerates in root canals after implantation of DPSC aggregates harvested from minipigs, consisting of an odontoblast-like layer, blood vessels, and nerves. Based on the preclinical trial with a large animal model, they further conducted a randomized clinical controlled trial to determine the therapeutic effect on immature permanent tooth injuries caused by trauma (Xuan et al., 2018). Those immature

TABLE 3 | Current preclinical and clinical studies of REPs based on dental MSCs.

Study/year	Type of dental MSCs	Experiment design						Results or outcomes	
		Animal model	Defects	Route of administration	Biomaterial/ scaffold	Growth factors	Observation period	Tissue regeneration	Effect evaluation and safety assessment
Preclinical studies									
Iohara et al., 2010	CD 105 + canine DPSCs	60 incisors; 15 dogs	whole pulp removal; enlargement of apical foramen to 0.7 mm	Autologous transplantation; root canal	Mixture of collagen type I & III	Stromal cell-derived factor-1 (SDF-1)	14-90 days	Functional dental pulp	HE: regenerative pulp with well vasculature and innervation on day 14
Iohara et al., 2012	Canine DPSCs	72 incisors; 18 dogs	The whole pulp tissue was removed, and the root canals were enlarged to open the apical foramen to 0.6mmin width in incisors	Autologous transplantation; root canal	Atelocollagen scaffold	Granulocyte-colony stimulating factor (G-CSF)	14-180 days	Functional dental pulp	Safety: no adverse effects on both the whole and local HE: regenerative pulp with well vasculature and innervation on day 14 RG: complete obliteration of the enlarged apical portion and lateral and coronal dentin formation Laser Doppler: functional recovery of pulpal blood flow after 90 days Pulp vitality: positive response on day 60 and day 180
Iohara et al., 2014	canine mobilized DPSCs	16 incisors; 4 dogs	Whole pulp removed, apical foramen enlarged to 0.5 mm	Autologous transplantation; root canal	Atelocollagen scaffold	Granulocyte-colony stimulating factor (G-CSF)	14-120 days	Functional dental pulp	HE: regenerative pulp with well vasculature and innervation on day 14 RG: complete obliteration of the enlarged apical portion and lateral and coronal dentin formation
Nakashima and Iohara, 2014	Canine mobilized DPSCs	-	Root canals after pulpectomy	Autologous transplantation; root canal	Drug-approved collagen	Granulocyte-colony stimulating factor (G-CSF)	14-180 days	Pulp-like tissue	Safety: no adverse effects, no inflammatory cells infiltrated, and no internal or external resorption of the tooth HE: pulplike tissue with well vasculature and innervation was regenerated 14 days RG: complete obliteration of the enlarged apical portion and lateral and coronal dentin formation
El Ashiry et al., 2018	Canine DPSCs	36 incisors; 12 dogs	Pulps from crown and root	Autologous transplantation; root canal	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 (BMP7)	1-4 months	Vascularized pulp- dentin like tissue	HE: delicate fibrous tissue resembling the pulp tissue inside the root canal containing multiple large and small blood vessels; newly formed dentin-like tissue with dentinal tubule-like structures along the dentinal walls of the root canal; the regenerated dentin-like tissue did not form well-organized dentinal tubules RGE: closure of the root apex, thickening of the root canal wall, and prolongation of the root lengthening

(Continued)

TABLE 3 | Continued

Study/year	Type of dental MSCs	Experiment design						Results or outcomes	
		Animal model	Defects	Route of administration	Biomaterial/ scaffold	Growth factors	Observation period	Tissue regeneration	Effect evaluation and safety assessment
Xuan et al., 2018	Pig DPSCs	minipigs	Empty root canals after pulpectomy	DPSC aggregates; autologous transplantation; root canals	-	-	3 months	Whole pulp tissue	HE: regenerated pulp tissue containing an odontoblast layer and blood vessels IHC: NeuN

Study/year	Type of dental MSCs	Experiment design						Results or outcomes	
		No. of subjects (teeth)	Defects	Route of administration	Biomaterial/ scaffold	Growth factors	Observation period	Tissue regeneration	Effect evaluation and safety assessment
Clinical studies									
Nakashima et al., 2017	Human mobilized DPSCs	5 teeth (2 incisors, 3 premolars); 5 patients with irreversible pulpitis	Root canals after pulpectomy	Autologous transplantation; root canal	Atelocollagen scaffold	Granulocyte colony-stimulating factor (G-CSF)	1, 2, 4, 12, and 24/28/32 weeks	Pulp-like tissue	Safety:no adverse events; no postoperative pain, including percussion pain and tenderness; no significant changes in the periapical areas EPT: positive responses after 4 weeks in 4 patients; 1 patient demonstrated a negative response after 24 weeks RG: obliteration of the enlarged apical portion at 24/28 weeks in 3 patients CBCT: lateral dentin formation at 28 weeks in 3 patients MRI: regenerated tissue in the root canal after 24 weeks was similar to that of normal dental pulp in 4 patients
Xuan et al., 2018	Human DPSCs	26 incisors; 36 patients	Dental trauma with pulp necrosis	Two hDPSC Aggregates; Autologous implantation; Root canals	-	Extracellular matrix	1, 3, 6, 9, 12, and 24 months	Whole dental pulp	Safety: no significant side effects after 12 months HE: regeneration of 3D whole dental pulp tissue Digital RVG: no inflammation at the periapical area and continued root development after 24 months EPT: decrease in sensation thresholds CBCT: apical foramen width decreased, the length of the treated tooth root increased Laser Doppler: increase in vascular formation

HE, hematoxylin and eosin staining; IHC, immunohistochemical staining; RG, radiographic examination; RVG, radiovisiography; EPT, electric pulp vitality testing; CBCT, cone beam computed tomography.

necrotic permanent teeth were transplanted autologously with DPSCs collected from primary teeth. Taking apexification as a control group, DPSC-treated immature permanent teeth presented with eliminated apical periodontitis and continued root formation during two years' follow-up. This was indicated by decreased apical foramen width and increased root length via CBCT, and dentin thickness increased via 3-D reconstruction. The viability of DPSC-treated teeth was validated by laser Doppler flowmetry and electric pulp testing, which showed an increase in vascular formation and decrease in sensation thresholds compared with controls. More excitingly, histological analysis of further traumatized teeth showed regeneration of pulp–dentin complex with an odontoblast layer. Thus, this study demonstrates better efficacy and safety of DPSCs implantation in cell-based REPs, in which 3-D dental pulp tissue with vasculature and innervation was regenerated. Besides, the efficacy and safety of allogenic umbilical cord MSCs have been proved in a preclinical trial (Brizuela et al., 2020). Other cell populations, such as SCAP or non-dental cells might also be useful in cell-based REPs. Considering the accessibility of cell sources, allogeneic cell sources are more usable.

Challenge for Cell-Based REPs

Cell-based REPs show promising outcomes in pulp–dentin regeneration. Several cell-based REPs are at the stage of clinical studies (Nakashima et al., 2017; Xuan et al., 2018), but transplantation of stem cells is still not recommended by either the American Association of Endodontists [AAE] (2018) or the European Society of Endodontology (ESE) (Galler et al., 2016). Multiple problems needed to be resolved before clinical application of stem cell transplantation, including isolation of stem cells, expansion of cells *in vitro*, practice facilities with good manufacturing, skill of clinicians, training of chair-side assistants, and high cost (Huang et al., 2013). MSCs are one of the most important elements in regenerative endodontics. However, their source and potency are still restrained due to the limitation of our available knowledge. *In vitro*- cultured human somatic stem cells, such as DPSCs, will end up with replicative senescence, a terminal state, after limited cell divisions (Kang et al., 2004). It is suggested that there is a notable elevation of senescent DPSCs cultured *in vitro* and an obvious reduction of odontogenic differentiation potential that may be attributed to loss of stem cell marker, Bmi1 (Mehrazarin et al., 2011). Besides, a large number of cell doublings with homogeneous loss of differentiation potential are required for cell transplantation with *ex vivo* expansion of DPSCs. Due to the aging-related change in DPSCs in the dental tissue of aged patients, the accessibility of DPSCs suitable for regeneration is restricted to immature permanent teeth of young patients. Therefore, lack of DPSCs from pulp tissue would make cell-based REPs in adult permanent teeth difficult to achieve. The *ex vivo* expansion of autologous MSCs in dental appointments with high time restrictions requires practice facilities with good manufacturing, and the procedure is always accompanied by high costs. In this context, allogeneic DPSCs may serve as a potential alternative, which can be produced in high volume and manipulated ready for REPs in the clinic. The immunomodulatory effects of allogeneic MSCs are suggested to be of importance in inflammatory disorders. Allogenic umbilical cord MSCs have been used for mature permanent teeth with combination of plasma-derived biomaterials, showing acceptable safety and efficacy in a phase 1/2 clinical trial (Brizuela et al., 2020). Although transplantation of allogeneic MSCs in REPs shows promising prospects, more research is needed regarding immunogenicity, long-term outcomes, and safety.

CONCLUSION

Cell-free REPs, including revascularization and cell homing with molecules recruiting endogenous MSCs, are successful in resolving apical periodontitis and arrested root formation, which are eventually clinical regenerative endodontics and widely applied in treating immature permanent teeth with necrotic pulp. However, histological studies show that pulp–dentin complex is absent in these cases although some studies show a positive response to vitality testing. Instead, cell-based REPs with dental MSCs have shown potential with pulp–dentin regeneration in large animal studies and clinical trials through cell transplantation. Before clinical translation of cell-based REPs, more research is still needed regarding isolation of stem cells, expansion of cells *in vitro*, good practice facilities, skills of clinicians, training of assistants, and reduction of costs. It is hoped that, when cell-based REPs realize true regeneration, they can be applied to the management of necrotic immature permanent teeth, resulting in long-term survival of patients' natural teeth and dentition.

AUTHOR CONTRIBUTIONS

DC and MW conceived the idea and designed the work. SY made the figures. XZ, YL, and YP integrated the materials. LZ revised the manuscript. DC wrote the manuscript. MW revised the manuscript critically. All authors have read and approved the final manuscript.

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A Dedifferentiation Strategy to Enhance the Osteogenic Potential of Dental Derived Stem Cells

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Dental stem cells (DSCs) holds the ability to differentiate into numerous cell types. This property makes these cells particularly appropriate for therapeutic use in regenerative medicine. We report evidence that when DSCs undergo osteogenic differentiation, the osteoblast-like cells can be reverted back to a stem-like state and then further differentiated toward the osteogenic phenotype again, without gene manipulation. We have investigated two different MSCs types, both from dental tissues: dental follicle progenitor stem cells (DFPCs) and dental pulp stem cells (DPSCs). After osteogenic differentiation, both DFPCs and DPSCs can be reverted to a naïve stem cell-like status; importantly, dedifferentiated DSCs showed a greater potential to further differentiate toward the osteogenic phenotype. Our report aims to demonstrate for the first time that it is possible, under physiological conditions, to control the dedifferentiation of DSCs and that the rerouting of cell fate could potentially be used to enhance their osteogenic therapeutic potential. Significantly, this study first validates the use of dedifferentiated DSCs as an alternative source for bone tissue engineering.

Keywords: dental stem cells (DSCs), dental follicle progenitor stem cells (DFPCs), dental pulp stem cells (DPSCs), stem cell fate, dedifferentiation

INTRODUCTION

Bone loss occurring after trauma or diseases is one of the main issues affecting the quality of life of many people worldwide (Paduano et al., 2017). Up to now, autologous bone transplantation has been considered the gold standard for the treatment for bone defects; however, it has several limitations, such as morbidity at the donor site and the limited availability of grafting material (Paduano et al., 2017). Therefore, new strategies are needed for the treatment and repair of bone defects.

Mesenchymal stem cells (MSCs) of dental origin, due to their multilineage differentiation ability, are considered a suitable cell source for clinical applications (Chatzivasileiou et al., 2013;

Paduano et al., 2016; Ballini et al., 2017, 2018). However, there are several issues still to overcome before DSCs can be used in bone cell therapy. Dental stem cells (DSCs), like other MSC types, can exhibit a relatively low cell survival rate and differentiation potential *in vivo,* and this can significantly reduce their effectiveness in stem cell therapy, and thus their clinical usage (Liu et al., 2011; Rui et al., 2015). Consequently, it is of considerable interest to explore novel strategies to improve the regenerative commitment of DSCs. In this context, several researchers have demonstrated that the dedifferentiation process provides an interesting strategy for improving the ultimate differentiation potential of MSCs *in vivo* (Liu et al., 2011).

It has been hypothesised that fully differentiated mammalian cells are unable to undertake reverse cell differentiation, a process known as "dedifferentiation" (Zhang et al., 2010). However, recent evidence has shown that specific cells, such as epidermal cells, could be stimulated to dedifferentiate when exposed to specific stimuli, resulting in the reversion of cells to a less differentiated and more pluripotent state (Zhang et al., 2010). Indeed, it is also well known that somatic cells of human origin can be induced to dedifferentiate into induced pluripotent stem cells (iPSCs) by introducing the Yamanaka transcription factors *Klf4, c-Myc*, *Sox-2,* and *Oct4* (Pennock et al., 2015). However, until now, dedifferentiation of cells has been mainly obtained by nuclear reprogramming, which is a non-physiological process.

Recently, dedifferentiation has been considered for therapeutic application to enable the rerouting of cell fate by inducing the reversion of differentiated cells to a less differentiated state. This is subsequently characterised by an increased differentiation potential (Odelberg et al., 2000). In 2005, a study demonstrated that MSCs isolated from rat bone marrow, without gene manipulation, could be reprogrammed *in vitro* through osteogenic differentiation and dedifferentiation, resulting in cells having an increased capacity to form ectopic bone in nude mice (Rui et al., 2015). Following this dedifferentiation process, MSCs can be reverted to more primitive stem cells with improved osteogenic potential, cell survival, migratory capacity, colony-forming ability and increased expression of *Oct4*, *Sox2,* and *Nanog* (Rui et al., 2015). Furthermore, a more recent study has provided evidence that MSCs reprogrammed through neuronal differentiation and dedifferentiation are more therapeutically efficacious (Liu et al., 2011). These findings are clearly of significant interest as they potentially provide a physiological approach to overcome the limitations of iPSC, such as their genomic instability, tumorigenicity and immunogenicity (Lu and Zhao, 2013).

While some evidence shows that differentiated MSCs possess the ability to dedifferentiate into immature progenitors without genetic manipulation (Zhang et al., 2010; Sawa et al., 2019), no studies have shown whether DSCs have the ability to dedifferentiation into a stem cell-like state. Thus, this study investigated if DSCs can be dedifferentiated into a stem cell-like state without gene manipulation and if the dedifferentiation process can efficiently enhance the osteogenic capacity of DSCs *in vitro*.

This study aimed at evaluating the dedifferentiation strategy and its potential for enhancing the therapeutic efficacy of DSCs for bone-regenerative medicine applications.

RESULTS

Osteogenic Dedifferentiated DFPCs

DFPCs used in this study express the typical MSC surface markers and possess the ability to form colony-forming units (CFU-F) and the multilineage differentiation potential (**Supplementary Figures 1–3**).

The methods used to drive the osteogenic differentiation, dedifferentiation and redifferentiation of DFPCs are schematically illustrated in **Figure 1A**. Briefly, undifferentiated DFPCs were induced in osteogenic medium for 10 days (Osteo-DFPCs), washed in PBS, and then re-incubated with the basal medium for a further 10 days (Dediff-DFPCs); redifferentiation was subsequently achieved using the osteogenic medium for another 10 days (Rediff-DFPCs, **Figure 1A**). To characterise the osteogenic commitment of DFPCs in each differentiation state, calcium deposits were assayed by Alizarin Red staining (**Figure 1B**). Withdrawal of osteogenic medium for 10 days reverted DFPC-derived osteogenic-like cells (Osteo-DFPCs) to cells with characteristic mesenchymal morphology and with reduced formation of mineralised nodules (Dediff-DFPCs). Intriguingly, these Dediff-DFPCs derived from differentiated osteogenic cultures (Osteo-DFPCs) could be re-induced into an osteogenic phenotype on re-exposure to osteogenic medium for 10 days (Rediff-DFPCs).

Importantly, results revealed that after 10 days of osteogenic induction medium, the presence of mineralisation was observed in both Rediff-DFPCs and Osteo-DFPCs. However, we detected a significantly higher Alizarin Red staining in Rediff-DFPCs than Osteo-DFPCs (**Figure 1B**). Also, spectrophotometric quantification of alizarin red staining indicated a higher mineralisation and calcium uptake in the Rediff-DFPCs than for the Osteo-DFPCs (**Figure 1C**). Importantly, Dediff-DFPCs retained their immunophenotype similar to that of undifferentiated DFPCs (**Figure 1D**).

Considering these interesting results, we subsequently compared osteogenic differentiation between Rediff-DFPCs and DFPCs cultured for 30 days in osteogenic medium (Osteo 30 days-DFPCs), the method widely used to differentiate MSCs toward an osteogenic lineage. Transcript levels of osteogenic-related genes were also assessed by qRT-PCR. As shown in **Figure 1E**, results demonstrated that the early inducer of osteogenic commitment runt-related transcription factor 2 (*Runx-2*), as well as the mature osteoblast marker osteocalcin (*OSC*) and bone-related protein osteonectin (*ON*), were significantly increased in Rediff-DFPCs compared with DFPCs cultured for 30 days in osteogenic medium (Osteo-30 days-DFPCs).

Taken together, these data demonstrated the enhanced expression of osteogenic-related transcripts in the Rediff-DFPCs derived from Dediff-DFPCs compared with Osteo 30 days-DFPCs. These data indicated that Dediff-DFPCs maintained

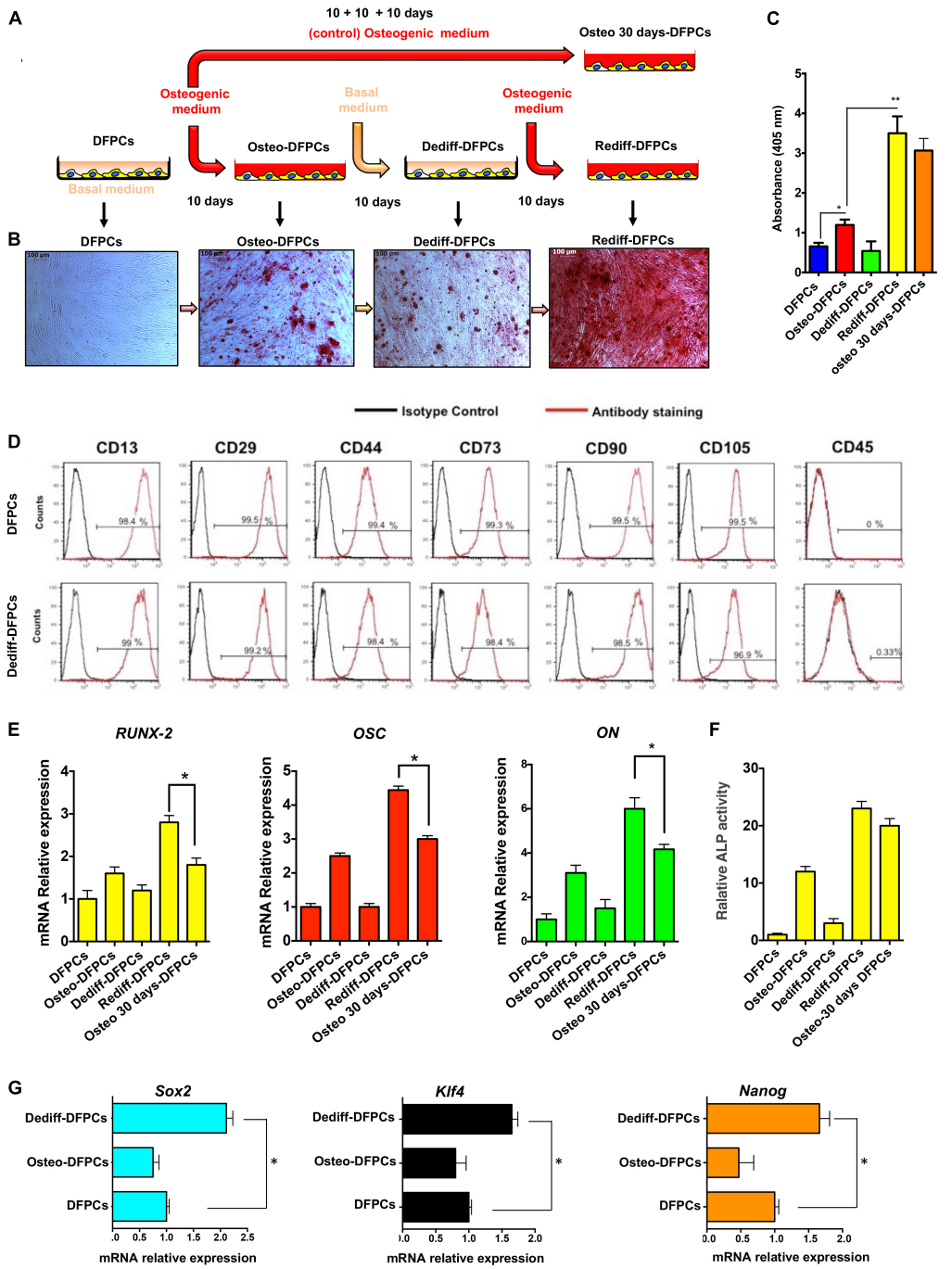

FIGURE 1 | Osteogenic differentiation (Osteo-DFPCs), dedifferentiation (Dediff-DFPCs), and redifferentiation (Rediff-DFSCs) of DFPCs. **(A)** Schematic representation showing the technique for deriving Osteo-DFPCs, Dediff-DFPCs, and Rediff-DFPCs as described in the Materials and Methods. Briefly, undifferentiated DFPCs were stimulated for 10 days in osteogenic medium, rinsed in PBS, and then re-incubated for another 10 days with basal medium; redifferentiation was subsequently achieved using the osteogenic medium for another 10 days. **(B)** Alizarin Red stained images showing the presence of calcium deposition produced by DFPCs. The untreated DFPCs, Osteo-DFPCs, Dediff-DFPCs, and Rediff-DFPCs were incubated with (-MEM or osteogenic induced medium following the schematic provided in **(A)**, then DSCs were fixed and subjected to Alizarin Red staining. **(C)** Alizarin red quantification showing calcium deposition in Osteo-DFPCs, Dediff-DFPCs, and Rediff-DFPCs compared to DFPCs control. $^{*}P < 0.05$ and $^{**}P < 0.01$. DFPCs cultured in osteogenic medium for 30 days were used as a positive control for osteogenic differentiation (Osteo 30 days-DFPCs). **(D)** Cell surface markers of DFPCs and Dediff-DFPCs. **(E)** Relative mRNA expression levels of Runx2, OSC, and ON assayed by qRT-PCR in Osteo-DFPCs, Dediff-DFPCs, and Rediff-DFPCs compared to DFPCs control. HPRT mRNA levels were used for normalisation. The data are shown as mean ± SD ($n = 3$), $^{*}P < 0.05$, compared to Osteo 30 days-DFPCs. **(F)** Relative ALP activity in Osteo-DFPCs, Dediff-DFPCs and Rediff-DFPCs compared to DFPCs control. **(G)** mRNA expression of the stemness-associated genes Sox2, Klf4, and Nanog in Dediff-DFPCs and Osteo-DFPCs compared with DFPCs. Data are shown as fold increase with respect to mRNA level expressed in undifferentiated DFPCs. $^{*}P < 0.05$.

osteogenic commitment, and therefore, a higher capacity to redifferentiate toward the osteogenic phenotype. In addition, as displayed in **Figure 1F**, Rediff-DFPCs also showed an increase of ALP activity compared with Osteo 30 days-DFPCs.

As *Sox2*, *KLf4*, and *Nanog* are key pluripotent genes necessary for reprogramming iPSCs and embryonic stem cell (ESC) pluripotency, we investigated their expression using qRT-PCR. Interestingly, results showed a significantly higher expression of *Sox2*, *KLf4*, and *Nanog* in Dediff-DFPCs than untreated DFPCs, suggesting a more primitive phenotype of dedifferentiated DFPCs (Dediff-DFPCs, **Figure 1G**). These results indicated that the stemness genes Sox2, Klf4, and Nanog play a crucial role in maintaining the self-renewal state in Dediff-DFPCs.

Interestingly, Dediff-DFPCs exhibited advantages in proliferation and clonogenicity over untreated DFPCs (**Supplementary Figure 5**).

Dedifferentiated DPSCs Exhibit Enhanced Osteogenic Differentiation Capabilities

As DPSCs are the most widely studied and well-characterised DSC type [3,4], we investigated the osteogenic differentiation capacity of dedifferentiated DPSCs (Dediff-DPSCs). As observed previously for DFPCs, DPSCs used in this study also express the typical MSC surface markers and possess the ability to form colony-forming units (CFU-F) and the multilineage differentiation potential (**Supplementary Figures 2–4**).

Importantly, we compared the osteogenic potential of Rediff-DPSCs not only with that of undifferentiated DPSCs but also with that of DPSCs grown in osteogenic medium for 30 days (Osteo 30 days-DPSCs), the standard method used to differentiate DPSCs into osteogenic-like cells *in vitro* (Marrelli et al., 2013; Barry et al., 2016*)*.

The approach used to stimulate osteogenic differentiation, dedifferentiation, redifferentiation and osteogenic differentiation for 30 days of DPSCs (Osteo 30 days-DPSCs) is schematically identical to **Figure 1A**. As observed previously for DFPCs, the Dediff-DPSCs derived from differentiated osteogenic cultures (Osteo-DPSCs) could also be re-induced into an osteogenic phenotype following re-exposure to osteogenic medium for 10 days (Rediff-DPSCs).

Importantly, results showed that after osteogenic medium induction, Alizarin Red positive stained calcium nodules formed in the Rediff-DFPCs were significantly greater than those observed in Osteo 30 days-DPSCs (**Figure 2A**). Spectrophotometric quantification of alizarin red staining demonstrated higher mineralisation in the Rediff-DPSCs than Osteo 30 days-DPSCs (**Figure 2B**). These data demonstrate that dedifferentiated DPSCs possesses greater potential for redifferentiation toward the osteogenic phenotype. Importantly, Dediff-DPSCs retained their immunophenotype similar to that of undifferentiated DPSCs (**Figure 2C**).

In addition, we observed that the mRNA expression levels of *osteonectin* (*ON*) and *osteopontin* (OPN), two markers of osteogenic differentiation, as well as transcript levels of two important markers of dental pulp cell differentiation, including *Dmp-1* and *Dspp* are were considerably increased during osteogenic differentiation (Osteo-DPSCs). Notably, the mRNA expression levels of all these genes decreased during the withdrawal of the osteogenic stimuli (Dediff-DPSCs, **Figure 2D**).

In addition, as displayed in **Figure 2E**, Rediff-DPSCs also showed an increase of ALP activity compared with Osteo 30 days-DPSCs.

The progression from osteogenic differentiated to undifferentiated cells is associated with a significant reduction in the expression of *ON, OPN, Dmp-1,* and *Dspp*. Notably, the mRNA levels of these markers were again upregulated in Rediff-DFPCs, cultures that were re-exposed to osteogenic medium.

As *Sox2*, *Klf4,* and *Nanog* are key pluripotent genes necessary for reprogramming iPSCs and ESC pluripotency (Pennock et al., 2015), we investigated their expression using a qRT-PCR assay. Interestingly, results showed a significantly higher expression of *Sox2, Klf4,* and *Nanog* in Dediff-DPSCs with respect to untreated DPSCs, demonstrating a more primitive phenotype of dedifferentiated DPSCs (Dediff-DPSCs, **Figure 2F**). The mRNA expression levels of these pluripotent genes were decreased during osteogenic differentiation (Osteo-DPSCs), whereas they were increased during the withdrawal of the osteogenic stimuli (Dediff-DPSCs). These results indicated that these stemness genes play a crucial role in maintaining the self-renewal state in Dediff-DPSCs. As observed previously for DFPCs, Dediff-DPSCs also exhibited advantages in proliferation and clonogenicity over untreated DPSCs (**Supplementary Figure 5**).

DISCUSSION

In our study, we analysed the osteogenic differentiation potential of redifferentiated DSCs (DFPCs and DPSCs) compared to osteogenic differentiated DSCs. Results showed that following osteogenic differentiation and dedifferentiation *in vitro*, both DSC types exhibited enhanced osteogenic differentiation potential. We also observed that the transcription factors *Sox2, Klf4,* and *Nanog*, key pluripotent genes, were upregulated after the dedifferentiation process of DSCs. Both dedifferentiated DSCs were characterised by an elevated mRNA expression level of *Sox2, Klf4,* and *Nanog*. It is well known that these transcription factors are essential for the maintenance of pluripotency, and therefore, we hypothesise that they could play a crucial role in the dedifferentiation process in these cells. Although it is well known that pluripotent genes such as *Sox2* and *Nanog* are critical for the maintenance of the pluripotency of embryonic stem (ES) cells and induced pluripotent stem cells (iPSC), it has been shown that these genes play a similar role also in adult stem cells (Rui et al., 2015). For example, the overexpression of *Nanog* and *Oct4* or Sox2 can improve the stemness and osteogenesis of human bone marrow MSCs (Go et al., 2008; Liu et al., 2009). Therefore, pluripotent genes are not only essential for self-renewal and pluripotency of ES cells and iPSCs, but also for MSCs such as DSCs.

Similar results were previously reported by Rui et al. (2015) who studied dedifferentiated bone marrow stem

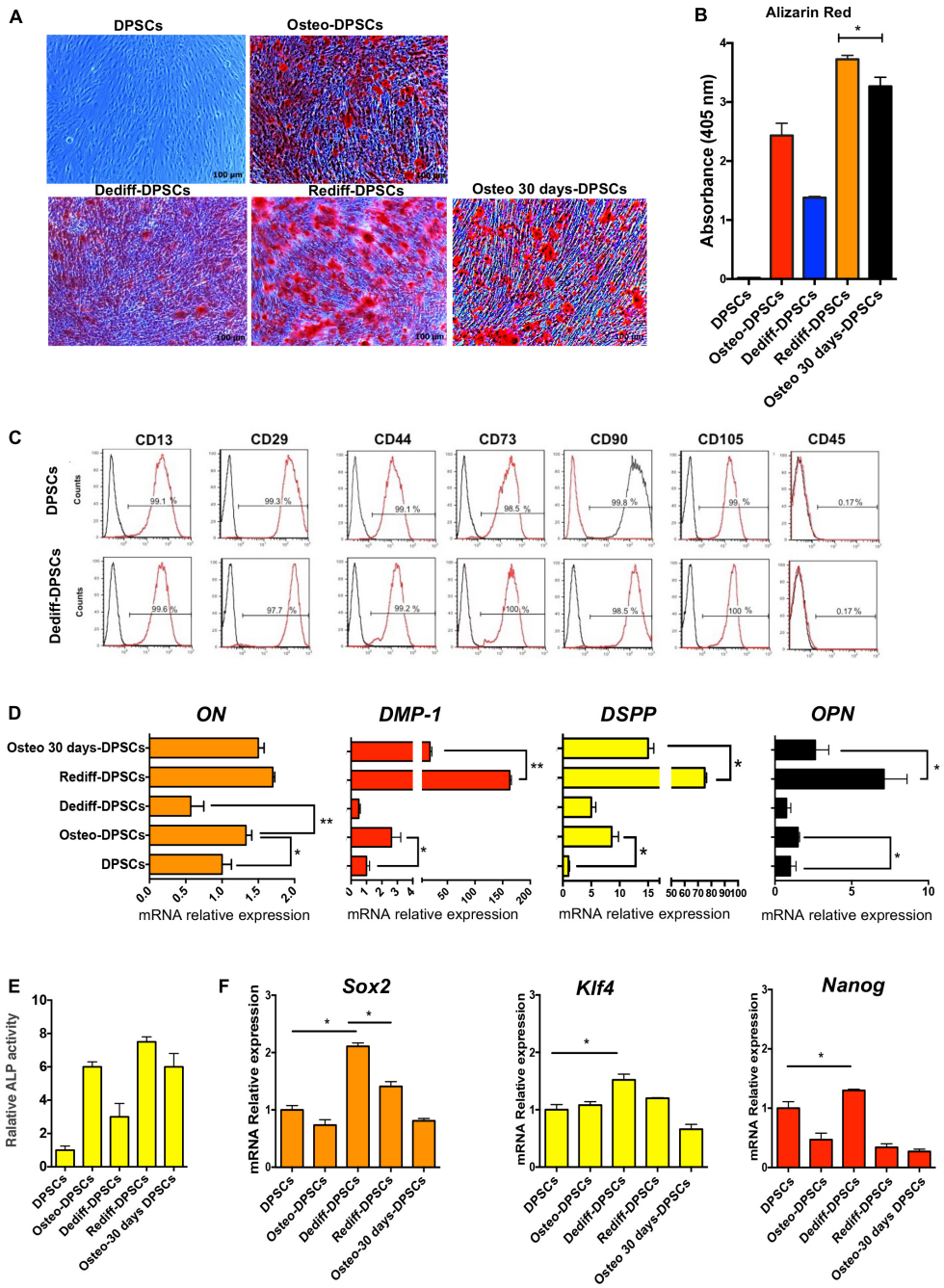

FIGURE 2 | Osteogenic differentiation (Osteo-DPSCs), dedifferentiation (Dediff-DPSCs) redifferentiation (Rediff-DPSCs) of DPSCs and osteogenic differentiation for 30 days (Osteo 30 days-DPSCs) used as control. **(A)** Alizarin red stain images showing calcium deposition in DPSCs, Osteo-DPSCs, Dediff-DPSCs, Rediff-DPSCs, and Osteo 30 days-DPSCs. **(B)** Alizarin red quantification showing mineralised deposits in Osteo-DPSCs, Dediff-DPSCs, Rediff-DPSCs, and Osteo 30 days-DPSCs. **(C)** Cell surface markers of DPSCs and Dediff-DPSCs. **(D)** Relative expression levels of *ON, DMP-1, DSPP,* and *OPN* assayed by qRT-PCR in DPSCs, Osteo-DPSCs, Dediff-DPSCs, Rediff-DPSCs, and Osteo 30 days-DPSCs. mRNA *HPRT* levels were used as control. Results are shown as mean ± SD ($n = 3$), $^{*}P < 0.05$; $^{**}P < 0.01$. **(E)** Relative ALP activity in Osteo-DPSCs, Dediff-DPSCs, and Rediff-DPSCs compared to DPSCs control. **(F)** mRNA expression of the stemness-associated genes *Sox2, Klf4, and Nanog* in Osteo-DPSCs, Dediff-DPSCs, Rediff-DPSCs, and Osteo 30 days-DPSCs with respect to DPSCs. $^{*}P < 0.05$.

cells (BMSCs). In their work, they demonstrated that the mRNA expression levels of *Sox2* and *Nanog* were increased in dedifferentiated BMSCs compared with untreated cells. Our results, together with those described above, suggest that the improvement of MSC potency observed during the dedifferentiation process could be related to the increased expression of *Sox2* and *Nanog*. Indeed, the induced alteration of gene expression observed in dedifferentiated

DSCs could confer an extended differentiation potential to these cells.

Importantly, here we also demonstrated for the first time that the modulation of culture conditions by altering the levels of osteogenic inducing factors could be used in human DSCs to induce the dedifferentiation process. Interestingly, this dedifferentiation strategy can increase the osteogenic differentiation capacity of DSCs compared with the standard method to differentiate DFPCs or DPSCs into osteogenic-like cells *in vitro*. Importantly, Rui et al. obtained similar results to ours and demonstrated that this differentiation strategy could be used to enhance the osteogenic therapeutic potential of BMSCs (Pennock et al., 2015; Rui et al., 2015).

Interestingly, it has been shown that epigenetic changes can regulate stem cell differentiation and dedifferentiation. Indeed, particular patterns of histone modifications and DNA methylation have an essential role in stimulating DPSC differentiation toward the osteogenic lineage (Paino et al., 2014). Two independent studies have demonstrated that histone deacetylase (HDAC) inhibition with valproic acid significantly improved mineralised matrix formation by improving the expression of bone glycoproteins involved in the formation of the mineralised matrix (Paino et al., 2014; Duncan et al., 2016). However, HDAC inhibitors could not easily be utilised *in vivo* to enhance the osteogenic differentiation potential of DPSCs. Thus, the development of an alternative strategy, such as the dedifferentiation process described here, which is able to reinforce the osteogenic differentiation potential of cells is needed.

While *in vitro* evidence indicates that dedifferentiated SCs provide a promising cell source for clinical applications, no clinical studies have yet been performed using this approach. Here, we have identified an alternative method to enhance the osteogenic potential of DSCs that could provide hypothetical clinical benefits with respect to the use of undifferentiated cells.

Naïve DSCs, like other MSC types, exhibit relatively low cell survival rates and differentiation potential *in vivo*. This finding can significantly reduce the effectiveness of stem cell therapies, and thus their clinical usage. To avoid this problem, we propose the use of cells having a higher osteogenic potential that can be relatively easily derived by using a physiological approach. The dedifferentiation process can be considered a physiological mechanism since it exists in various tissues and organs from animals, amphibians and plants (Yao and Wang, 2020). For example, cardiomyocytes and Schwann cells can dedifferentiate during cardiac regeneration and nerve injury, respectively (Yao and Wang, 2020). Thus, dedifferentiation is mechanistically associated with natural regeneration since it is part of the physiological response to injury in numerous organs (Merrell and Stanger, 2016).

Thus, due to their enhanced ability to differentiate toward the osteogenic commitment phenotype, instead of using naïve DSCs, we propose the use of dedifferentiated DSCs for bone therapeutic purposes.

The data generated in this study has the potential to be translated for the development of novel and more efficacious therapeutic approaches for human bone and dental tissue regeneration. Despite the scientific literature reporting several

TABLE 1 | Primer details used for qRT-PCR analysis.

Gene symbol		Sequence (5′–3′)	NCBI accession number
Runx-2[a]	**Forward:**	ATGTGTGTTTGTTTCAGCAGCA	NM_001278478.2
	Reverse:	TCCCTAAAGTCACTCGGTATGTGTA	
Klf4[b]	**Forward:**	CCATCTTTCTCCACGTTCG	NM_004235.4
	Reverse:	AGTCGCTTCATGTGGGAG	
Sox2[c]	**Forward:**	GACTTCACATGTCCCAGCACTA	NM_003106.3
	Reverse:	CTCTTTTGCACCCCTCCCATT	
Nanog[d]	**Forward:**	ATTCAGGACAGCCCTGATTCTTC	NM_024865.3
	Reverse:	TTTTTGCGACACTCTTCTCTGC	
HPRT[e]	**Forward:**	TGACACTGGCAAAACAATGCA	NM_000194.2
	Reverse:	GGTCCTTTTCACCAGCAAGCT	
ON[f]	**Forward:**	TGCATGTGTCTTAGTCTTAGTCACC	NM_001309443.2
	Reverse:	GCTAACTTAGTGCTTACAGGAACCA	
OSC[g]	**Forward:**	TGAGAGCCCTCACACTCCTC	NM_199173.6
	Reverse:	ACCTTTGCTGGACTCTGCAC	
DMP-1[h]	**Forward:**	GTGAGTGAGTCCAGGGGAGATAA	NM_004407.3
	Reverse:	TTTTGAGTGGGAGAGTGTGTGC	
DSPP[i]	**Forward:**	CTGTTGGGAAGAGCCAAGATAAG	NM_014208.3
	Reverse:	CCAAGATCATTCCATGTTGTCCT	
OPN[j]	**Forward:**	CAGTTGTCCCCACAGTAGACAC	NM_001040058.1
	Reverse:	GTGATGTCCTCGTCTGTAGCATC	

[a]*Runx-2, RUNX family transcription factor;* [b]*Klf4, kruppel-like factor 4;* [c]*Sox2, SRY-related HMG-box 2;* [d]*Nanog, homeobox transcription factor Nanog;* [e]*HPRT, hypoxanthine phosphoribosyltransferase;* [f]*ON, osteonectin;* [g]*OSC, osteocalcin;* [h]*DMP-1, dentin matrix acid phosphoprotein-1;* [i]*DSPP, dentin sialophosphoprotein;* [j]*OPN, osteopontin.*

tissue engineering strategies involving MSCs (Paduano et al., 2016, 2017; Posa et al., 2016; Ballini et al., 2017, 2018; Marrelli et al., 2018) or biomimetic materials (Marrelli et al., 2013; Barry et al., 2016; Tatullo et al., 2018), biomedical researchers continue to face key difficulties which hinder translational approaches for bone regeneration, including issues relating to patient safety following extended cell manipulation (Marrelli et al., 2013; Cantore et al., 2016; Duncan et al., 2016; Marrazzo et al., 2016; Spagnuolo et al., 2020). Importantly, the approach described here requires a relatively simple and safe methodology that does not modify the biological characteristics of the cells.

A limitation of this study is the current lack of evaluation of the multi-differentiation potential of dedifferentiated DSCs. Currently, we have only focused on the evaluation of the osteogenic differentiation potential of dedifferentiated DSCs. We believe that the knowledge of DSC physiology in terms of dedifferentiation and redifferentiation could enhance the development of novel and more competent therapeutic strategies for human bone regeneration. The significance of this study is its exploration of the possibility to use dedifferentiated DSCs as an alternative stem cell source for bone tissue engineering. Importantly, our strategy requires the use of good manufacturing procedures that do not alter the biological features of these accessible cells and thus, it would also be ethically acceptable.

It is essential to underline that both types of DSCs studied were isolated using an enzymatic digestion strategy. Therefore, although we observed that the population of dedifferentiated DSCs possesses a phenotype distinct from their undifferentiated counterpart, we are aware that there is phenotypic heterogeneity in each DSC population. Consequently, the heterogeneity of the DSC population could make the generalisation of findings difficult, and therefore, future studies are necessary to generate a standardised protocol.

CONCLUSION

Our results show for the first time that under physiological conditions, it is possible to induce the dedifferentiation of DSCs. Importantly, dedifferentiated DSCs exhibit an enhanced potential for osteogenic differentiation compared with their undifferentiated counterpart. Therefore, the dedifferentiation strategy could be potentially used to enhance the osteogenic therapeutic potential of DSCs. Furthermore, *in vivo* studies are necessary to evaluate the therapeutic efficacy of dedifferentiated DSCs *in vivo* and shed light on the molecular mechanisms involved in DSC dedifferentiation. In conclusion, we provide a novel approach in dental stem cell biology, in which the rerouting of DSCs fate could offer new therapeutic opportunities.

MATERIALS AND METHODS

Isolation and Application of Dental Stem Cells

In this study, two types of human dental stem (DSCs) were studied and included: dental follicle progenitor stem cells (DFPCs) and dental pulp stem cells (DPSCs). Freshly isolated DFPCs and DPSCs were regarded as being at the undifferentiated stage.

DFPCs and DPSCs were isolated as previously described (Chatzivasileiou et al., 2013; Marrelli et al., 2015; Tatullo et al., 2015a,b; Paduano et al., 2016, 2017). DSCs were obtained from healthy volunteers, who provided informed consent at the *Calabrodental* Dental Clinic (Crotone, Italy). The study was performed under guidelines approved by the Ethical Committee at the Calabrodental Dental Clinic (Ethical agreement number CBD-001/TRI/2020). Briefly, impacted third molars were rinsed twice in PBS containing streptomycin (100 μg/mL) and penicillin (100 U/mL). Dental pulp tissues were separated from the crown and root, and the freshly extracted dental follicle was separated from the mineralised tooth. Collected dental pulp tissue and dental follicles were minced and immersed in a solution containing dispase (4 mg/mL) and type I collagenase (3 mg/mL) at 37°C for 60 min. Each cell suspension was filtered using Falcon strainers (70 μM) and incubated in basal media comprised of alpha-MEM culture medium containing 10% FBS (Invitrogen, Carlsbad, California, United States), streptomycin (100 mg/mL), glutamine (2 mM) and penicillin (100 U/mL). Both DSCs were incubated at 37°C with 5% CO_2.

Osteogenic Differentiation, Dedifferentiation and Re-differentiation Analysis of DSCs

Cells were plated into 100-mm cell culture dishes at a cell density of 3×10^5 (DFPCs) and 4×10^5 DPSCs until reaching a confluence of 60–70%. Then, to induce osteogenic differentiation, undifferentiated (freshly isolated) DFPCs and DPSCs were grown in osteogenic medium containing α-MEM (Sigma, St. Louis, MO, United States) supplemented with 50 μg/mL L-ascorbic acid, 5 mM β-Glycerophosphate, 10 nM dexamethasone and 20% FBS (Invitrogen, Carlsbad, CA, United States), for 10 days (Osteo-DFPCs and Osteo-DPSCs) according to previously described procedures (Marrazzo et al., 2016; Paduano et al., 2017). Subsequently, the osteogenic medium was replenished with basal media, and subsequently, cells were grown for a further 10 days (Dediff-DFPCs and Dediff-DPSCs). These cells were considered in a dedifferentiated stage (Dediff).

The basal medium was then again replaced with osteogenic medium for 10 days (Rediff-DFPCs and Rediff-DPSCs). Importantly, DFPCs and DPSCs cultured in osteogenic medium for 30 days were used as a positive control for osteogenic differentiation (Osteo 30 days-DPSCs). Mineralisation of DFPCs and DPSCs were observed on day 10 (Osteo-DFPCs and Osteo-DPSCs), at day 20 (Dediff-DFPCs and Dediff-DPSCs) and day 30 (Rediff-DFPCs, Rediff-DPSCs, Osteo 30 days-DPSCs. Since DFPCs exhibit a proliferative rate higher than DPSCs, instead of at 10 days, they can be induced toward the osteogenic lineage in the presence of osteogenic medium for 7 days.

Briefly, on the indicated day, cells were rinsed with PBS and fixed with paraformaldehyde 4% (Sigma, St. Louis, MO, United States) at room temperature for 20 min. A 5 mg/mL concentrated solution of Alizarin Red (Sigma, St. Louis, Missouri, United States) was added to both cell types for 30 min, and the quantification of Alizarin Red was obtained as we have previously

described elsewhere [16]. Briefly, cell samples were cultivated in 10% acetic acid and boiled for 10 min. After centrifugation, samples were evaluated at a wavelength of 405 nm using a Multiskan Go Spectrophotometer (Thermo Fisher Scientific, Waltham, MA, United States) (Paduano et al., 2017).

Gene Expression Analysis

RNA was isolated from DFPCs and DPSCs according to the *Purelink RNA mini kit* protocol (Applied Biosystem, United Kingdom), and the concentrations of extracted RNA were calculated using a spectrophotometer (Multiskan Go, Thermo Fisher Scientific, Waltham, Massachusetts, United States). Gene expression levels were measured by qRT-PCR, as previously described (Paino et al., 2014; Paduano et al., 2016, 2017; Tatullo et al., 2017, 2018). Briefly, RNA (200 ng) was reverse-transcribed according to the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, California, United States). Subsequently, cDNA samples were amplified by qRT-PCR using specific primers supplemented with SYBR Green Master Mix. qRT-PCR reactions were performed using the following conditions: initial denaturation step at 95°C for 10 min, followed by 40 cycles of 10 s at 95°C and 1 min at 60°C. The specificity of PCR products was checked by melting curve analysis. After amplification, mRNA expression levels of the target genes were calculated by the $2\text{-}\Delta\Delta Ct$ method. Hypoxanthine phosphoribosyltransferase (HRPT) was used as a control to normalise the levels of mRNA in all samples. See **Table 1** for primer details.

Alkaline Phosphatase (ALP) Assay

ALP activity was analysed using the alkaline phosphatase assay kit (ABCAM). Briefly, for each condition, cells were lysed and centrifuged. Supernatants were collected for the detection of ALP activity as described by the manufacturer. Absorbance was measured at 405 nm, and values were normalised to the total protein per volume of lysate.

Statistical Analysis

Data are shown as mean ± SD from three independent experiments in triplicate. Results were analysed by GraphPad Prism software, and values were indicated statistically significant when $^{*}P < 0.05$ and $^{**}P < 0.01$.

DATA AVAILABILITY STATEMENT

The authors acknowledge that the data presented in this study must be deposited and made publicly available in an acceptable repository, prior to publication. Frontiers cannot accept a manuscript that does not adhere to our open data policies.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Committee at the Calabrodental Dental Clinic (Ethical agreement number CBD-001/TRI/2020. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

FP and MT conceived the study. FP, EA, BM, and MI performed the experiments. FP, EA, MT, and PC analysed and interpreted the data. FP, MT, GS, and DM wrote the manuscript. DD, IM, and PC supervised the project. All authors contributed to the article and approved the submitted version.

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DEDICATION

This article is dedicated to the memory of Massimo Marrelli, who died in Crotone, Italy, on October 27, 2018.

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.668558/full#supplementary-material

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Conflict of Interest: DM was employed by company EMS—Elite Medical Service Ltd.

The remaining 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
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Dental-Derived Mesenchymal Stem Cells: State of the Art

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Mesenchymal stem cells (MSCs) could be identified in mammalian teeth. Currently, dental-derived MSCs (DMSCs) has become a collective term for all the MSCs isolated from dental pulp, periodontal ligament, dental follicle, apical papilla, and even gingiva. These DMSCs possess similar multipotent potential as bone marrow-derived MSCs, including differentiation into cells that have the characteristics of odontoblasts, cementoblasts, osteoblasts, chondrocytes, myocytes, epithelial cells, neural cells, hepatocytes, and adipocytes. Besides, DMSCs also have powerful immunomodulatory functions, which enable them to orchestrate the surrounding immune microenvironment. These properties enable DMSCs to have a promising approach in injury repair, tissue regeneration, and treatment of various diseases. This review outlines the most recent advances in DMSCs' functions and applications and enlightens how these advances are paving the path for DMSC-based therapies.

Keywords: mesenchymal stem cells, tooth regeneration, tissue engineering, bone defect reconstruction, immune modulation

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INTRODUCTION

By revisiting history, the concept of mesenchymal stem cells (MSCs) and some relevant fundamental studies can be traced back to several decades ago (Bianco et al., 2008), which were associated with the breakthrough finding of an intrinsic osteogenic potential of bone marrow cells, as was revealed by heterotopic transplantation (Friedenstein et al., 1968). Subsequently, bone marrow stromal cells, a minor yet distinguishable subpopulation of bone marrow cells, were pointed out to possess osteogenic potential, as well as nonhematopoietic origin, rapid adherence to culture dishes, and colony-forming ability (Friedenstein et al., 1970). In 1991, the term MSCs was first coined by Caplan, proposing their multipotent differentiation ability to give rise to mesodermal lineage (Caplan, 1991). Thereafter, MSCs have been well known for their multipotency (Pittenger et al., 1999) and identified in a variety of tissues and organs since the formulation of the terminology, including, but not limited to, bone marrow, liver, heart, adipose tissue, and scalp tissue (Zuk et al., 2002; Shih et al., 2005; Beltrami et al., 2007).

In 2000, a clonogenic population of dental pulp cells was first isolated by Gronthos et al. (2000) and identified as MSCs due to similar properties as bone marrow stromal cells (BMSCs)

in terms of immunoreactivity profile, as well as the capacity for self-renewal and the potential for multidirectional differentiation. Since then, more and more dental-derived cells have been found to possess stem cell properties and were named according to their tissue of origin, including dental pulp stem cells (DPSCs) (Gronthos et al., 2000), stem cells from human exfoliated deciduous teeth (SHEDs) (Miura et al., 2003), periodontal ligament stem cells (PDLSCs) (Seo et al., 2004), dental follicle stem cells (DFSCs) (Handa et al., 2002; Morsczeck et al., 2005), stem cells from apical papilla (SCAPs) (Sonoyama et al., 2006, 2008), and gingival mesenchymal stem cells (GMSCs) (Zhang et al., 2009) (**Figure 1**). Overall, all the abovementioned dental-derived stem cell populations can be termed collectively as dental-derived mesenchymal stem cells (DMSCs) (Sharpe, 2016). In this review, we will focus on introducing and discussing the most up-to-date advances of DMSCs concerning functions, applications, and beyond.

DIFFERENTIATION AND FUNCTION

Dentinogenesis

Odontoblasts are extremely specialized cells involved in the deposition and mineralization of the dentin matrix, namely, dentinogenesis or dentin formation. The functions of odontoblasts include the formation and regeneration of the dentin–pulp complex (Kawashima and Okiji, 2016). DPSCs and SHEDs are two subpopulations of DMSCs that perform as major resources of odontoblasts during tertiary dentin formation upon postnatal injury since the preexisting odontoblast cannot produce reparative dentin (Tatullo et al., 2015; Mortada and Mortada, 2018). The standard procedure for odontogenic differentiation can be induced *in vitro* by culturing DMSCs in odontogenic medium containing dexamethasone, β-glycerophosphate, and ascorbic acid (Langenbach and Handschel, 2013). Under such culture conditions, DMSCs subsequently express osteoblast-associated markers, including alkaline phosphatase (ALP), collagen type 1 (COL1), osteopontin (OPN), osteocalcin (OCN), dentin matrix acid phosphoprotein 1 (DMP1), matrix extracellular phosphoglycoprotein (MEPE), and dentin sialophosphoprotein (DSPP). These markers are equally widely used as odontoblasts-specific differentiation markers in plenty of studies (Dong et al., 2019; Iezzi et al., 2019). Besides the abovementioned markers, previously unrecognized odontoblast markers have been identified, such as NOTUM and SALL1 (Krivanek et al., 2020), by single-cell RNA sequencing (scRNA-seq), which is a seminal technique developed in recent years and enables researchers to explore transcriptional profiles at the level of a single cell with unprecedented resolution. Interestingly, scRNA-seq uncovered novel functions of some well-defined odontoblast markers as well, for instance, the role of DSPP in amelogenesis (Chiba et al., 2020).

To date, researchers have explored different induction conditions, which aim to promote or impair odontogenic differentiation. Allogeneic fibrin clot (AFC) serum exhibited sufficient cytokines as well as growth factors to induce odontogenic differentiation of human DPSCs and human PDLSCs *in vitro* (Cao et al., 2020). *Sapindus mukorossi* seed oil has also been proven to increase the ALP activity and the secretion of mineralized nodules of DPSCs, and can promote osteogenic or odontogenic differentiation and matrix vesicle secretion of DPSCs under odontogenic induction (Shiu et al., 2020). In contrast, alcohol can suppress DPSC odontogenic differentiation and mineralization, by decreasing the ALP activity, attenuating the formation of mineralized nodules, and suppressing the expression of odontoblastic markers, including ALP, DSPP, DMP1, and OCN. Mechanistically, alcohol negatively regulates odontogenic differentiation through the mechanistic target of rapamycin (mTOR) signaling in a dose-dependent manner (Qin et al., 2017). Hence, quite a few elements are associated with the process of odontogenic differentiation.

Also, some other signaling molecules participate in the odontogenic differentiation process. Copine 7 (CPNE7) works as a diffusible signaling molecule and was proven to induce non-dental MSCs to differentiate into odontoblasts *in vitro via* the control of DSPP expression (Oh et al., 2015). Also, CPNE7 has been demonstrated to promote the formation of dentin-like tissues *in vivo* (Oh et al., 2015). A similar pro-odontogenic effect was found in endothelin-1 (ET-1), which can promote the odontoblastic differentiation of DPSCs (Liu M. et al., 2018). Receptor tyrosine kinases (RTKs) played a pivotal role in cell fate decisions, of which ephrin receptors (Eph) make up the largest known subgroup. After odontogenic induction, EphrinB2 (ligand) and its cognate receptors EphB2 and EphB4 were upregulated in DPSCs in both gene and protein levels, and meanwhile, EphrinB2 signaling showed enhanced effects on osteogenic/odontogenic differentiation of human DPSCs (Heng et al., 2018). Other regulatory molecules are lysine demethylase 6B (KDM6B) (Xu et al., 2013) and pentraxin-3 (PTX3) (Kim Y. et al., 2019). Mechanistically, KDM6B was recruited to BMP2 promoters, leading to the removal of epigenetic marks H3K27me3, activation of *BMP2* transcription, and thus, odontogenic differentiation of DMSCs (Xu et al., 2013). As for PTX3, it was indicated to be positively correlated with osteogenic or odontogenic differentiation of human DPSCs (Kim Y. et al., 2019). The relationship and interaction between different molecules are still unraveled, and it remains to be further explored and elucidated for the underlying mechanisms of odontogenic differentiation.

Apart from culture conditions and signaling molecules, RNA was demonstrated to have an impact on the process of odontogenic differentiation. For instance, miR-223-3p knockdown was proven to increase SMAD3 and inhibit odontogenic differentiation (Huang X. et al., 2019). Upregulated miR-21 was revealed to associate with increased odontogenic differentiation of DPSCs in a tumor necrosis factor-α (TNF-α)-mediated manner (Xu et al., 2018). Meanwhile, miR-143-5p and hsa-let-7c showed the inhibition of odontogenic differentiation of DMSCs (Ma et al., 2016; Liu G.X. et al., 2018; Zhan et al., 2018). Long non-coding RNAs (lncRNAs) have been reported to involve in dentin development and facilitate the odontogenic differentiation of human DPSCs (Zeng et al., 2018). To conclude, odontogenic differentiation and dentinogenesis are governed by a variety of signaling molecules, growth factors, miRNAs,

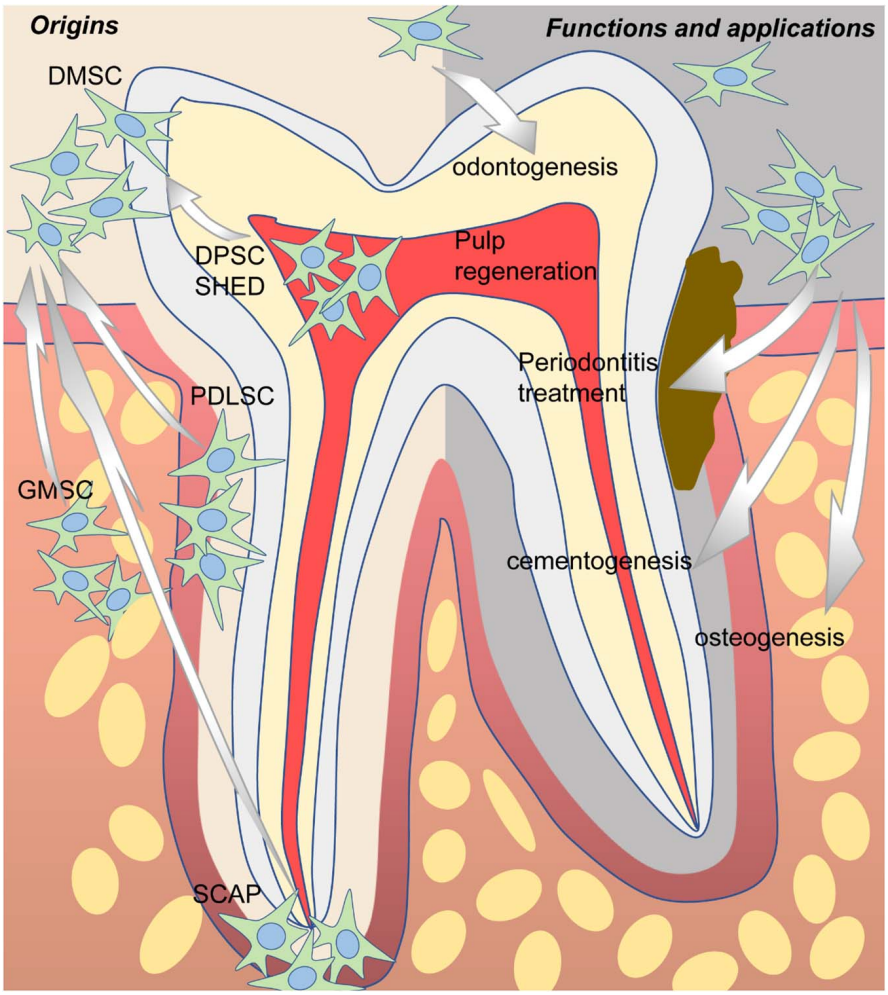

FIGURE 1 | The origins, potential functions, and applications of dental-derived mesenchymal stem cells (DMSCs).

and specific culturing conditions, resulting in a complex and intertwined regulatory network.

Cementogenesis

Cementogenesis refers to the process of cementum formation, which covers the roots of teeth by cementoblasts. Cementoblasts, whose biological function is cementogenesis, are the key cell type responsible for anchoring the periodontal ligament to the tooth (Zhao et al., 2021). In general, cementoblasts can be found within periodontal tissue and play a crucial role in periodontal complex stabilization and regeneration. Furthermore, it has been validated that DMSCs, especially PDLSCs and DFSCs, can differentiate into cementoblasts (Hyun et al., 2017; Zhai et al., 2019; Yang J.W. et al., 2020).

Similarly, extensive studies have been conducted to better understand cementogenic differentiation concerning RNA interference and signaling pathways. Ets variant 1 (ETV1), a differentiation-related transcription factor that reciprocally regulates miR-628-5p and miR-383 coordinately in a feedback loop, is thought to be necessary for cementogenesis (Iwata et al., 2021). Another study focused on miR-361-3p, which decreased during cementoblastic differentiation, while inhibition of miR-361-3p resulted in increased cementoblastic differentiation (Liao et al., 2019). Moreover, the Wnt/β-catenin (WNT) pathway was blocked by forced expression of miR-361-3p in cementoblastic differentiation, whereas multiple other pathways are notably activated, including the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38/MAPK), phosphoinositide 3-kinase (PI3K) protein kinase B (AKT) (PI3K/AKT), and nuclear factor kappa-light-chain-enhancer of activated B-cells (NF-κB) pathways (Liao et al., 2019).

As for signaling pathways, canonical WNT signaling might positively regulate the expression of cementogenic-related markers of DFSCs, including runt-related transcription factor 2 (RUNX2), ALP, OCN, cementum attachment protein (CAP), and cementum protein (CEMP) (Du et al., 2012; Nemoto et al., 2016). A potential link between WNT signaling and the plasminogen-plasmin pathway to control cementogenic differentiation has been reported (Martins et al., 2020). Besides, the PI3K/AKT and

JNK pathways have also been identified to participate in the enhanced cementogenesis of PDLSCs, which can be activated by M2 macrophages through cytokine and/or chemokine secretion, in particular, interleukin-10 (IL-10) and vascular endothelial growth factor (VEGF) (Li et al., 2019).

However, the characterization of DMSCs committed to undergo osteoblast/cementoblast differentiation remains largely unknown (Saito et al., 2014). Some proteins, which have been demonstrated to play vital roles in other lineage commitment and differentiation, can influence the cementoblast lineage cells as well. For instance, Sirtuin 6 (SIRT6) is essential for osteogenic differentiation, but it also inhibits glucose transporter 1 (GLUT1), a glucose transporter necessary in cementogenesis, and inhibited cementoblast differentiation by activating the AMP-activated protein kinase (AMPK) pathway (Huang L. et al., 2019). Furthermore, another good example is amelogenins. Amelogenins are crucial components of developing extracellular enamel matrix, which direct the organization and mineralization of enamel. Studies have demonstrated that C-terminus of amelogenins induces division, differentiation, and maturation of MSCs, especially in cementoblastic lineage cells (Kunimatsu et al., 2017, 2018). Additionally, cementoblastic differentiation of mesenchymal progenitor cells is tightly maintained by an autocrine system mediated by parathyroid hormone (PTH)-related peptide (PTHrP) and the PTH/PTHrP receptor, thereby preventing DMSCs from premature differentiation into cementoblasts (Takahashi et al., 2019). However, there is still insufficient research investigating this aspect, which requires more studies before clinical applications.

Osteogenesis

Osteogenesis, in brief, is the process of bone formation by osteoblasts. Among all subpopulations of DMSCs, DPSCs, and PDLSCs are most extensively studied. DPSCs cultured *in vitro* can differentiate into osteoblast-like cells in addition to its well-known potential to differentiate into odontoblasts (Mortada and Mortada, 2018) (**Figure 2**). This process is deemed to be regulated by various pathways, though not completely elucidated. The BMP-4/Smad signaling pathway is essential for the osteogenic differentiation of DPSCs, which can be suppressed by tumor necrosis factor-inducible protein-6 (TSG-6) (Wang Y. et al., 2020). The impaired osteogenic capacity of DPSCs in the inflammatory microenvironment can be rescued by WNT4 overexpression, which subsequently may function by affecting JNK signaling pathways (Zhong et al., 2019, 2020). LncRNAs, emerging as novel regulators, might play an important role in the osteogenesis of DPSCs by regulating gene expression. Transcriptome microarray identified a series of differentially expressed lncRNAs during osteogenic differentiation in human DPSCs (Zhang et al., 2017). Meanwhile, different lncRNAs may affect the expression of the same target oppositely, for example, RUNX2. To illustrate, lncRNA MEG3 inhibited human DPSCs osteogenic differentiation and promoted RUNX2 degradation via miR-543/SMURF1/RUNX2 regulatory network (Zhao et al., 2020c), whereas lncRNA LINC00968 promoted osteogenic differentiation and bone formation *in vitro* and *in vivo* and upregulated RUNX2 expression through competitive binding of miR-3658 (Liao et al., 2020). Besides, some chemical compounds, such as berberine (BBR), were recently identified to contribute to the osteogenic differentiation of DPSCs (Xin et al., 2020).

Like DPSCs, PDLSCs can also differentiate into osteoblast-like cells and highly expressed osteogenesis-related markers (Seo et al., 2004). Likewise, lncRNAs are associated with the osteogenic differentiation process of PDLSCs (Qu et al., 2016). Depleting lncRNA ANCR in progenitor-containing cell populations led to rapid differentiation gene induction (Kretz et al., 2012). A recent study shows that the silencing of a novel circular RNA (circRNA), circCDK8, can promote osteogenic differentiation of PDLSCs *via* repressing endoplasmic reticulum (ER) stress, autophagy, and apoptosis in a hypoxic microenvironment (Zheng et al., 2021). Furthermore, the effects of many chemical compounds on PDLSCs have been explored. Curcumin, a natural phenolic product derived from the turmeric rhizome, has been indicated to have a variety of biological functions including anti-inflammation, anti-cancer, and antioxidation (Aggarwal et al., 2007; Anand et al., 2007). Recent works have shown that curcumin may be a promising substance to promote the osteogenic differentiation in human PDLSCs by increasing early growth response protein 1 (EGR1) expression (Shi et al., 2021) and activating PI3K/AKT/Nrf2 signaling pathway (Xiong et al., 2020). Fraxinellone, a programmed death-ligand 1 (PD-L1) inhibitor, could alleviate inflammation and strengthen osteogenic differentiation of lipopolysaccharide (LPS)-stimulated PDLSCs through BMP2/Smad pathway (Fu et al., 2021), suggesting the possibility of fraxinellone in bone tissue regeneration. Rutin, a bioflavonoid distributed in fruits and vegetables, was found to significantly enhance the proliferation and osteogenic differentiation of PDLSCs through PI3K/AKT/mTOR signal pathway (Zhao et al., 2020a). In addition, both rutin and another herbal extraction, resveratrol, can protect human PDLSCs from TNF-α-induced damage to osteogenesis (Yuan et al., 2020; Zhao et al., 2020b). The recombinant chimeric protein of angiopoietin-1 (ANG-1) and a short domain of cartilage oligomeric matrix protein (COMP) could also exert the osteogenic role via PI3K/AKT pathway (Kook et al., 2014). Besides biological or chemical factors, physical stimulus, such as laser irradiation, may affect bone formation as well (Abramovitch-Gottlib et al., 2005; Kushibiki et al., 2015). However, the evidence seems inadequate to draw a conclusion, as no difference is observed between positive control and laser-irradiated groups (Pinheiro et al., 2018; Gholami et al., 2020). Further pertinent studies are needed to validate the efficacy of physical interventions on osteogenesis.

Although studies on DPSCs and PDLSCs are accumulated the most, other subpopulations of DMSCs have also been tested for their osteogenic potential. Both DFSCs and SHEDs possess osteogenesis capacity, while SHEDs are considered prospective seeding cells for use in stem cell-mediated bioengineered tooth root regeneration because of their easy accessibility (Yang X. et al., 2019). SCAPs have osteogenic potential as well and can be enhanced by secreted frizzled-related protein 2 (SFRP2), an antagonist of the canonical WNT signaling pathway (Yu et al., 2016). Last but not the least, transplantation of GMSCs effectively contributed to bone defect regeneration, which suggests their

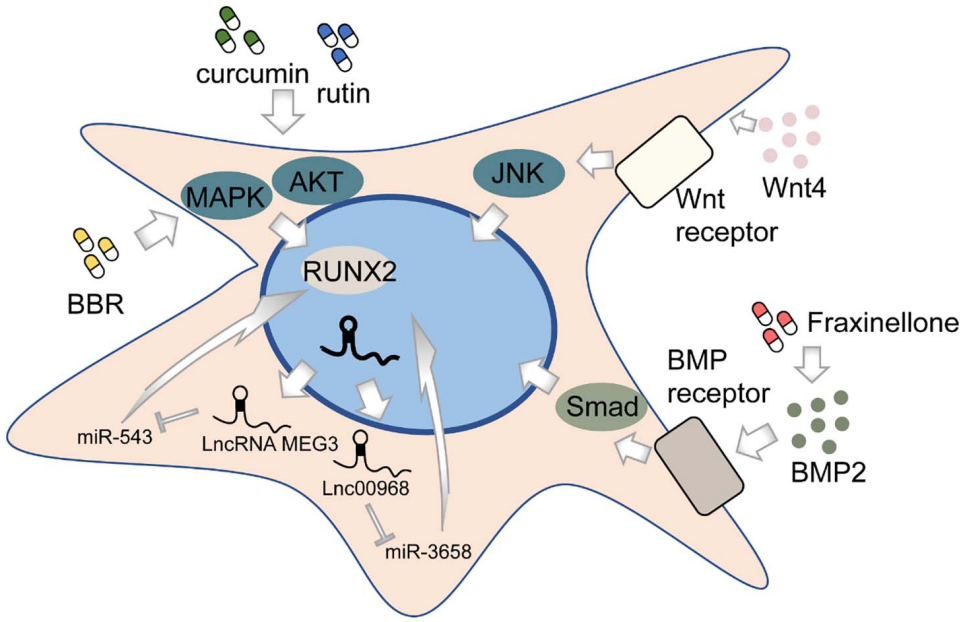

FIGURE 2 | The regulation of osteogenic differentiation of DMSCs.

strong osteogenic potential (Tomar et al., 2010; Wang et al., 2011; Yang et al., 2013).

Chondrogenesis

Chondrogenesis, namely, the formation of cartilage, is directed by chondrocytes. MSCs have chondrogenic potential, which is usually clarified by culturing in the chondrogenic medium as a micromass (Ichinose et al., 2005) and evaluated by Alcian blue staining. The expression level of chondrogenic markers, for instance, collagen II (COL2), collagen V (COL5), and sex-determining region Y box protein 9 (SOX9) can further confirm the differentiation status. Previously, primary synovial MSCs were proof of chondrogenic ability (Kohno et al., 2017). As expected, DMSCs were also validated to have the chondrogenic capacity (Huang et al., 2009). It has been reported that inhibition of ERK1/2 inhibited chondrogenic differentiation of DPSCs (Ba et al., 2017), whereas contradictory findings were shown previously in another paper (Dai et al., 2012), which suggests a complex regulatory mechanism underlying chondrogenic differentiation process. Distal-less homeobox 5 (DLX5) and homeobox C8 (HOXC8) enhanced the chondrogenic differentiation of SCAPs, and their overexpression upregulated the transcriptional activity of COL2, COL5, and SOX9 (Yang H. et al., 2020). Fas cell surface death receptor ligand (FasL) stimulation brought chondrogenic differentiation of human PDLSCs to a higher level, with the evidence of more collagen deposition and the presence of acid mucins and glycosaminoglycans (Pisciotta et al., 2020). Epigenetic modifications also play a role in regulating chondrogenic differentiation. For example, the upregulation of KDM6A could promote chondrogenesis of PDLSCs by demethylation of H3K27me3 (Wang et al., 2018). Interestingly, an extract of *Enterococcus faecium* (*E. faecium*), called L-15, has been reported to improve chondrogenic differentiation of human DPSCs (Kim H. et al., 2019). Briefly, *E. faecium* is a commensal in the gastrointestinal tract, and researchers found that its L-15 extract promoted the expression of early-stage chondrogenic markers, including SOX9, collagen type II alpha 1 (COL2A1), and aggrecan (ACAN), and suggested that L-15 at a concentration of 50 μg/ml was safe for *in vitro* chondrogenic induction (Kim H. et al., 2019).

Myogenesis

The process of generating muscle or the formation of muscular tissue is recognized as myogenesis. Due to the potential versatility and easy accessibility, DMSCs are considered to be applicable as precursors for myogenic differentiation. Several studies have demonstrated that DPSCs possess the capacity of differentiating into smooth muscle cells (SMCs) *in vitro* under myogenic induction (Song et al., 2016; Jiang et al., 2019; Ha et al., 2021). Most of the studies focus on bladder SMCs as well as vascular SMCs (Song et al., 2016). The mechanism of the myogenic differentiation process of DPSCs remains unclear. The canonical WNT signaling pathway is well known to play an essential role in cell fate determination. Indeed, WNT signaling is involved in the process of DPSC differentiation into SMCs, in combination with multiple necessary growth factors, including transforming growth factor-beta (TGF-β1), basic fibroblast growth factor (bFGF, also known as FGF2), epidermal growth factor (EGF), hepatocyte growth factor (HGF), platelet-derived growth factor (PDGF), and VEGF (Jiang et al., 2019). To be noted, TGF-β1 may be one of the most important supplements to induce DMSCs to differentiate into SMCs. Mechanistically, TGF-β1 positively controls the SMC differentiation process of periodontal

ligament-derived cells, possibly at the early stage of the lineage commitment, via Smad- and p38/MAPK-dependent manners (Yoshida et al., 2012). In contrast, fibroblast growth factor 1 (FGF1, also known as aFGF) suppressed the SMC differentiation of periodontal ligament-derived cells via ERK1/2 signaling (Takahashi et al., 2012).

Angiogenesis

Angiogenesis refers to the establishment of new blood vessels from the preexisting vasculatures, occurs throughout life, and consists of four stages (**Figure 3**). In short, the endothelial cells (ECs) receive pro-angiogenic signals (stage I), followed by vascular fenestration (stage II), then partial endothelial to mesenchymal transition (EndoMT) (stage III), and in the end, new blood vessels sprouting (stage IV) (Zimta et al., 2019). DMSCs can secrete a wide range of pro-angiogenic factors including VEGF, FGF2, and PDGF in stage I, which bind to their corresponding receptors on ECs (Tonnesen et al., 2000; Tran-Hung et al., 2008). Furthermore, DPSCs can express monocyte chemoattractant protein-1 (MCP-1), also known as C–C motif chemokine ligand 2 (CCL2), to activate the ECs and stimulate ECs migration in the local microenvironment (Bronckaers et al., 2013). DPSCs, when co-cultured with the ECs, could stimulate EC proliferation through the p38/MAPK pathway and the secretion of FGF2 and VEGF in stage II (Tran-Hung et al., 2006). In stage III, the activation of the PI3K/AKT and MEK/ERK signaling pathways stimulates ECs residing in the interior layer of a blood vessel (Song and Finley, 2020). In a recent study, the gene-modified DPSCs, transfected with VEGF or stromal cell-derived factor 1α (SDF-1α) using lentiviral particles, were more capable of proliferation and stimulating the sprouting of the capillary tube-like structures in stage IV (Zhu et al., 2019). For a successful induction of local neoangiogenesis, two major cell populations must be taken into account: stem cells and vascular ECs. The cellular communication between these two types of cells could be mediated by exosomes. Especially, these extracellular vesicles are loaded with microRNA species, including miR-15/16, miR-31, miR-145, miR-221/222, miR-320a, miR-126, and miR-424 in angiogenesis-related progress (Gonzalez-King et al., 2017).

Dental-derived mesenchymal stem cells not only stimulate blood vessel formation via paracrine angiogenic factors but also participate directly in angiogenesis by differentiating into ECs (d'Aquino et al., 2007). As for the endothelial differentiation potential of DMSCs, DPSCs are able to differentiate into endothelial-like cells in the presence of VEGF. Previous work showed successful endothelial induction of DPSCs under a certain cell plating concentration when cultured in a specialized VEGF-added and serum-free culture medium (Karbanová et al., 2011). Also, DPSCs can functionally resemble perivascular supporting cells and induce more blood vessels when directly co-cultured with ECs (Janebodin et al., 2013; Saghiri and Asatourian, 2015). Intriguingly, DPSCs can also produce anti-angiogenic factors, including endostatin, insulin-like growth factor-binding protein 3 (IGFBP3), urokinase-type plasminogen activator (uPA), tissue inhibitor matrix metalloproteinase 1 (TIMP-1), and plasminogen activator inhibitor 1 (PAI-1) (Bronckaers et al., 2013). Collectively, these studies indicate that DMSCs could be potentially applied in the vascularization of regenerated dental tissues.

Neurogenesis

Neurogenesis is defined as a process of generating functional new neurons from neural stem cells and precursors; DMSCs are cranial neural crest derived, with huge potential in neural repair and nerve regeneration, which could function similarly as Schwann cells (Pisciotta et al., 2013) (**Figure 4**). DMSCs possess the capacity for both neuronal and glial differentiation. In fact, it has been demonstrated that DMSCs express specific neural markers like nestin, glial fibrillary acidic protein (GFAP), β-III-tubulin, synaptophysin, and S100 protein (Sonoyama et al., 2008; Martens et al., 2012). Furthermore, the percentage of cells expressing neural markers in DMSCs is relatively higher than BMSCs. It has been reported that human DPSCs express significantly higher levels of neurotrophins and elicit more extensive innervation than human BMSCs (Pagella et al., 2020). DMSCs, in particular DFSCs, could differentiate into mature neurons and oligodendrocytes following spinal cord injury (Yang et al., 2017). Under neurogenic induction, DPSCs could also form a stellate neuron-like phenotype, which resembled the characteristics of functional neurons (Wang et al., 2019). Besides morphology, DPSCs also exhibited the capacity to generate a sodium current when exposed to a neurogenic medium, which was functionally consistent with neuronal cells (Arthur et al., 2008). Additionally, brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3) can reprogram human DPSCs to neurogenic and gliogenic neural crest progenitors. Human DPSCs cultured in a serum-free medium with BDNF and NT-3 exhibited a greater potential to differentiate toward neuronal and Schwann glial lineage cells (Luzuriaga et al., 2019). Furthermore, DPSCs and SHEDs treated with BDNF, NT-3, and glial cell line-derived neurotrophic factor (GDNF) could differentiate into spiral ganglion neuron-like cells, which express neuron-specific β-III-tubulin, GATA-binding protein 3 (GATA3), and tropomyosin receptor kinase B (TrkB), protein markers of spiral ganglion neurons (Gonmanee et al., 2018). As for the aspect of function, intracellular calcium dynamics could be observed, which reflected neurotransmitter release (Gonmanee et al., 2018, 2020). DPSCs transfected with the human *Olig2* gene, an essential transcription factor for lineage determination of oligodendrocytes, would differentiate into oligodendrocyte progenitors (Askari et al., 2015). Taken together, DMSCs have their inherent advantages in neural repair and neurogenesis, such as they derived from the cranial neural crest, as well as their neurotrophic and neuroprotective properties, which suggest that DMSCs may be an ideal cell source for neural tissue regeneration.

Hepatogenesis

The process of giving rise to or forming liver tissue is known as hepatogenesis, which also includes the structural and functional development of the liver. Over a decade ago, the expression of the liver-specific gene *Cyp7a1* revealed hepatogenic differentiation in embryoid body-derived stem cells

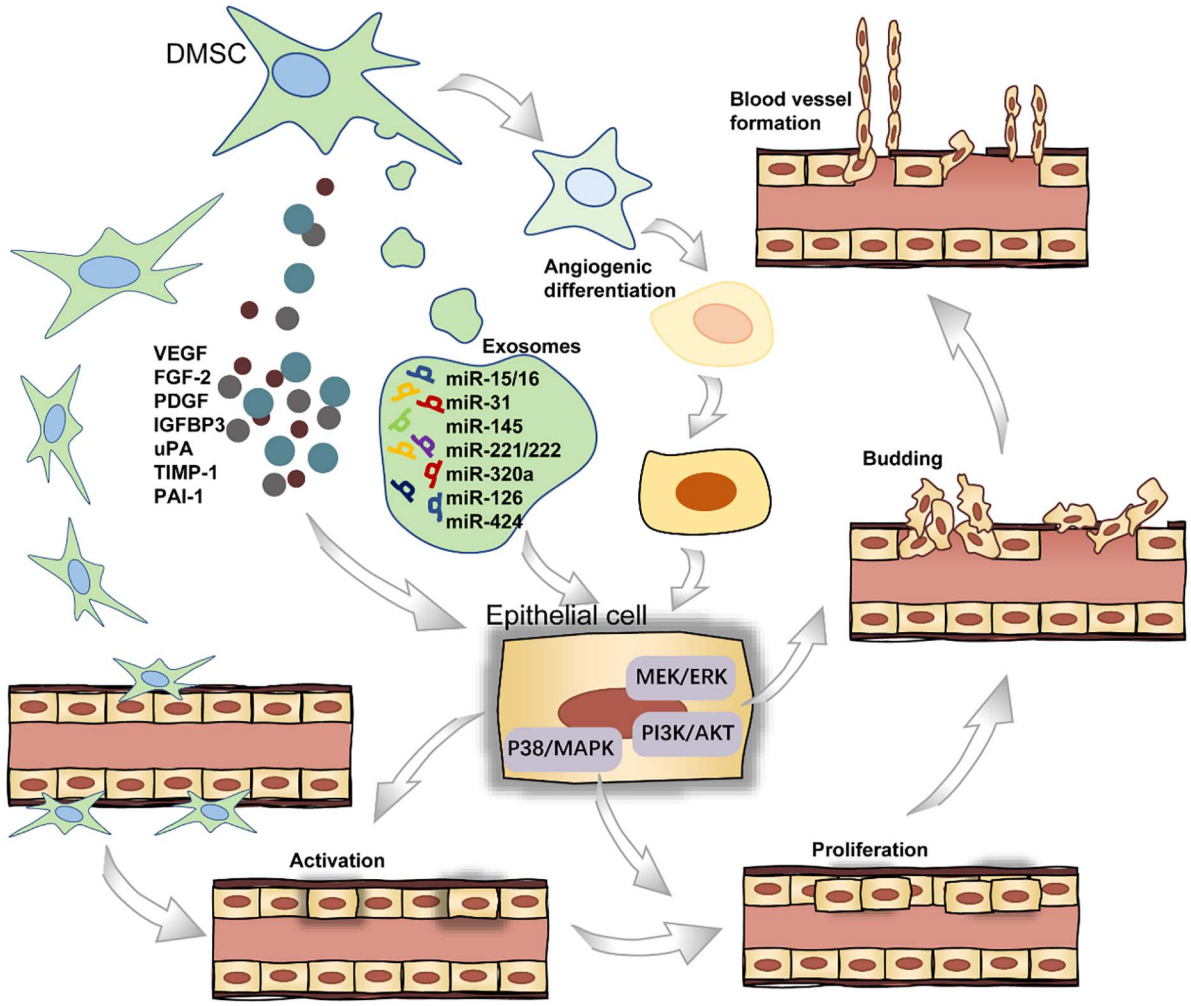

FIGURE 3 | The role of DMSCs in blood vessel formation.

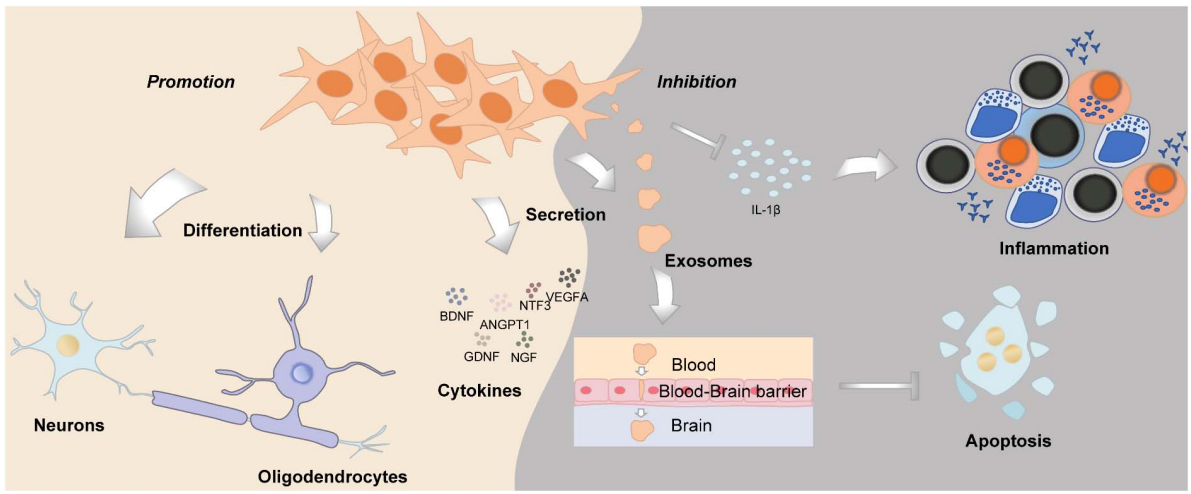

FIGURE 4 | The role of DMSCs in neurogenesis and neuroregeneration.

(Asahina et al., 2004, 2008). In more recent years, a research group reported that SHEDs supplemented with hepatic growth factors, including dexamethasone, insulin–transferrin–selenium-X (ITS-X), and oncostatin, were proven to have quite a few cells positive for specific hepatic markers including α-fetoprotein (AFP), albumin, and hepatic nuclear factor 4α (HNF-4α) after induction (Ishkitiev et al., 2010). To be noted, the hepatogenic-differentiated SHEDs could store glycogen and produce urea, which suggested the cells commenced to function as hepatocytes (Ishkitiev et al., 2010). Later on, the same group demonstrated that both SHEDs and DPSCs could differentiate into high-purity hepatocyte-like cells in a serum-free medium, which might serve as a novel source for hepatic lineage differentiation for transplantation in the future (Ishkitiev et al., 2012). Melatonin could promote hepatic differentiation of human DPSCs by modulating various signaling pathways (Cho et al., 2015). Actually, not only DPSCs but also DFSCs, SCAPs, and BMSCs all had hepatogenic potential. Among these MSCs, DPSCs reflected the best hepatogenic potential compared with the other three cell types (Kumar et al., 2017). More importantly, recent studies from different groups confirmed that cryopreserved human DPSCs could differentiate into functional hepatocyte-like cells (Chen et al., 2016; Han et al., 2017), which implicated the promising potential of a personal DMSC banking as a possible source of tailor-made hepatocytes in the future (Ohkoshi et al., 2018).

Adipogenesis

Adipogenesis is the formation of adipocytes, fat cells, from precursor stem cells. Currently, published data are conflicting about the potential and the time required for DMSCs to achieve adipogenic differentiation *in vitro*. During the adipogenesis of DMSCs, related components of the peroxisome proliferator-activated receptor (PPAR) signaling pathway, including PPAR gamma (PPAR-γ, also known as PPARG), fatty acid-binding protein 4 (FABP4), and the CCAAT/enhancer-binding protein (C/EBP) family were significantly upregulated (Nozaki and Ohura, 2011). Although some studies have reported that DMSCs can achieve adipogenic differentiation as efficiently as other MSCs, sometimes their conclusions were based solely on microscopic images of a few differentiated cells without further confirmation (Gronthos et al., 2000; Isobe et al., 2016). Meanwhile, DPSCs cultured in the adipogenic induction medium did not show cytoplasmic lipid accumulation and had low expression levels of adipogenic-related genes (Fracaro et al., 2020). A recent study disclosed the gene expression profile of DMSCs, which supported their limited capacity of differentiating into adipocytes (Fracaro et al., 2020). However, it seems that the adipogenic limitation is not insurmountable. As is revealed in another study, PIN1, a peptidyl-prolyl *cis/trans* isomerase, was identified to serve as a key regulator during adipogenesis of DMSCs. The overexpression of PIN1 via adenoviral infection in human DPSCs increased adipogenic differentiation while inhibiting odontogenic differentiation (Lee et al., 2014). In summary, we may have to admit that DMSCs do have difficulties in adipogenesis, but undoubtedly, DMSCs still possess adipogenic differentiation potential.

Immunomodulation

The oral cavity is the habitat of various microorganisms, and host–microbe homeostasis is an important aspect in the maintenance of oral health. Oral diseases are usually related to the imbalance of microbial flora and the invasion of microorganisms into oral tissues, sometimes even leading to systemic diseases. DMSCs may participate in immunomodulation by interacting with both innate and adaptive immune cells in their microenvironment (Andrukhov et al., 2019; Wang M. et al., 2020) (**Figure 5**).

Periodontal ligament stem cells, isolated from periodontal ligament tissues, exert a unique immunomodulatory property (Yu et al., 2019) and are capable of modulating a variety of immune cells. To illustrate, PDLSCs could suppress T-cell proliferation and disrupt T-cell differentiation into regulatory T-cells (Tregs) after being treated with D-mannose (Guo et al., 2018). PDLSCs also cause T-cell proliferation disorders through downregulating the expression of non-classical major histocompatibility complex (MHC)-like glycoprotein CD1b on dendritic cells (DCs) (Shin et al., 2017). Furthermore, PDLSCs also reduce the apoptosis of polymorphonuclear neutrophils (PMNs), the most abundant circulating blood leukocytes, through IL-6 secretion (Wang Q. et al., 2017). Moreover, PDLSCs inhibited B-cell activation through cell–cell interaction, which is primarily mediated by PD-1 and PD-L1 (Liu et al., 2013). As for macrophages, PDLSCs could potentiate the polarization toward M2 macrophages by stimulating arginase-1 (ARG1), CD163, and IL-10 and inhibiting TNF-α (Liu et al., 2019). Plus, PDLSCs could suppress the expression of TNF-α by macrophages, contributing to periodontal tissue regeneration (Nagata et al., 2017).

Besides PDLSCs, there are plenty of studies investigating the immunomodulatory function of other DMSCs subpopulations. Researchers found that the exosomes derived from DPSCs had immunosuppressive properties, even stronger than the exosomes derived from BMSCs (Ji et al., 2019). DPSCs are of low immunogenicity, could inhibit the proliferation of lymphocytes via TGF-β1, and regulate the production of cytokines *in vitro* (Ding et al., 2015). In the context of allogeneic stem cell-based therapies, DPSCs inhibited peripheral blood mononuclear cell proliferation in a non-cell contact manner (Wada et al., 2009).

In recent years, GMSCs gradually attract more attention for tissue engineering applications due to their easy accessibility and excellent immunomodulatory property (Fawzy El-Sayed and Dörfer, 2016). Analogous to PDLSCs, GMSCs were reported to be able to polarize macrophages into the M2 phenotype *via* enhanced secretion of IL-6 and granulocyte–macrophage colony-stimulating factor (GM-CSF) (Zhang et al., 2010). In addition, GMSCs could significantly inhibit the maturation and differentiation of DCs derived from peripheral blood monocytes and could suppress a variety of inflammatory cytokines as well (Su et al., 2011). Most recently, a comprehensive study conducted through scRNA-seq also validated that some sub-clusters of GMSCs showed a potential role in immunomodulation. However, these clusters appeared to display pro-inflammatory

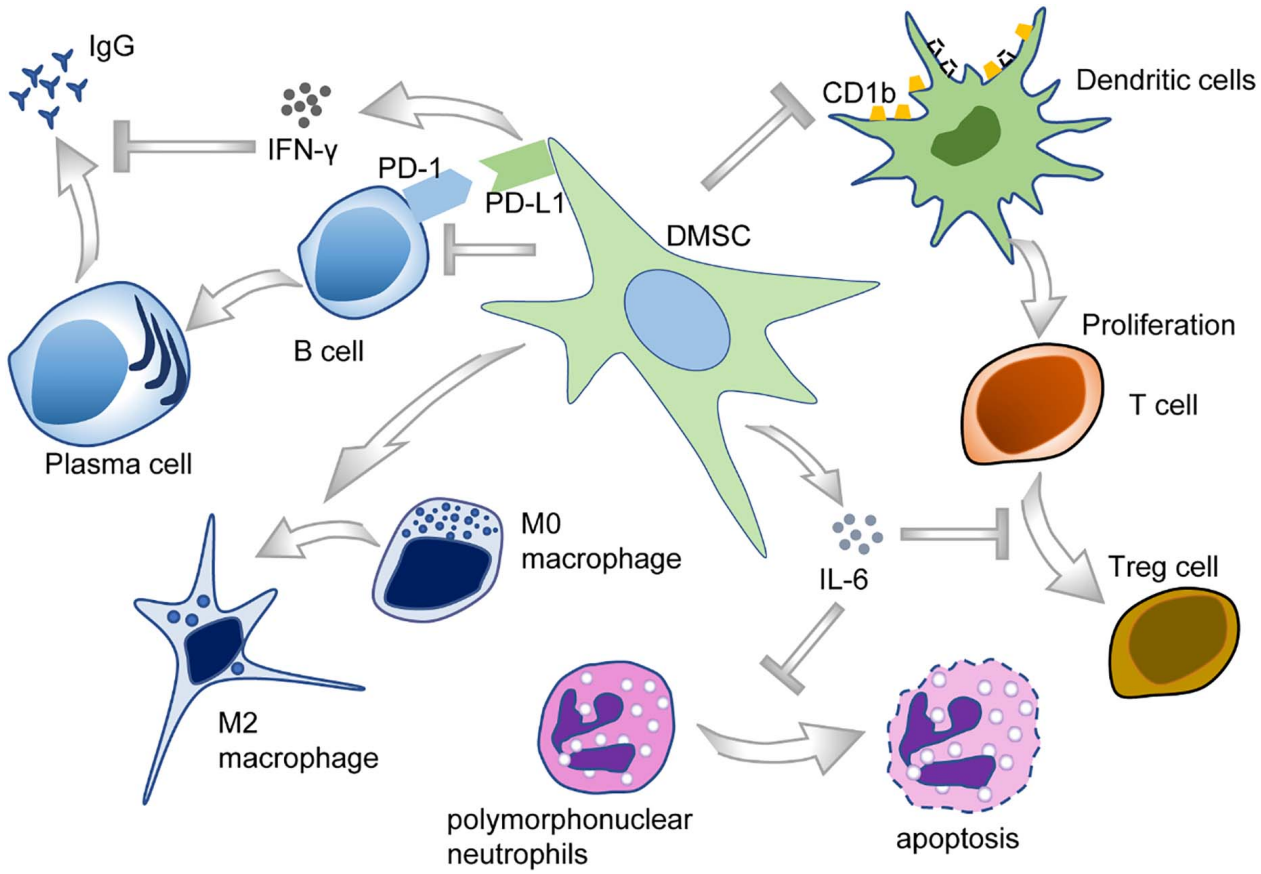

FIGURE 5 | The immunomodulatory functions of DMSCs.

nature, with enrichment for gene ontology (GO) terms such as T-cell activation, cytokine-mediated signaling pathway, and interferon-gamma (IFN-γ) signaling (Caetano et al., 2021). Given the characteristics of the oral cavity, a complex ecosystem containing numerous microorganisms with different pathogenic potentials, it would be interesting to examine whether DMSCs possess direct antimicrobial activity like BMSCs.

APPLICATION

Reconstruction of Bone Defects

Considering the prominent osteogenic differentiation ability, DMSCs are widely used to repair bone defects in combination with different types of scaffolds. Recently, a research group evaluated the efficacy of bone regeneration by using pre-differentiated GMSCs together with a self-assembling hydrogel scaffold to repair the critical size maxillary alveolar bone defect in a rat model. At 4 and 8 weeks after surgery, they observed a significant enhancement in bone regeneration compared with the control (Kandalam et al., 2020). DFSCs have also exhibited the properties of promoting new bone formation combined with treated dentin matrix (TDM) particles, a kind of scaffolding material, in a rat calvarial bone defect model (Yang H. et al., 2019).

Together with DMSCs and scaffolds, some chemical compounds were administered simultaneously to assess their pro-osteogenic functions in bone tissue engineering. As was mentioned earlier in this review, curcumin has been proven to activate osteogenic differentiation of DMSCs *in vitro* (Xiong et al., 2020). Researchers prepared guided bone regeneration (GBR) membrane with curcumin and aspirin addition. By utilizing this novel GBR membrane and DPSCs, bone regeneration was promoted, and meanwhile, antimicrobial effects were observed (Ghavimi et al., 2020). A similar result was found by another group that aspirin, in tetra-PEG hydrogel-based aspirin-sustained release system, could enhance the osteogenic capacity of PDLSCs (Zhang et al., 2019).

As a novel therapeutic option, tissue engineering strategies and their optimization attract attention and investigation continuously (Bajestan et al., 2017). Recently, researchers for the first time used deciduous DPSCs associated with a hydroxyapatite-collagen sponge to treat alveolar defects of cleft lip and palate patients and achieved satisfactory bone healing results (Tanikawa et al., 2020). In the dental research field, researchers also give attention to the regeneration of periodontal defects with DMSCs. GMSCs in combination with β-calcium triphosphate (β-TCP) scaffold could successfully reduce the vertical pocket depth and improve the cellularity

of regenerated periodontal tissues (Abdal-Wahab et al., 2020). However, another similar study by using PDLSCs and a xenogeneic bone substitute (XBS) demonstrated no statistically significant differences concerning healing and re-ossification whether PDLSCs were present or not (Sánchez et al., 2020), which suggests that we should interpret experimental results with extra caution when elucidating the precise functions and effects of DMSCs.

Tooth Regeneration

As is aforementioned, DMSCs are clonogenic cells and possess multiple differentiation potentials, which are conceived as a suitable cell source for dental tissue engineering and regeneration. Indeed, DMSCs are of profound significance in the regeneration of enamel, dentin, and pulp tissues. Transplantation of DPSCs *in situ* with granulocyte-colony-stimulating factor (G-CSF) has already been proven to result in the successful regeneration of vascularized pulp tissue in canine teeth (Iohara et al., 2013). A more pronounced volume of dental pulp-like tissue with a higher capillary density can be derived through a similar process *in vitro* with bFGF and G-CSF (Takeuchi et al., 2015). Researchers have also reported the successful regeneration of vascularized pulp-like tissue in an ectopic root transplantation model (Johnston et al., 2008). The shape of root canals and the size of the apical foreman are the key elements to focus on when judging the results. DPSCs derived from inflammatory dental pulp tissue have similar biological characteristics compared with those from normal dental pulp and could mediate pulp and dentin regeneration as well (Ling et al., 2020). The development of organoid or spheroid techniques for functional tooth regeneration has progressed rapidly. Several studies have demonstrated that organoids or spheroids of dental pulp cells were beneficial in the expression of VEGF (Hsieh et al., 2018), the capacity of angiogenesis (Janjić et al., 2018), and the formation of vascular dental pulp-like tissue (Dissanayaka et al., 2014). Hence, organoids or spheroids might constitute a practical and promising option for future clinical regenerative therapy (Oshima et al., 2017).

Different biocompatible scaffolds have been widely explored. Collagen can help SHEDs shape themselves into capillary-like microvessels (Sakai et al., 2010). Platelet-rich fibrin and chitosan both have been shown to contribute to dental pulp tissue engineering (Huangqin and Mingwen, 2007; Zhang et al., 2013). Polylactic acid (PLA) and polyglycolic acid (PGA) scaffolds are synthetic polymers that have been demonstrated to support cell proliferation and angiogenesis when attaining sufficient cell density (Woo et al., 2009). Another artificial biomaterial, polyhydroxybutyrate (PHB)/chitosan/nano-bioglass (nBG) nanofiber scaffold, has been revealed to promote SHEDs to differentiate into odontoblast-like cells and thereafter dentin formation (Khoroushi et al., 2018). Among all the biomaterials, fibrin appeared to be the most promising scaffold materials for dental pulp tissue engineering (Galler et al., 2018). Its advantages include easy handling, reasonable price, high cytocompatibility, and facilitation of pulp-like tissue formation.

For dentin mineralization and regeneration, the underlying mechanism is complicated and not clear yet. Therefore, most related studies concentrated on describing histological outcomes without digging into mechanisms. A study has proved that DPSCs organized in spheres are suitable for dental tissue engineering, which is capable of differentiating into odontoblast-like cells, inducing mineral formation, and display the possibility of "dentinal filling" of the root canal (Neunzehn et al., 2017). In addition, the microbiota appears to have an impact on dentinogenesis (Su et al., 2020) as well as some fabricated materials. To illustrate, nano-monetite hydrosol (nMH) contributed to dentin remineralization and acid resistance, but the interaction between nMH and DPSCs has not been discussed (Tan et al., 2020). Human β-defensin 4 (HBD4) controlled the intensity of dental pulp inflammation in a rat model of reversible pulpitis and induced the formation of restorative dentin (Zhai et al., 2020). For future research, studies should be conducted to reveal the interaction of DMSCs and scaffolds, and, more importantly, the underlying mechanisms in the context of tooth regeneration *in vivo*.

Treatment of Periodontitis

Case definition of periodontal disease in epidemiological studies is a challenge (Peres et al., 2019), but is generally manifested with the loss of periodontal supporting tissues, including periodontal ligament, cementum, and alveolar bone. The ultimate goal of periodontal therapy is to achieve the regeneration of periodontal tissues. All regenerative therapies have shown that the formation of new cementum is a common critical step because the deposition of cementum precedes the attachment of new periodontal ligament fibers to the root surface (Han et al., 2014; Nuñez et al., 2019). So far, DMSC-based therapy has become one of the most promising approaches to periodontal tissue regeneration.

The periodontal ligament is the major component of the periodontium connecting the root surface of the tooth and alveolar bone. It is comprised of heterogeneous cell populations, which include fibroblasts, undifferentiated MSCs, and epithelial cells, such as the epithelial cell rests of Malassez. PDLSCs can be isolated from adults' periodontal ligament tissues with the ability to generate alveolar bone, periodontal ligament, and cementum, as well as the potential of self-renewal (Martínez et al., 2011; Tomokiyo et al., 2018). Previous studies suggested that TGF-β1 played a crucial role in the fibroblastic differentiation process of PDLSCs (Fujii et al., 2010). Moreover, VEGF can stimulate the osteogenic differentiation of PDLSCs *in vitro* and induce the mineralization of bone structure (Lee et al., 2012).

Gingival mesenchymal stem cells have been proven to have multi-directional differentiation ability. One group demonstrated that transplanted GMSCs could accurately reach the periodontal injury sites and facilitate periodontal tissue regeneration by tail vein injection (Sun et al., 2019). A similar phenomenon was also observed in damaged periodontal tissues in a dog model (Yu et al., 2013). Another group used miniature pigs to establish an experimental periodontitis model and delivered SCAPs by local injection for treatment purposes, which verified the superior regenerative effect of periodontal tissue in the SCAPs treatment group (Li et al., 2018). Although the two groups of researchers both have proven that DMSCs effectively

promote the regeneration of periodontal tissues, they applied different delivery approaches to injecting DMSCs. Plus, the mechanisms of how GMSCs or SCAPs promote periodontal tissue regeneration are not uncovered yet. Studies should put sufficient effort into clarifying the functions and mechanisms of DMSCs during periodontal tissue regeneration. As for DPSCs, the therapeutic effects are vaguer, though DPSCs indeed have the potential to differentiate into adipogenic, osteogenic, chondrogenic, and neural cells. Some studies have concluded that DPSCs successfully accomplish periodontal tissue repair and regeneration (Liu et al., 2011); however, some studies suggested that DPSCs hardly ever repaired the defects of periodontal tissue (Park et al., 2011). On the contrary, PDLSCs might be a more favorable candidate for clinical application than DFSCs and DPSCs (Park et al., 2011). Different conclusions indicate that the specific role of each subpopulation of DMSCs in periodontal tissue regeneration is still waiting for more detailed and in-depth exploration.

Repair of Cartilage Injury

The chondrogenic potential of DMSCs is usually investigated in the presence of biological materials. Platelet-rich plasma (PRP) is a promising biomaterial that can be used as tissue engineering scaffolds (Pietrzak and Eppley, 2005). TGF-β1, as a conventional additive for chondrogenic differentiation, combined with a 10% PRP conditioned medium significantly potentiated chondrogenesis of DPSCs *in vitro* (Salkın et al., 2021). Recently, it has also been reported that nanoscale thermosensitive hydrogel scaffolds carrying human DPSCs could promote cartilage formation both *in vitro* and *in vivo* (Talaat et al., 2020). Moreover, the store condition of MSCs matters in tissue engineering. Researchers found that long-term cryopreservation in 95% FBS with 5% DMSO could maintain the chondrogenic capacity as well as the colony-forming ability of MSCs (Fujisawa et al., 2019).

Furthermore, there are some applications of DMSCs related to cartilage repair in osteoarthritis (OA). OA is a degenerative and inflammatory joint disorder, which takes place when the cartilage between the ends of bones gradually deteriorates. Recently, researchers revealed the protective effects of SHED-derived conditioned medium on OA chondrocytes. They found that OA chondrocytes exhibited an enhanced ability of anti-inflammation as well as a higher level of ACAN and COL2 when treated with SHED-derived conditioned medium (Muhammad et al., 2020). In addition, DPSC-derived conditioned medium could increase the survival and proliferation of immature murine articular chondrocytes *in vitro*; meanwhile, DPSCs directly underwent chondrogenesis as well (Lo Monaco et al., 2020).

Wound Healing

Wound healing is a complicated and meticulously organized process that restores the integrity and function of the damaged tissue. The process of wound healing involves the interaction of different cell types, including endothelial cells, platelets, inflammatory cells, and fibroblasts, etc. These cells secrete VEGF, bFGF, PDGF, TGF-β, placental growth factor (PlGF), and tissue inhibitor of metalloproteinases 2 (TIMP-2) as well as other factors to regulate the healing process and modulate the extracellular matrix components. Researchers revealed that bFGF-treated SHEDs could significantly increase collagen fibril coverage in wounds and, thus, promote the healing process (Nishino et al., 2011). Moreover, wound healing facilitated by DMSCs is not merely confined to fibroblastic proliferation and/or collagen accumulation. A recent study showed that DPSC treatment stimulated re-epithelialization and ameliorated collagen deposition in healing wounds; in dystrophic mice, DPSC treatment resulted in reduced fibrosis and collagen content, and infiltration of higher numbers of proangiogenic M2-like macrophages, which causes more satisfactory healing status (Martínez-Sarrà et al., 2017). Preclinical studies suggest that high-intensity laser therapy and photobiomodulation therapy may collaborate with DMSCs to soften cells, reorganize cytoskeleton, and improve the efficacy of wound healing (Daigo et al., 2020; Malthiery et al., 2020).

Repair or Regeneration of Neural Tissue

Dental-derived mesenchymal stem cells can be used as an ideal source for neural tissue repair and regeneration. All types of DMSCs can express BDNF, GDNF, nerve growth factor (NGF), NT-3, angiopoietin 1 (ANGPT1), and VEGF, participating in neural repair by exerting paracrine effects (Kolar et al., 2017; Ullah et al., 2017). Moreover, the expression levels of the abovementioned markers are highly increased following neural induction (Yamamoto et al., 2016). DMSCs promote axon regeneration through their neurotrophic functions, which could enhance the growth rate of Schwann cells and promote angiogenesis (Yamamoto et al., 2016; Ullah et al., 2017). Several studies have reported that BDNF plays an important role in neurite outgrowth and axonal targeting, which is regulated by DMSCs (De Almeida et al., 2014; Kolar et al., 2017). Moreover, transplantation of GMSCs positively modulated peripheral nerve regeneration and remyelination of Schwann cells, by the antagonistic myelination regulators (Zhang et al., 2016).

Dental-derived mesenchymal stem cells differentiate into neuron-like cells and replace the damaged cells. For instance, DPSC-derived oligodendrocytes significantly increased the myelination of peripheral nerves *in vivo*, suggesting the possibility of their use in demyelinating diseases (Askari et al., 2015). Moreover, DPSCs can differentiate into retinal ganglion cells, which also suggested the potential for the treatment of neurodegenerative diseases such as glaucoma (Bray et al., 2014; Roozafzoon et al., 2015). Additionally, DPSCs and SHEDs co-cultured with auditory brainstem slice (ABS) possess the ability to differentiate into spiral ganglion neuron-like cells, revealing the possibility of being a therapeutic approach for sensorineural hearing loss patients (Gonmanee et al., 2020). Meanwhile, the therapeutic effects of DMSCs have been tested in ischemic vascular diseases and observed neuroregeneration (Zhang Q. et al., 2018). In the rat model of middle cerebral artery occlusion (MCAO), DPSCs were administered intravenously and were found to migrate into ischemic areas and function as neuron-like cells, and consequently reducing infarct range.

Dental-derived mesenchymal stem cells can improve neural repair while suppressing neural cell apoptosis. Spinal cord injury

(SCI) often causes a broad range of dysfunction, due to the loss of neurons and glia as well as the limited axonal regeneration after SCI. Transplanting human DPSCs into the completely transected adult rat spinal cord led to the remarkable recovery of hind limb locomotor functions (Sakai et al., 2012). DPSCs displayed three kinds of neuroregenerative properties in SCI recovery. First, the apoptosis of neurons, astrocytes, and oligodendrocytes, induced by SCI, was inhibited, which improved the preservation of neuronal filaments and myelin sheaths. Second, multiple axon growth inhibitors were directly inhibited by DPSCs via paracrine mechanisms. Third, DPSCs replaced lost cells through differentiating into mature oligodendrocytes. Another group showed that SHEDs improved motor function after SCI, by reducing TNF-α expression, the cystic cavity, and the glial scar (Nicola et al., 2016).

Furthermore, DMSCs have the potential to restore neurons in neurodegenerative diseases of the central nervous system (CNS), such as Alzheimer's disease (AD) (Mita et al., 2015; Wang F. et al., 2017) and Parkinson's disease (PD) (Chen et al., 2020b). DPSCs could inhibit the phosphorylation of tau protein and promote the proliferation of neural stem cells in an animal model of AD (Wang F. et al., 2017). The exosomes secreted by DMSCs are able to cross blood–brain barriers (Jiang and Gao, 2017; Stanko et al., 2018). Some exosomes can reduce cytotoxicity and apoptosis triggered by amyloid-beta (Aβ) peptide (Ahmed et al., 2016). Thus, when translated into clinical application, the administration of DMSC-conditioned medium or DMSC-derived exosomes might be more practicable and safer. DMSCs coupled with three-dimensional frames had an enhanced ability to facilitate facial nerve recovery and regeneration after injury (Sasaki et al., 2014; Zhang Q. et al., 2018). Local delivery of GMSC-derived exosomes could markedly improve sciatic nerve regeneration and functional recovery (Mao et al., 2019). Additionally, DPSCs have superior migration potential toward the neurodegenerative milieu compared with BMSCs (Senthilkumar et al., 2020). Collectively, these studies indicate that DMSC-derived exosomes and/or DMSC-conditioned medium can be effective in neuroregeneration, through regulating anti-inflammatory, neurogenic, anti-apoptotic, angiogenic, and osteogenic mediators.

Treatment of Cardiovascular Diseases

Stroke is one of the most prevalent cardiovascular diseases worldwide. Recently, the advances in stem cell-based therapies to treat stroke have been reviewed (Suda et al., 2020). In a clinical trial, autologous BMSCs transplantation by intravenous infusion has been proven to be a feasible and safe therapy that could improve functional recovery in patients with severe cerebral infarcts (Bang et al., 2005). As for DMSCs, systemic delivery of DPSCs after reperfusion could reduce ischemic brain damage and improve functional recovery in a rat model (Nito et al., 2018). DPSC transplantation significantly reduced microglial activation and the expression of pro-inflammatory cytokines after reperfusion (Nito et al., 2018). Therefore, some researchers indicated that DPSCs might be a better choice of stem cell-based therapy for ischemic stroke than BMSCs (Song et al., 2017). Considering the neural differentiation potential of DPSCs, some studies delineated the transcriptional profile of DPSCs migrated to ischemic areas, which expressed neural markers, such as β-III-tubulin, doublecortin, nestin, and neurofilament (Zhang X. et al., 2018; Lan et al., 2019; Chen et al., 2020a). More randomized, double-blind, placebo-controlled, multicenter clinical trials are required to carefully evaluate the safety and efficacy of DMSCs in the treatment of ischemic stroke patients.

Ischemic heart diseases may cause irreversible damage to heart tissues, such as myocardial infarction (Song et al., 2017). In recent years, studies have demonstrated that DMSCs promoted the survival of cardiomyocytes in response to hypoxia and serum deprivation (Song et al., 2017). Furthermore, SHED-derived conditioned medium attenuated the LPS-induced expression of pro-inflammatory mediators. In brief, the cellular mechanism was the suppression of apoptosis and inflammatory reactions of cardiac myocytes (Yamaguchi et al., 2015).

Treatment of Autoimmune Diseases

Systemic lupus erythematosus (SLE) is a prototypic autoimmune disease, with abnormalities in the innate immune system as well as the adaptive immune system. In a recent study, GMSCs were found to limit the development of autoantibodies and proteinuria, which decreased the frequency of plasma B cells and lupus nephritis histopathological score by directly inhibiting B-cell activation, proliferation, and differentiation (Dang et al., 2020).

Multiple sclerosis (MS) is a long-lasting progressive autoimmune neurological disorder of the CNS. Immune cell invasion, axonal injury, and myelin sheath deformation are the common hallmarks of MS, which eventually cause neurological disability (Frohman et al., 2006). The immunosuppressive role of PDLSC-derived conditioned medium and exosomes might partly attribute to the presence of soluble immunomodulatory factors, NALP3 inflammasome inactivation, and, thus, resulting in the alleviation of MS (Soundara Rajan et al., 2017). DMSCs can also modulate inflammatory, oxidative stress, and apoptotic pathways in an experimental model of MS (Giacoppo et al., 2017).

Treatment of Liver Diseases

Liver cirrhosis, characterized by extensive fibrosis and the replacement of normal liver architecture into abnormal nodules due to diffused degeneration and death of hepatocytes, is the terminal stage of a variety of chronic liver diseases (Tsochatzis et al., 2008). As was aforementioned, DMSCs have the potential of differentiating into hepatocyte-like cells. For therapeutic applications, it has been reported that SHED-derived hepatocyte-like cells were positive for all examined hepatic markers (Yokoyama et al., 2019). Moreover, SHED-derived hepatocyte transplantation eliminated liver fibrosis and restored liver structure in rats (Yokoyama et al., 2019). Unfortunately, the number of *in vivo* experiments and preclinical studies remains very limited.

Treatment of Urinary Diseases

Bladder augmentation or replacement is required in a variety of urological disorders, which may be caused by cancer, spinal

cord injury, etc. SMC regeneration is an essential step in tissue engineering of the urinary bladder (Song et al., 2016). In a rat model of stress urinary incontinence with pudendal nerve transected, human DPSCs were engrafted in the external urethral sphincter, the thickness of which was mostly recovered 4 weeks later. DPSCs committed toward myogenic lineage *in vivo* promoted the formation of blood vessels and resulted in an appreciable recovery of urinary continence (Zordani et al., 2019). However, to generate functional, contractile, and mature SMCs through DMSCs, still there is a long way to go before making it a clinically available approach.

CONCLUSION

Dental-derived mesenchymal stem cells are comprised of several distinct subpopulations, including, but not limited to, DPSCs, SHEDs, PDLSCs, DFSCs, SCAPs, and GMSCs, with multi-directional differentiation potentials as well as immunomodulatory functions. Besides, each subpopulation of DMSCs is not equivalent in terms of their biological properties. Hereby, to the best of our knowledge, we have thoroughly reviewed the multipotency of DMSCs, including odontogenic, cementogenic, osteogenic, chondrogenic, myogenic, neurogenic, angiogenic, hepatogenic, and adipogenic differentiation. However, we have noticed that numerous studies concerning the differentiation of DMSCs are conducted *in vitro* in the presence of different induction culture media, regardless of DMSCs' specific origin, natural environment, and real behaviors *in vivo*.

Despite the possible discrepancy between the *in vitro* and *in vivo* differentiation potentials, we still believe that DMSCs are *bona fide* multipotent stem cells and feasible for a variety of clinical applications, such as soft and hard tissue engineering, tooth regeneration, and treatment of degenerative diseases. To this end, more experiments are a prerequisite for clinical translation. Not only more basic research is needed to unravel the regulatory mechanisms but also more preclinical and clinical studies are required to optimize and ensure the efficacy of DMSC-based therapy.

AUTHOR CONTRIBUTIONS

YM, BL, and ZZ contributed to the design, review, and proofreading of the manuscript. BL, TO, and YC contributed to the material collections and analysis. YC and YM contributed to the design of the figures. All authors agreed with the submission of the final version of the manuscript.

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Conflict 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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Silencing VEGFR-2 Hampers Odontoblastic Differentiation of Dental Pulp Stem Cells

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Dental pulp stem cells (DPSCs) are a source of postnatal stem cells essential for maintenance and regeneration of dentin and pulp tissues. Previous *in vivo* transplantation studies have shown that DPSCs are able to give rise to odontoblast-like cells, form dentin/pulp-like structures, and induce blood vessel formation. Importantly, dentin formation is closely associated to blood vessels. We have previously demonstrated that DPSC-induced angiogenesis is VEGFR-2-dependent. VEGFR-2 may play an important role in odontoblast differentiation of DPSCs, tooth formation and regeneration. Nevertheless, the role of VEGFR-2 signaling in odontoblast differentiation of DPSCs is still not well understood. Thus, in this study we aimed to determine the role of VEGFR-2 in odontoblast differentiation of DPSCs by knocking down the expression of VEGFR-2 in DPSCs and studying their odontoblast differentiation capacity *in vitro* and *in vivo*. Isolation and characterization of murine DPSCs was performed as previously described. DPSCs were induced by VEGFR-2 shRNA viral vectors transfection (MOI = 10:1) to silence the expression of VEGFR-2. The GFP+ expression in CopGFP DPSCs was used as a surrogate to measure the efficiency of transfection and verification that the viral vector does not affect the expression of VEGFR-2. The efficiency of viral transfection was shown by significant reduction in the levels of VEGFR-2 based on the Q-RT-PCR and immunofluorescence in VEGFR-2 knockdown DPSCs, compared to normal DPSCs. VEGFR-2 shRNA DPSCs expressed not only very low level of VEGFR-2, but also that of its ligand, VEGF-A, compared to CopGFP DPSCs in both transcriptional and translational levels. *In vitro* differentiation of DPSCs in osteo-odontogenic media supplemented with BMP-2 (100 ng/ml) for 21 days demonstrated that CopGFP DPSCs, but not VEGFR-2 shRNA DPSCs, were positive for alkaline phosphatase (ALP) staining and formed mineralized nodules demonstrated by positive Alizarin Red S staining. The expression levels of dentin matrix proteins, dentin matrix protein-1 (*Dmp1*), dentin sialoprotein (*Dspp*), and bone sialoprotein (*Bsp*), were also up-regulated in differentiated CopGFP DPSCs, compared to those in VEGFR-2 shRNA DPSCs, suggesting an impairment of odontoblast differentiation in VEGFR-2 shRNA DPSCs. *In vivo* subcutaneous transplantation of DPSCs with hydroxyapatite (HAp/TCP)

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for 5 weeks demonstrated that CopGFP DPSCs were able to differentiate into elongated and polarized odontoblast-like cells forming loose connective tissue resembling pulp-like structures with abundant blood vessels, as demonstrated by H&E, Alizarin Red S, and dentin matrix staining. On the other hand, in VEGFR-2 shRNA DPSC transplants, odontoblast-like cells were not observed. Collagen fibers were seen in replacement of dentin/pulp-like structures. These results indicate that VEGFR-2 may play an important role in dentin regeneration and highlight the potential of VEGFR-2 modulation to enhance dentin regeneration and tissue engineering as a promising clinical application.

Keywords: VEGFR-2, dental pulp stem cells, tooth development, tooth regeneration, dental pulp, angiogenesis, VEGF, odontoblasts

INTRODUCTION

Dental pulp stem cells (DPSCs), a type of neural crest derived mesenchymal stem cells, have been isolated from the dental pulp, a loose connective tissue located in the center innermost part of tooth structure (Janebodin et al., 2011). Compare to other types of stem cells, this dental tissue-derived stem cell is considered a promising source of adult stem cells for regenerative dentistry and medicine given its accessibility, tremendous expansion and differentiation capacity. Isolation of DPSCs is a less invasive method compared to other sources of mesenchymal stem cells. DPSCs can be generated from various sources of dental tissues considered biological wastes such as wisdom teeth, embedded teeth, supernumerary teeth and inflamed teeth (Gronthos et al., 2000; Huang et al., 2008; Tomasello et al., 2017; Paz et al., 2018). Additionally, several studies have demonstrated its potential in tissue regeneration both *in vitro* and *in vivo* animal models (Gronthos et al., 2002; Victor and Reiter, 2017; Kabatas et al., 2018; Yamada et al., 2019; Sui et al., 2020).

We and others have shown that DPSCs exhibited *in vitro* multipotential capacity by differentiating to a variety of cell types such as osteoblasts, chondrocytes, adipocytes, neurons and smooth muscle cells (Gronthos et al., 2000; Janebodin et al., 2011; Shi et al., 2020). Moreover, *in vivo* studies revealed that DPSC transplants exhibit versatile ability for tissue regeneration in various specific conditions and animal models (Kerkis et al., 2008; Martinez-Sarra et al., 2017; Fernandes et al., 2018; Ullah et al., 2018). Research interest in DPSCs has increased with a hope to manipulate this stem cell for potential future clinical applications relating to both dental and non-dental tissue regeneration and diseases (Paz et al., 2018; Yamada et al., 2019).

The potential of DPSCs for regeneration purposes is not only based on their intrinsic differentiation capacity, but also their paracrine function such as c, neurotrophic, and immunomodulating abilities (Bronckaers et al., 2013; Luo et al., 2018; Andrukhov et al., 2019). Vascular endothelial growth factor (VEGF) signaling has been shown important for DPSCs-induced angiogenesis (Janebodin et al., 2013). Recently, VEGF has been shown to be important for the development and repair of skeletal tissue such as bone and cartilage (Hu and Olsen, 2016b). Osteogenic cells and hypertrophic chondrocytes express high levels of VEGF during intramembranous and intracartilaginous bone formation, respectively (Jacobsen et al., 2008; Berendsen and Olsen, 2014; Duan et al., 2016). In addition, several studies suggest that VEGF can stimulate osteoblast differentiation (Hu and Olsen, 2016a). Dysfunction of VEGF signaling results in defective osteoblast differentiation and osteogenesis (Liu et al., 2012). VEGF is notably expressed in multiple cell types including endothelial cells, osteoblasts, bone marrow and dental mesenchymal stem cells (Liu et al., 2012; Janebodin et al., 2013; Buettmann et al., 2019).

Dental pulp stem cells are important for dentin and tooth regeneration. Previous *in vivo* transplantation studies have shown that DPSCs were able to give rise to odontoblast-like cells (OLCs), form dentin/pulp-like structures, and induce blood vessel formation. Dentin formation was also found closely related to blood vessels (Janebodin et al., 2011). We have previously shown that DPSC-induced angiogenesis is VEGFR-2-dependent (Janebodin et al., 2013). However, the role of VEGFR-2 signaling in odontoblast differentiation of DPSCs is still not well understood.

In this present study, we aimed to investigate the role of VEGFR-2 signaling for odontoblastic differentiation of DPSCs. Murine DPSCs were isolated and well characterized from neonatal dental pulp tissue of first and second mandibular molar teeth. DPSCs were expanded and the expression of VEGFR-2 was silenced using a specific VEGFR-2 shRNA viral vector. The efficiency of silencing was evaluated in VEGFR-2 shRNA treated DPSCs compared to the control DPSCs transfected by CopGFP shRNA by determining both transcriptional and translational levels. Afterward, the odontogenic differentiation capacity of DPSCs in these two groups was examined both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Mouse Housing and Husbandry

All mouse experiments were performed in accordance with approved Institutional Animal Care and Use Committee (IACUC) guidelines, University of Washington. Mice were originally purchased from the Jackson Laboratory. Mice were housed in a specific-pathogen-free (SPF) environment in ventilated cages with filter tops (Allentown Inc., Allentown, NJ, United States). A maximum of 5 mice were housed together

in the same cage. Mice were maintained in standard light/dark cycle (6:00 a.m. lights on, 6:00 p.m. lights off), standard food (5058 mouse breeding food), standard temperature (68–79°F), and no environmental restriction. Mice were allocated randomly to control and experimental groups.

Isolation, Culture and Characterization of Murine DPSCs

Dental pulp tissues were dissected and pooled from 5-day-old neonatal murine teeth. DPSCs were isolated, cultured, expanded, and characterized as previously described (Janebodin et al., 2011). Briefly, dental pulp from lower molar teeth was gently isolated and kept in stem cell media described below. The tissue was washed with phosphate buffer saline (PBS) (HyClone). To release cells, the extracellular matrix was digested with Dispase II (1.2 units/ml), Collagenase IV (2 mg/ml) (Worthington) supplemented with $CaCl_2$ (2 mM) in PBS for 1 h at 37°C. Then, an equal volume of stem cell media was added to the digested tissue prior to filtering through 70 mm nylon cell strainers (BD Falcon) and centrifuging at 300 × *g* for 10 min at room temperature. Cells were subsequently resuspended in stem cell media and single cell suspensions were plated at a density of 1000 cells/cm^2.

Cells were cultured at 37°C under 5% O_2 and 5% CO_2 in stem cell media, containing a final concentration of 60% low-glucose DMEM (Gibco, Invitrogen), 40% MCDB201 (Sigma), 2% fetal calf serum (HyClone), insulin-transferrin-selenium (ITS) (Sigma), linoleic acid with bovine serum albumin (LA-BSA) (Sigma), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 100 units/ml penicillin with 100 mg/ml streptomycin (HyClone), and 1×10^3 units/ml leukemia-inhibitory factor (LIF-ESGRO, Millipore), 10 ng/mL EGF (Sigma) and 10 ng/mL PDGF-BB (R&D) (Breyer et al., 2006). Once more than 50% cell confluent, they were detached with 0.25% trypsin-EDTA (Invitrogen) and replated at a 1:4 dilution under the same culture condition with fresh media. Differentiation capacity of cultured cells was determined by *in vitro* multi-differentiation to bone, cartilage and fat cells as previously described (Gronthos et al., 1994; Gregoire, 2001; Dahlin et al., 2014). The osteo-odontogenic media contains serum-free media with 10% FBS, 10 mM β-glycerophosphate, 0.2 mM L-ascorbic acid, and 100 nM dexamethasone. The chondrogenic media contains serum-free high-glucose DMEM, 1% ITS+ premix (BD Biosciences), 50 mg/mL ascorbic acid, 100 nM dexamethasone, supplemented with 10 ng/ml TGF-β3 (Shenandoah Biotech). The adipogenic media contains serum-free media supplemented with 10% horse serum, 100 μM indomethacin (Alfa Aesar), 0.5 mM 3-isobutyl-1-methyl-xanthine (ACROS) and 1 μM dexamethasone.

Silencing VEGFR-2 in Murine DPSCs

Murine DPSCs were stably transfected with VEGFR-2 shRNA to silence the expression of VEGFR-2 as per manufacturer's protocol. Briefly, cells (1000 cells/cm^2) were plated and cultured in stem cell media for 24 h. DPSCs were allowed to proliferate until 70% confluent before viral transfection. Then, cells were cultured in stem cell media with polybrene (5 μg/ml) to increase binding between the pseudoviral capsid and the cell membrane. Cells were infected by the VEGFR-2 shRNA lentiviral particles (Santa Cruz Biotechnology, Santa Cruz, CA, United States) with 10:1 of multiplicity of infection (MOI), then mixed gently, and incubated overnight. CopGFP control lentiviral particles (Santa Cruz Biotechnology) were also used as control to evaluate transduction efficiency at the similar MOI used in VEGFR-2 shRNA lentiviral particles. After overnight transduction, infected cells were replaced with stem cell media without polybrene and incubated overnight. To select stable infected cells expressing the shRNA, we expanded cells one more time in stem cell media overnight, and then selected them by exposure to puromycin dihydrochloride (5 μg/ml) (Sigma). The infected cells were cultured in puromycin-containing stem cell media until the resistant cells were identified. The homogeneous population of resistant cells was observed by the expression of Green Fluorescence Protein (GFP) in cells transduced with CopGFP control lentiviral particles. To determine the knockdown efficacy, the level of VEGFR-2 and VEGF-A in both silencing and control groups was determined by mRNA and protein levels through real-time PCR and immunofluorescence analyses.

Immunofluorescence

Cells were fixed with 4% formaldehyde/PBS for 5 min, washed with 1% BSA in 0.1% Triton-X 100/PBS, and stained with primary antibodies as described in **Supplementary Table 1** incubated overnight at 4°C. Goat-derived Alexa 594-conjugated secondary antibodies (Invitrogen) were diluted at 1:800 and incubated for 1 h. Cells were stained with 4′, 6-diamine-2-phenylindol (DAPI) at 1:1000 to visualize the nuclei. All antibodies were diluted in 1% BSA in 0.1% Triton-X 100/PBS. We used IgG isotype from the species made for the primary antibody (0.1 ug/ml) (Vector Laboratories Inc., Burlingame, CA, United States), were included for all staining.

In vitro Osteo-Odontogenic Differentiation

VEGFR-2 shRNA treated DPSCs and CopGFP treated DPSCs (2×10^4 cells/cm^2, $n = 4$ wells/group) were incubated overnight in stem cell media at 37°C under 5% O_2 and 5% CO_2. After 24 h, each cell type was separately cultured in BMP-2 media, β-glycerophosphate plus ascorbic acid (BGP + Vit. C) media, VEGF media for 21 days to evaluate the osteo-odontogenic differentiation capacity with media change every 3 days. BMP-2 media is serum-free media containing 10% FBS, 100 ng/ml BMP-2 (Shenandoah Biotech), 0.2 mM L-ascorbic acid, and 100 nM dexamethasone (Hu et al., 2004). BGP + Vit. C media is serum-free media containing 10% FBS, 10 mM β-glycerophosphate (CalBiochem), 0.2 mM L-ascorbic acid, and 100 nM dexamethasone (Song et al., 2009). VEGF media is serum-free media containing 10 ng/ml VEGF (R&D) (Forgues et al., 2019). Serum-free media is 60% low-glucose DMEM, 40% MCDB201, ITS, LA-BSA, 10^{-9} M dexamethasone, 10^{-4} M ascorbic acid 2-phosphate, 100 units/ml penicillin with 100 mg/ml streptomycin. After differentiation, both cell

groups were separately divided into 2 subgroups for RNA collection and staining.

Alkaline Phosphatase Staining

After differentiation, cultured cells were stained by ALP staining kit (BioVision) as per manufacturer's protocol. Briefly, the media were carefully removed from the cultured cell wells. Cells were fixed with 4% formaldehyde/PBS for 15 min at room temperature. Then, 500 μl of wash buffer was gently added and carefully removed using a pipette. 250 μl of ALP Staining Reagent solution was carefully added to completely cover the cells in each well of a 24-well plate. Cells were incubated for 30 min at 37°C before washed gently with 500 μl of wash buffer for 3 times. 300 μl of wash buffer was added and stained cells were imaged using a light microscope.

Alizarin Red S Staining

Cultured cells were stained by Alizarin Red S staining kit (ScienCell Research Laboratories) as per manufacturer's protocol. Briefly, the media were carefully removed from the cultured cell wells and cells were washed three times with PBS. Cells were fixed with 4% formaldehyde/PBS for 15 min at room temperature. The fixative solution was removed and cells were washed three times with deionized water. The water was removed and 500 μl of 40 mM Alizarin Red S dye was added per well. Cells were incubated at room temperature for 30 min with gentle shaking. The dye was removed and cells were washed five times with deionized water before taking images. For tissue transplants, the sections were deparaffinized and hydrated to 70% ethanol. Then, the tissue sections were fixed with 10% formaldehyde/PBS for 15 min at room temperature before staining following the protocol described above.

Real-Time PCR Analysis

Dental pulp stem cells were extracted for total RNA by using the RNeasy Mini kit (Qiagen) according to the manufacturer's protocol. Quantity and purity of RNA was determined by 260/280 nm absorbance. First-strand cDNA was synthesized from 500 ng of RNA using the High Capacity cDNA synthesis kit (Applied Biosystems) per manufacturer's protocols using a randomized primer. Real-time PCR primers are included in **Supplementary Table 2**.

A 20 ng of cDNA for Q-RT-PCR were prepared using the SYBR green PCR master mix (Applied Biosystems). Reactions were processed by the ABI 7900HT PCR system with the following parameters: 50°C/2 min and 95°C/10 min, followed by 40 cycles of 95°C/15 s and 60°C/1 min. Results were analyzed using SDS 2.2 software and relative expression calculated using the comparative Ct method. The threshold cycle (Ct) value for each gene was normalized to the Ct value of GAPDH. The relative mRNA expression, presented as fold change difference, was calculated by the comparative Ct method according to the formula: $2^{-\Delta\Delta Ct}$, where ΔCt = Ct target–Ct GAPDH and ΔΔCt = ΔCt target–ΔCt calibrator. Each sample was run in triplicate reactions for each gene. Error bars represent the standard deviation calculated from the triplicate analysis of each sample.

In vivo Transplantation of DPSCs

In accordance with approved IACUC protocols, 1×10^6 of VEGFR-2 shRNA treated DPSCs and CopGFP treated DPSCs were separately transplanted into 1-month-old male *Rag1* null mice (Jackson Laboratory, Bar Harbor, ME, United States) by dorsal subcutaneous transplantation with hydroxyapatite tricalciumphosphate (HAp/TCP) (Zimmer) (n = 5 mice/cell type). Grafts were harvested after 5 weeks of transplantation. Transplanted tissues samples were fixed with 4% formaldehyde for 2 h then demineralized for 7 days in 10% EDTA at 4°C. Then, the transplants were embedded in paraffin and cut to 5 μm thick sections. Sections were analyzed by H&E, Alizarin Red S and immunohistochemistry staining.

Tissue Staining

Prior to staining procedures, paraffin-embedded tissue sections were deparaffinized and hydrated to 70% ethanol. Tissues were stained with H&E staining following the manufacturer's standard protocol. For immunohistochemistry, fixed tissue sections were permeabilized with 1% BSA in 0.1% Triton-X 100 (Sigma)/PBS for 10 min, inhibited endogenous peroxidase activity with 0.3% hydrogen peroxide in methanol for 30 min, and blocked non-specific binding sites with 10% goat or horse serum (Vector Laboratories Inc., Burlingame, CA, United States) for 1 h. Primary antibodies listed in **Supplementary Table 1** were used and incubated overnight at 4°C. Stained tissues were incubated with a biotinylated antibody at 1:100 (Vector Laboratories Inc., Burlingame, CA, United States) for 1 h, washed and treated with the Vectastain ABC kit and 3, 3′-diaminobenzidine (DAB) substrate kit according to manufacturer's protocol (Vector Laboratories Inc., Burlingame, CA, United States).

Statistical Analysis

The number of mice were calculated using power analysis based on previous pilot studies in our laboratory (statistical power of 0.90; p level = 0.05). Statistical analysis of the *in vitro* studies, histology, and molecular analysis results from three independent experiments were performed by the Student's t test. Data are presented as mean ± SD. p-values ≤ 0.001, 0.005, 0.05 was considered as statistically significant.

RESULTS

DPSCs Exhibited Self-Renewal and Multi-Differentiation Capacity *in vitro*

Dental pulp stem cells were isolated from neonatal murine pulp tissue of lower molar teeth. Cell suspension was plated in a low cell density as a single cell deposition. After 2 days of culture in stem cell media, several cell colonies were observed (**Figure 1A**). Later, larger colonies of cells were seen before cell trypsinization and expansion (**Figures 1B,C**). Compared to undifferentiated DPSCs (**Figure 1D**), cultures in specific differentiation media revealed DPSCs differentiation abilities to give rise to osteoblast-like cells (positive staining for BSP and DMP-1, **Figures 1E,F**),

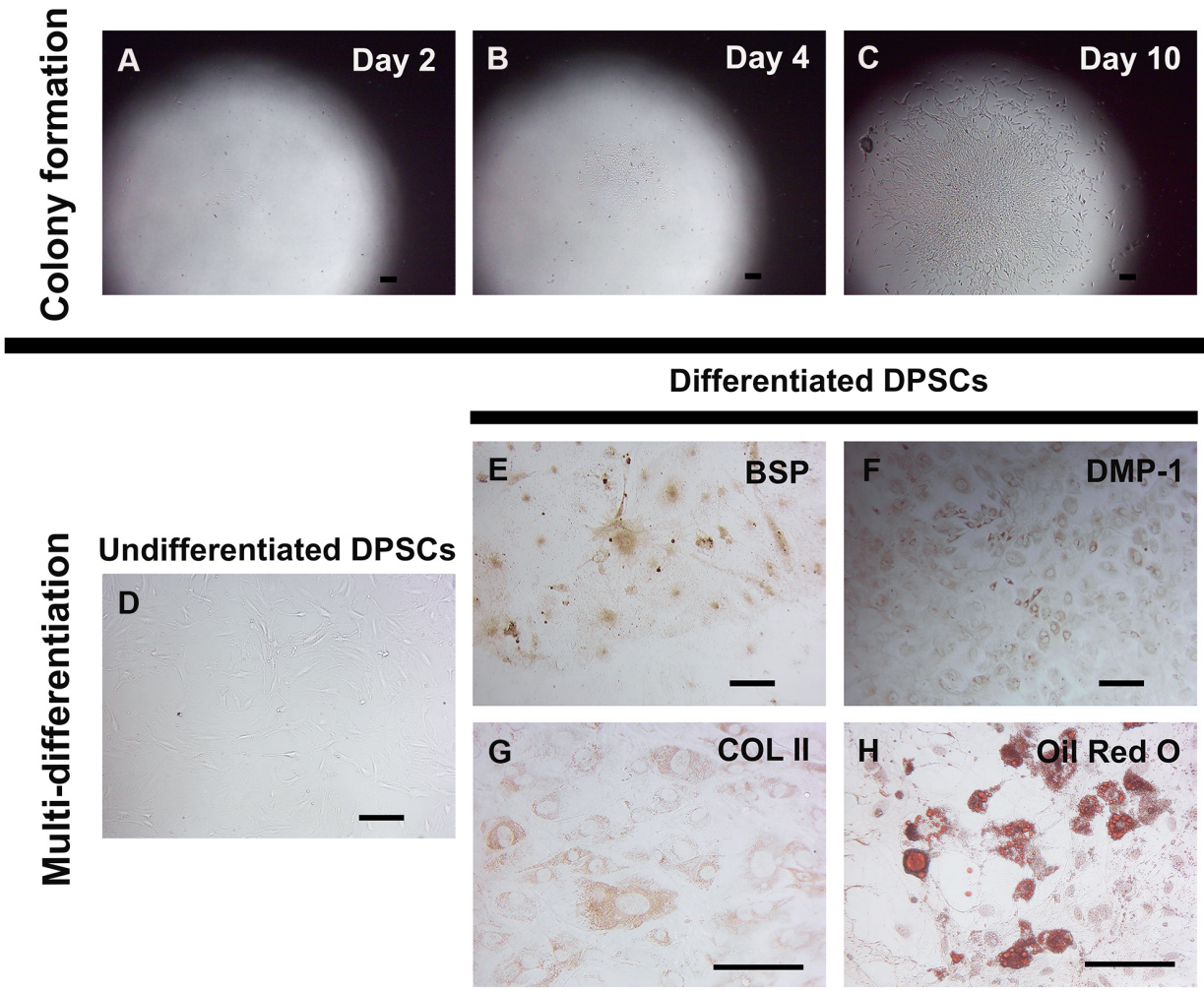

FIGURE 1 | Self-renewal and *in vitro* multi-differentiation of murine DPSCs. Murine DPSCs were isolated from dental pulp of neonatal lower molar teeth. A low density of cells (1000 cells/cm^2) were plated for several single cell depositions to observe a colony forming ability and characterize for their multi-differentiation. Representative figures show at the same spot of DPSC clone at different time points, Day 2 **(A)**, Day 4 **(B)**, and Day 10 **(C)**. The undifferentiated DPSC clone proliferated to gain the larger size, indicating its self-renewal property. The *in vitro* differentiation in various specific differentiation media demonstrates DPSC multi-differentiation potential. Undifferentiated DPSCs cultured in the stem cell media were spindle-shaped, indicating mesenchymal-like stem cell morphology **(D)**. Differentiated DPSCs cultured in the osteo-odontogenic media exhibited positive anti-BSP (in brown) **(E)** and anti-DMP-1 (in brown) **(F)** staining. Cultured in the chondrogenic media, differentiated DPSCs showed positive anti-COL II (in brown) **(G)** staining. Strongly positive Oil Red O staining (in red) **(H)** was seen in DPSC-derived lipid-containing cells in the adipogenic media. Scale bars indicate 100 μm.

chondrocyte-like cells (positive staining for COL II, **Figure 1G**), and adipocytes (positive staining with Oil Red O, **Figure 1H**).

Down-Regulation of VEGFR-2 Significantly Decreases Expression of VEGF-A in DPSCs

In order to investigate the potential role of VEGFR-2 signaling in osteo-odontogenic differentiation of DPSCs, VEGFR-2 shRNA was utilized to silence VEGFR-2 expression in murine DPSCs. The CopGFP shRNA was also used as a control to determine the efficiency of transfection. We targeted DPSCs with both VEGFR-2 and CopGFP shRNA separately and observed approximately 100% GFP expression in CopGFP (**Figures 2A,C**) but not in VEGFR-2 shRNA group (**Figures 2B,D**). CopGFP DPSCs expressed high levels of both VEGFR-2 and VEGF-A (**Figures 2E,G,K,L**). In contrast, an 8-fold decrease of *Vegfr-2* expression was shown in VEGFR-2 shRNA DPSCs compared to the CopGFP control DPSCs (***$p < 0.001$) (**Figure 2K**). The gene expression level corresponded to a dramatic decrease in VEGFR-2 immunofluorescence staining of VEGFR-2 shRNA DPSCs (**Figures 2F,L**). The quantification of the VEGFR-2 fluorescence intensity showed a statistically significant decrease in VEGFR-2 level in VEGFR-2 knockdown DPSCs, compared to normal DPSCs (**$p < 0.005$) (**Figure 2L**). In addition, VEGFR-2 shRNA DPSCs down-regulated VEGF-A gene and protein levels (*$p < 0.05$ and **$p < 0.005$, respectively), compared to the CopGFP control cells (**Figures 2H,K,L**). The rabbit IgG

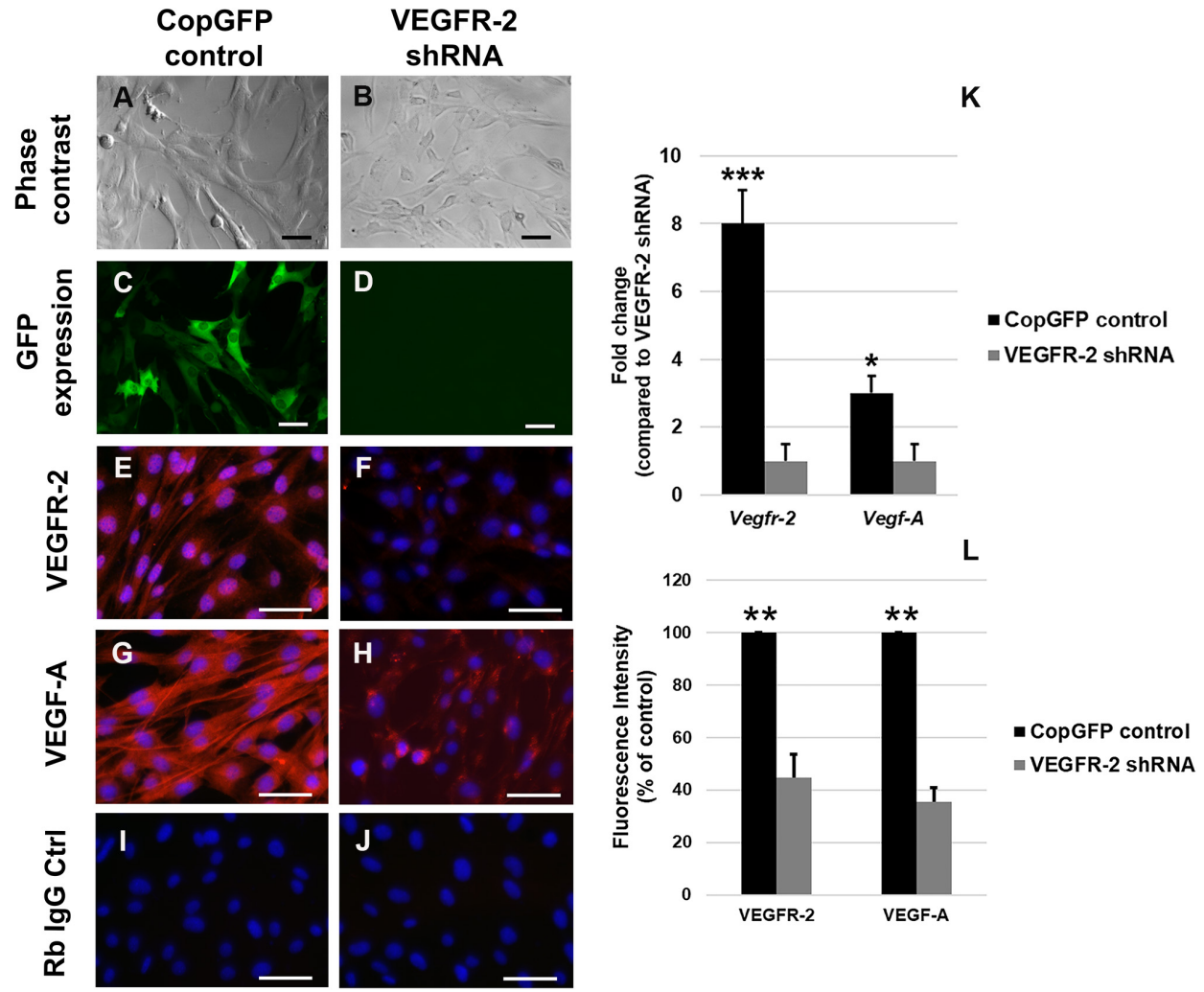

FIGURE 2 | Silencing of VEGFR-2 in murine DPSCs using the lentiviral vector. Murine DPSCs were transfected by either CopGFP control **(A)** or VEGFR-2 shRNA **(B)** lentiviral vectors with 10:1 of MOI (multiplicity of infection). After transfection, both groups exhibited their regular spindle shapes, presenting no toxicity of the viral vector. The 100% expression of GFP in CopGFP control **(C)** but not in VEGFR-2 shRNA group **(D)** was seen. The GFP+ expression in CopGFP DPSCs indicates the high efficiency of transfection. Immunofluorescence (in red) demonstrates strong positive staining of VEGFR-2 **(E)** and its ligand, VEGF-A **(G)** in CopGFP control DPSCs whereas there were very low expression of VEGFR-2 **(F)** and VEGF-A **(H)** in VEGFR-2 shRNA DPSCs. The rabbit IgG control (Rb IgG Ctrl) stained in CopGFP control **(I)** and VEGFR-2 shRNA DPSCs **(J)** were completely negative to confirm the specificity of antibodies. DAPI (in blue) was used for the nuclear staining. Scale bars indicate 100 μm. The bar graph **(K)** reveals that real-time PCR analysis showed higher expression of both *Vegfr-2* and *Vegf-A* in CopGFP control DPSCs, compared to that in VEGFR-2 shRNA treated DPSCs. Differences in gene expression is demonstrated as the fold change of that in VEGFR-2 shRNA DPSC group. GAPDH was used as the internal control. The bar graph **(L)** reveals the quantification of the VEGFR-2 and VEGF-A fluorescence intensity in both normal and VEGFR-2 knockdown DPSCs by using ImageJ software. The quantification showed a statistically significant decrease in VEGFR-2 and VEGF-A levels in VEGFR-2 knockdown DPSCs, compared to normal DPSCs. The results were shown as the fluorescence intensity (% of control). ***, **, * is used for significantly statistical difference at p-value < 0.001, 0.005, 0.05, respectively. The results represent the means ± SD ($n = 5$ from each group/experiment) of three independent experiments.

control (Rb IgG Ctrl) stained in CopGFP control (**Figure 2I**) and VEGFR-2 shRNA DPSCs (**Figure 2J**) were completely negative to confirm the specificity of antibodies.

VEGFR-2 Deficient DPSCs Lacked Osteo-Odontogenic Potential *in vitro*

CopGFP DPSCs cultured in BMP-2 media showed strongly positive staining of ALP enzyme (in black) (**Figures 3A,C,D**). In contrast, VEGFR-2 shRNA DPSCs exhibited significantly decreased ALP staining (**Figures 3B,E**). Few slightly ALP positive cells in VEGFR-2 shRNA DPSCs were observed (**Figure 3F**). In addition, abundant mineralized nodules were observed and positively stained Alizarin Red S (in red) in the CopGFP DPSCs (**Figures 3G,I**) while those nodules were absent in the VEGFR-2 shRNA DPSCs (**Figures 3H,K**). A cluster of cuboidal cells were seen, indicating osteo-OLCs (**Figure 3J**). Nevertheless, Alizarin Red S stained slightly positive in the cytoplasmic

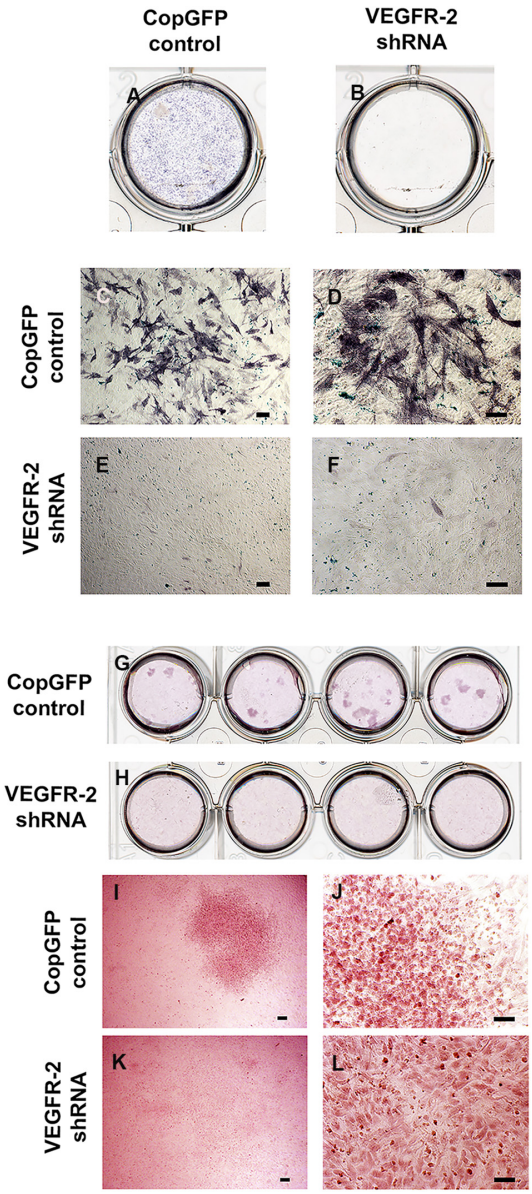

FIGURE 3 | Alkaline phosphatase and Alizarin Red S staining in CopGFP control and VEGFR-2 shRNA DPSCs after a culture in osteo-odontogenic BMP-2 media. Alkaline phosphatase staining demonstrated a dissimilar result between CopGFP control **(A)** and VEGFR-2 shRNA DPSCs **(B)** after osteo-odontogenic induction in BMP-2 media. Abundant positive alkaline phosphatase stained cells (in dark-bluish purple) were shown in CopGFP control DPSCs, suggesting DPSCs undergoing osteo-odontogenesis **(C,D)**. Few slightly positive stained for alkaline phosphatase staining were found in VEGFR-2 shRNA DPSCs **(E,F)**. Alizarin Red S staining demonstrated a different result between CopGFP control and VEGFR-2 shRNA DPSCs after osteo-odontogenic induction in BMP-2 media. Several Alizarin Red S positive mineralized nodules were detected in differentiated CopGFP control DPSCs **(G)**. There was absence of mineralized nodule found in differentiated VEGFR-2 shRNA DPSCs **(H)**. A representative figure reveals a low magnification of Alizarin Red S positive mineralized nodules in the CopGFP control **(I)**. The higher magnification of the nodule showed cuboidal-like cells, resembling osteo-odontoblast-like cells **(J)**. VEGFR-2 shRNA DPSCs did not show any mineralized nodule formation **(K)** but they were undifferentiated spindle-shaped **(L)**. Scale bars indicate 100 μm.

compartment of VEGFR-2 shRNA DPSCs due to the intracellular calcium contents (**Figure 3L**). Remarkably, VEGFR-2 shRNA DPSCs maintained a spindle-shaped morphology, resembling an undifferentiated state. Additionally, immunocytochemistry showed certain positive cells of anti-DMP-1 (**Figure 4A**) and anti-DSP (**Figure 4B**) staining (in brown) in differentiated CopGFP control DPSCs but slightly stained cells in VEGFR-2 shRNA DPSCs (**Figures 4D,E**). The rabbit IgG control (Rb IgG Ctrl) stained in CopGFP control (**Figure 4C**) and VEGFR-2 shRNA DPSCs (**Figure 4F**) were completely negative to confirm the specificity of antibodies. VEGFR-2 shRNA DPSCs also down-regulated the expression of *Dmp-1*, *Dspp*, and *Bsp*; however, only *Dmp-1* and *Bsp* showed a significant difference compared to the CopGFP DPSCs ($^*p < 0.05$) (**Figure 4G**). Cultured in BGP + Vit. C media, occasional strongly ALP positive cells (**Figures 5A,C,E**) were seen in the control DPSCs but not in the VEGFR-2 shRNA DPSCs (**Figures 5B,D,F**). Some Alizarin Red S positive nodules were present in the CopGFP cells whereas those were not detected in the VEGFR-2 shRNA cells (**Figure 5M**). Similarly, cultured in VEGF media, few ALP positive cells were present in only the CopGFP group (**Figures 5G,I,K**), not in the VEGFR-2 shRNA group (**Figures 5H,J,L**). However, there were no mineralized nodules observed in both CopGFP and VEGFR-2 shRNA groups (**Figure 5N**).

VEGFR-2 Deficient DPSCs Were Unable to Give Rise to Odontoblast-Like Cells and Form Pulp-Like Structures After Transplantation

H&E staining revealed the overall morphology of CopGFP DPSC transplants (**Figures 6A–C**) and VEGFR-2 shRNA DPSC transplants (**Figures 6G–I**). *In vivo* transplantation of CopGFP DPSCs demonstrated elongated and polarized cells (**Figures 6D–F**; arrowheads) running perpendicularly to hydroxyapatite/tricalcium phosphate (HAp/TCP), resembling the morphology of OLCs. In addition, a formation of pulp-like structure was revealed by the presence of loose connective tissue with capillaries containing RBCs (**Figures 6D–F**) in CopGFP DPSC transplants, mimicking pulp-like tissues in the natural teeth. In contrast, VEGFR-2 shRNA DPSCs transplants lacked OLCs and pulp-like structures. Instead, there was only a formation of disperse collagenous tissue (**Figures 6J–L**).

In the cross-section of the tooth, Alizarin Red S showed the strongly positive staining of dentin and odontoblasts; it also exhibited the histology of dental pulp which is a vascularized loose connective tissue (**Figure 7A**). A transplanted tissue generated by the CopGFP DPSCs stained positive for Alizarin Red S and also demonstrated the presence of OLCs (**Figures 7B,C**; arrowheads) with pulp-like–tissues (**Figures 7B,C**). In contrast, VEGFR-2 shRNA DPSC transplants showed disorganized tissues faintly positive for Alizarin Red S staining (**Figures 7D,E**). In the tooth section, anti-DMP-1 and anti-DSP staining was positive in dentin and intense in odontoblasts (**Figures 7F,G**). Interestingly, the transplanted tissues generated by the control cells also showed strong signal of immunohistochemistry for DMP-1 and DSP proteins in OLCs

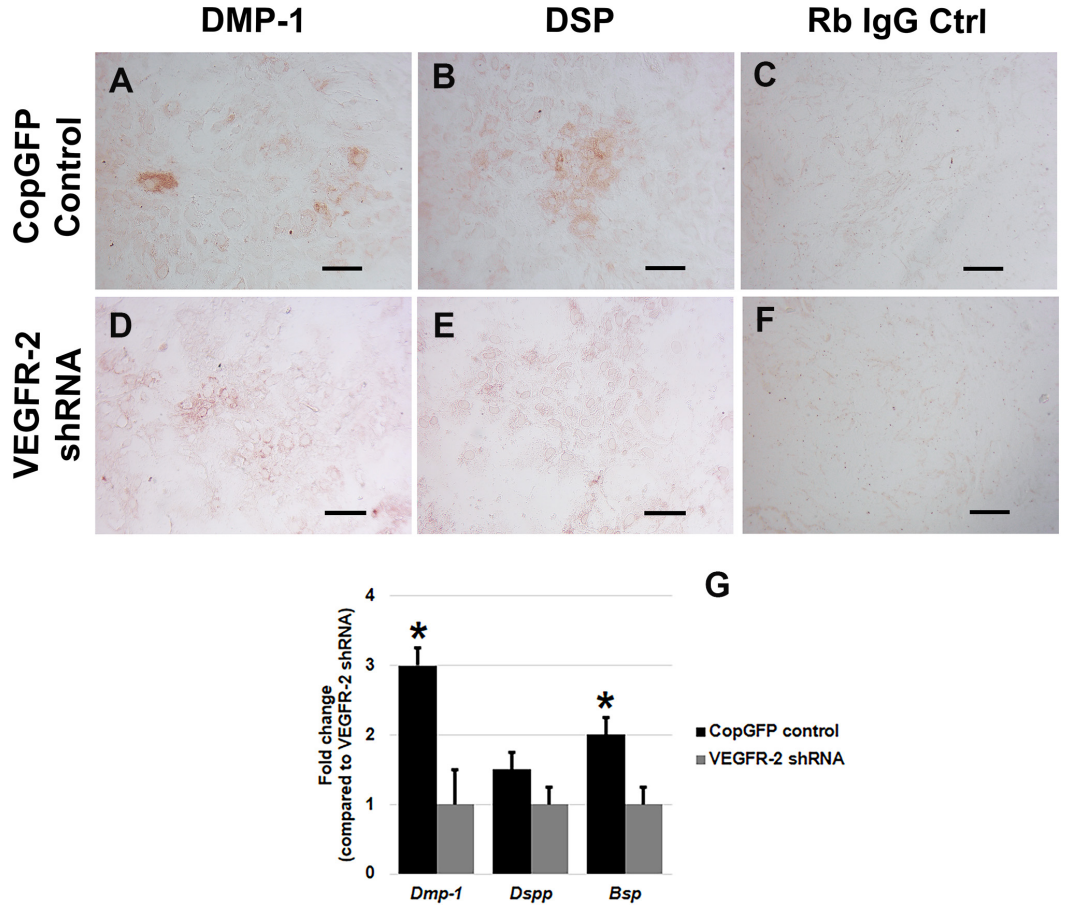

FIGURE 4 | Dentin matrix protein staining and osteo-odontogenic gene expression in CopGFP control and VEGFR-2 shRNA DPSCs after a culture in osteo-odontogenic BMP-2 media. Immunocytochemistry showed certain positive cells of anti-DMP-1 **(A)** and anti-DSP **(B)** staining (in brown) in differentiated CopGFP control DPSCs but slightly stained cells in VEGFR-2 shRNA DPSCs **(D,E)**. The rabbit IgG control (Rb IgG Ctrl) stained in CopGFP control **(C)** and VEGFR-2 shRNA DPSCs **(F)** were completely negative to confirm the specificity of antibodies. Scale bars indicate 100 μm. The bar graph reveals that real-time PCR analysis showed higher expression of *Dmp*-1, *Dspp*, and *Bsp* in differentiated CopGFP control DPSCs, compared to that in differentiated VEGFR-2 shRNA treated DPSCs **(G)**. Differences in gene expression is demonstrated as the fold change of that in differentiated VEGFR-2 shRNA DPSC group. GAPDH was used as the internal control. * is used for significantly statistical difference at p-value < 0.05. The results represent the means ± SD of three independent experiments.

(**Figures 7I,J**; arrowheads) and matrix (**Figures 7I,J**; asterisks). In contrast, we were not able to observe positive DMP-1 and DSP staining in the VEGFR-2 shRNA DPSC transplants (**Figures 7L,M**). Rb IgG control was used as a negative control (**Figures 7H,K,N**).

DISCUSSION

Dental pulp stem cells which are isolated from tooth pulp tissues have been shown to play an important role in dentin/pulp repair and regeneration (Gronthos et al., 2002; Paz et al., 2018). Recent studies have revealed that DPSCs have not only an odontogenic capacity but also an angiogenic potential (Martinez-Sarra et al., 2017; Yamada et al., 2019; Shi et al., 2020; Sui et al., 2020). We have previously shown that angiogenesis induced by DPSCs is regulated by VEGFR-2 signaling (Janebodin et al., 2013). However, the relevance of VEGFR-2 signaling in odontogenesis and dentinogenesis is still undetermined. In this study, we investigated the potential role of VEGFR-2 for odontogenic capacity of murine DPSCs both *in vitro* and *in vivo*.

First, DPSCs were isolated from mouse neonatal-derived lower molar teeth and examined for their stem cell properties as previous protocol (Janebodin et al., 2011). Plating cells at low density close to a single cell deposition revealed formation of several cell colonies. As expected, those colonies increased in size with longer culture time, highlighting their self-renewal capacity. Moreover, DPSCs cultured in various differentiation media exhibited characteristic morphological changes combined with the positive staining for osteoblastic, chondrogenic, and adipogenic. These results correspond to previous studies confirming the mesenchymal stem cell's properties of DPSCs (Gronthos et al., 2000; Huang et al., 2008; Janebodin et al., 2011; Paz et al., 2018).

Next, we silenced the expression of VEGFR-2 in DPSCs by using the VEGFR-2 shRNA lentiviral transfection. The shRNA

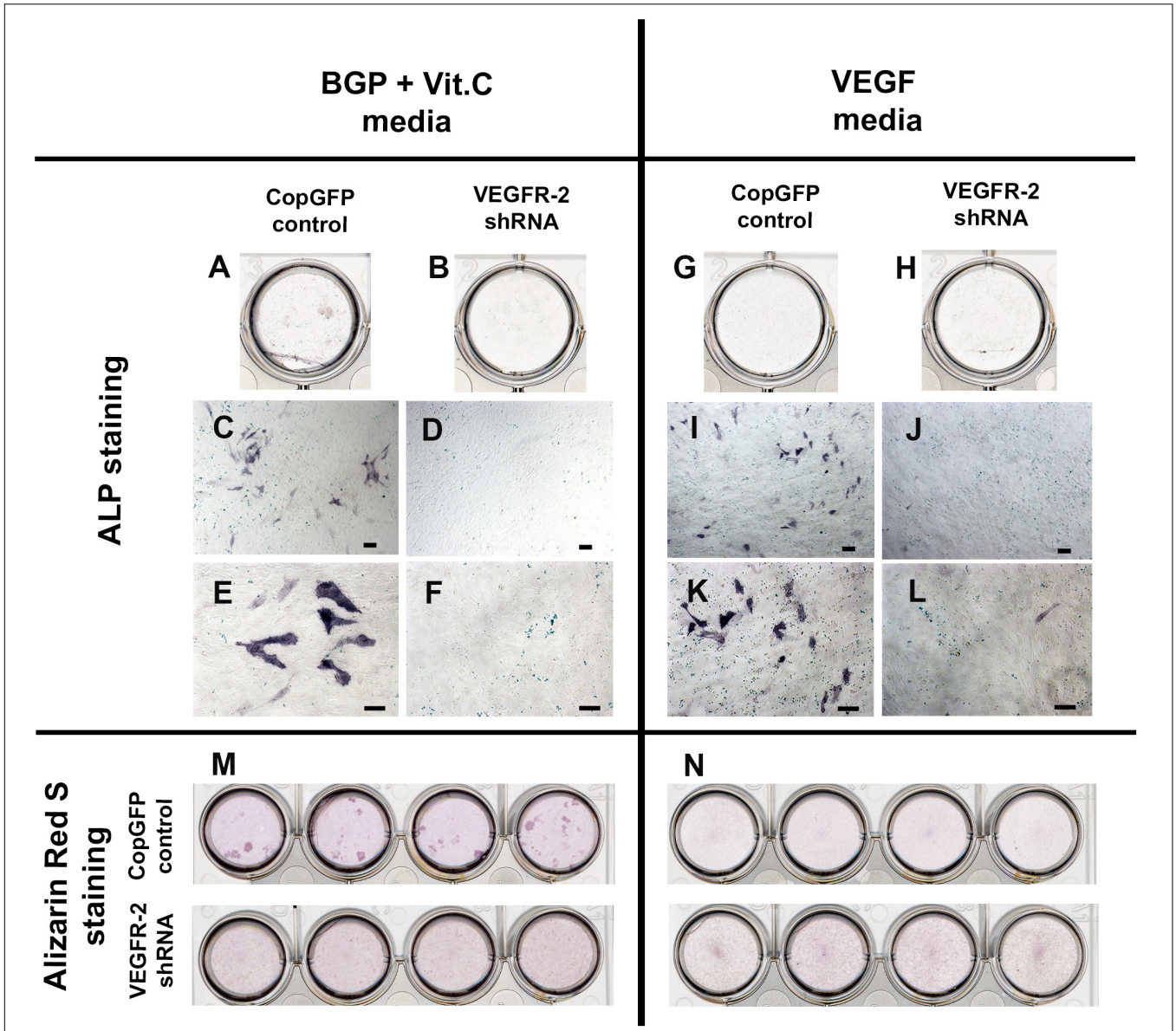

FIGURE 5 | Alkaline phosphatase and Alizarin Red S staining in CopGFP control and VEGFR-2 shRNA DPSCs after a culture in BGP + Vit. C media and VEGF media. After induction in BGP plus vitamin C, some differentiated CopGFP DPSCs **(A,C,E)** but not VEGFR-2 shRNA DPSCs **(B,D,F)** demonstrated positive staining of alkaline phosphatase. Alizarin Red S staining showed some positive mineralized nodules in CopGFP DPSCs but not VEGFR-2 shRNA DPSCs **(M)**. For VEGF induction, a similar results of alkaline phosphatase staining were observed in both CopGFP control **(G,I,K)** and VEGFR-2 shRNA DPSCs **(H,J,L)**, compared to the BGP plus vitamin C. Interestingly, no mineralized nodule was found in both cell groups after VEGF induction **(N)**. Scale bars indicate 100 μm.

(short hairpin RNA) delivery into mammalian cells is considered a powerful method to study gene functions via the cellular mechanism of RNA interference. This technique provides a stable integration of shRNA, resulting in a long-term knock down of an interested gene (Moore et al., 2010). To confirm the initial successful transfection, treated cells were selected through puromycin supplemented media. Furthermore, the CopGFP shRNA which is a non-specific viral vector conjugated with GFP was used as a control (Guryanova et al., 2006). Approximately 100% of DPSCs transfected with the CopGFP construct expressed GFP. Since both CopGFP and VEGFR-2 shRNA have the similar constructs, we assumed that transfection of DPSCs with VEGFR-2 shRNA was highly efficient. To prove that, real-time PCR and immunofluorescence were used to demonstrate that the VEGFR-2 shRNA DPSCs exhibited a decrease in VEGFR-2 gene and protein expression, compared to the CopGFP control. Importantly, the transcriptional and translational level of VEGF-A were also down-regulated. VEGF-A is a protein with a variety of biological functions especially in endothelial cells and pericytes such as vascular permeability, cell survival, proliferation and migration that are important in angiogenesis. VEGF-A binds to and activates VEGFR-1 and VEGFR-2 (Neufeld et al., 1999).

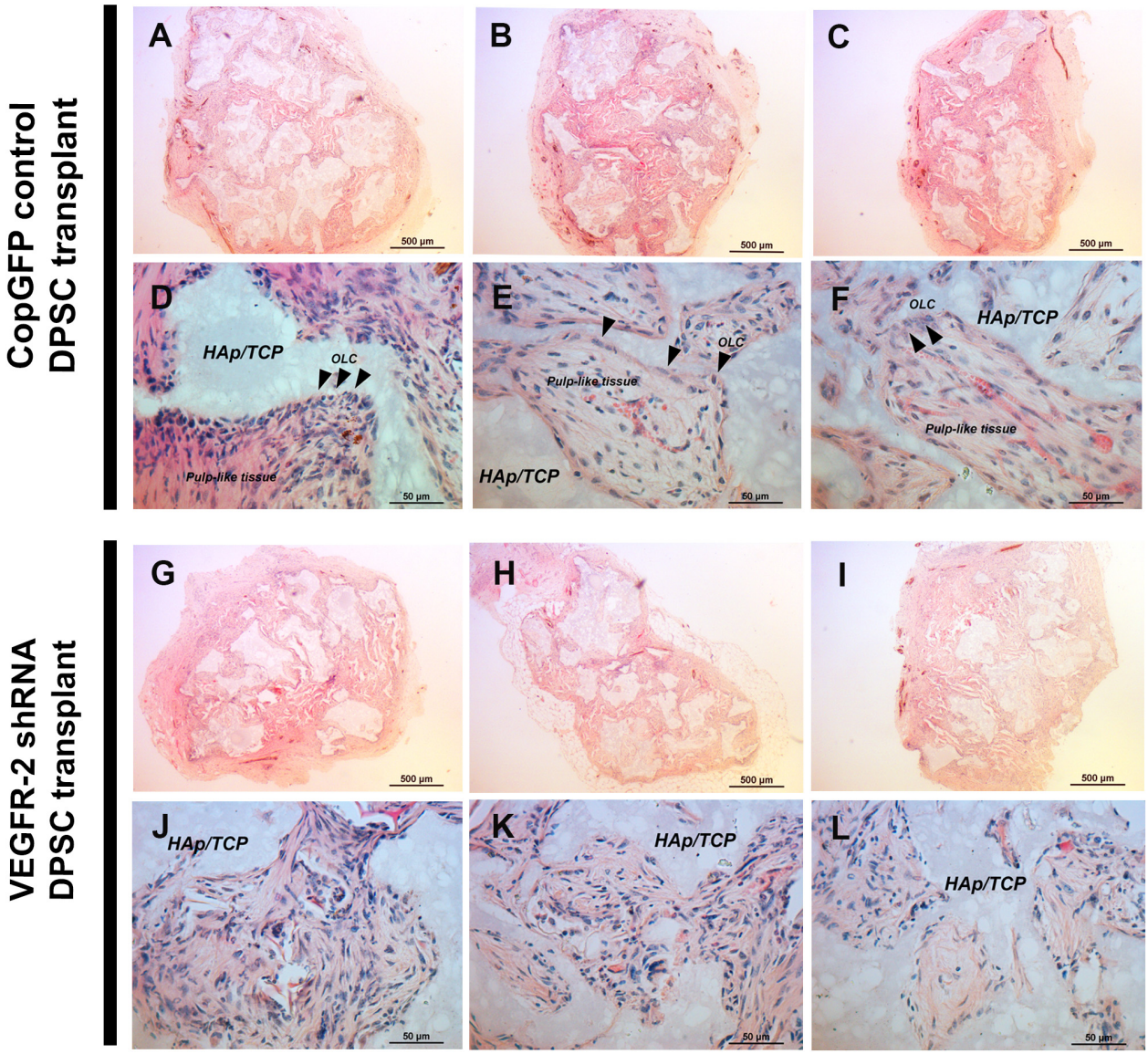

FIGURE 6 | *In vivo* transplantation of CopGFP control and VEGFR-2 shRNA DPSCs by H&E staining. 1 × 10^6 cells of CopGFP control and VEGFR-2 shRNA DPSCs were separately transplanted into 1-month-old male *Rag1* null mice by dorsal subcutaneous transplantation with hydroxyapatite tricalciumphosphate (HAp/TCP) (n = 5 mice per cell type). Transplanted tissues were harvested after 5 weeks, then proceeded and stained for analysis. H&E staining was performed to demonstrate the morphology of CopGFP DPSC transplants [**(A–C)**; low magnification of representative tissues] and VEGFR-2 shRNA DPSC transplants [**(G–I)**; low magnification of representative tissues]. Scale bars indicate 500 µm. CopGFP DPSC transplants exhibited elongated and polarized odontoblast-like cells (OLC) (arrowheads) and more organized loose connective tissue with abundant blood vessels mimicking pulp-like structures **(D–F)** while VEGFR-2 shRNA DPSC transplants showed an absence of odontoblast-like cells. Instead, disorganized fibrous collagenous tissue was seen **(J–L)**. OLC; odontoblast-like cells, HAp/TCP; hydroxyapatite/tricalciumphosphate. Scale bars indicate 50 µm.

Although VEGFR-2 has a low affinity for VEGF-A, its tyrosine kinase activity is stronger than that of VEGFR-1 (Eichmann and Simons, 2012; Berendsen and Olsen, 2014). Previous studies have shown that DPSCs, a neural crest-derived mesenchymal stem cell, have pericyte-like properties capable of inducing angiogenesis through VEGFR-2/VEGF-A pathway (Janebodin et al., 2013). This suggests that the down-regulation of VEGFR-2 may regulate expression of its ligand, VEGF-A. Nevertheless, the underlying mechanism needs further investigation.

The osteo-odontogenic capacity of CopGFP and VEGFR-2 shRNA DPSCs were examined by cultured in two types of osteo-odontogenic media, BMP-2 media and BGP plus vitamin C media. The CopGFP control cells exhibited higher osteo-odontogenic potential in both media, compared to the VEGFR-2 shRNA cells based on ALP and Alizarin Red S staining. However, CopGFP cells cultured in BMP-2 media showed higher number of differentiated cells compared to those in BGP plus vitamin C media. This result underscores BMP-2 as a powerful morphogen

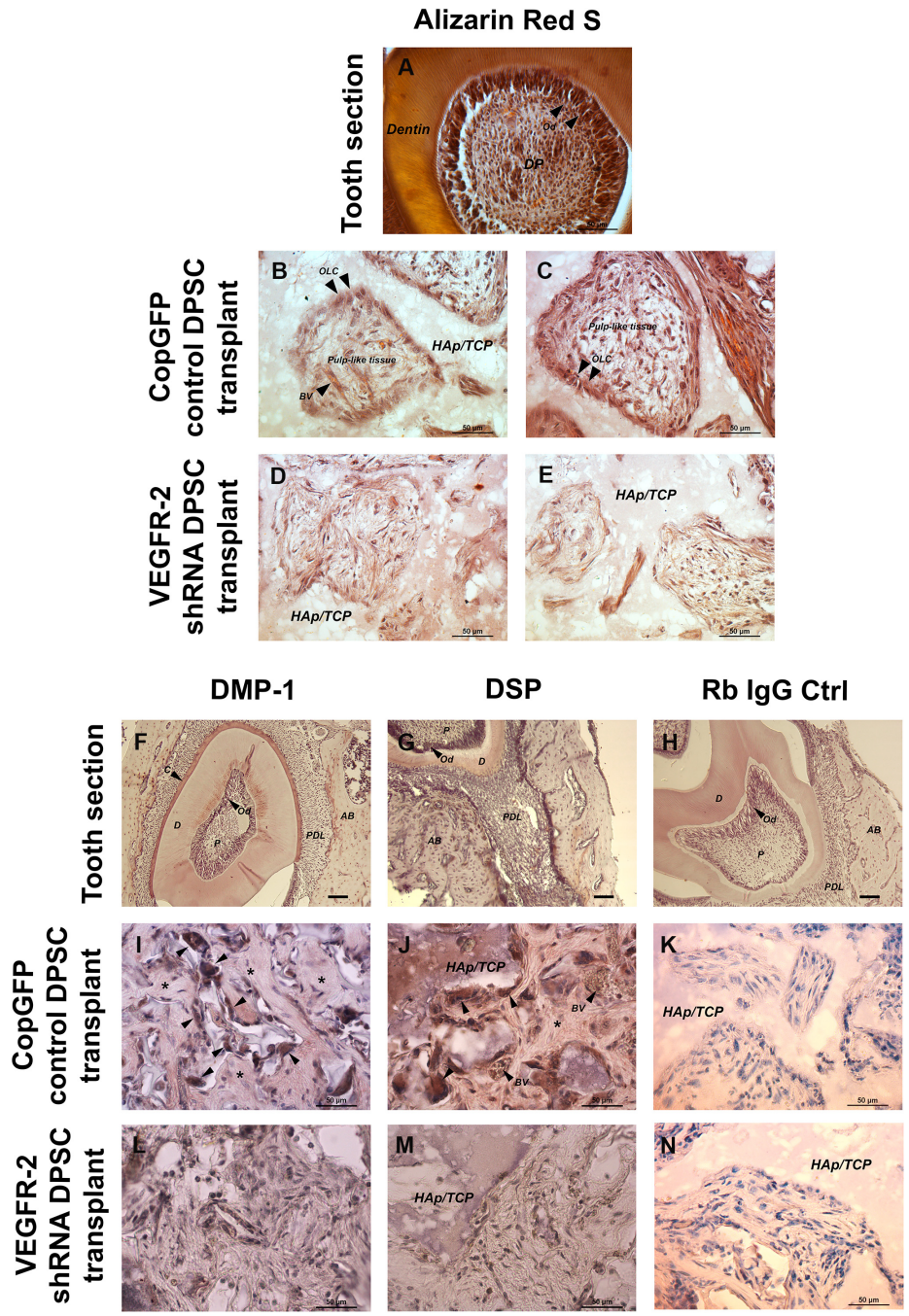

FIGURE 7 | *In vivo* transplantation of CopGFP control and VEGFR-2 shRNA DPSCs by Alizarin Red S staining and immunohistochemistry. Alizarin Red S staining was conducted to confirm the mineralized tissues, dental structures and morphology of CopGFP DPSC transplants and VEGFR-2 shRNA DPSC transplants. The cross-section of murine tooth was used as a positive control for Alizarin Red S staining. The positive area of Alizarin Red S showed in dentin, odontoblasts and some dental pulp regions **(A)**. A CopGFP control DPSC transplant revealed positive staining of fibrous structure surrounding HAp/TCP with elongated and polarized odontoblast-like cells (OLC) (arrowheads). Some positive odontoblast-like cells (OLC) (arrowheads) running perpendicular to HAp/TCP and pulp-like tissues with blood capillaries were seen in CopGFP DPSC transplants **(B,C)**. VEGFR-2 shRNA DPSC transplants only showed diffuse unpatterned cell arrangement with disorganized connective tissue **(D,E)**. Immunohistochemistry of anti-DMP-1 and anti-DSP staining was also conducted to determine the specific dentin protein expression in the transplant tissues. The tooth sections were used as a positive control. The expression of DMP-1 and DSP was seen in dentin, cementum and alveolar bone. Both dentin matrices were very strong in the odontoblast cell layer **(F,G)**. The CopGFP control DPSC transplants revealed the strongly positive anti-DMP-1 and anti-DSP staining in odontoblast-like cells (OCL) (arrowheads) **(I,J)**. Its matrices were slightly stained for both markers (asterisks). The VEGFR-2 shRNA DPSC transplants showed the negative anti-DMP-1 and anti-DSP staining **(L,M)**. The Rabbit IgG control (Rb IgG Ctrl) was used as a negative control to confirm the specificity of antibody **(H,K,N)**. AB; alveolar bone, C; cementum, D; dentin, HAp/TCP; hydroxyapatite/tricalciumphosphate, OLC; odontoblast-like cells, P; Pulp, PDL; periodontal ligament. Scale bars indicate 50 μm.

in osteoblast differentiation, bone development, formation and repair (Jorgensen et al., 2004; Marupanthorn et al., 2017). ALP plays an important role as an early osteogenic marker (Sabokbar et al., 1994). Alizarin Red S staining is a method to determine the mineralization or calcification which is a late hallmark for osteoblastic differentiation (Clark et al., 2015). Additionally, osteo-odontogenic genes, *Dmp-1*, *Dspp*, and *Bsp*, were examined. Those markers have been previously reported as important factors for bone and dentin formation (Hwang et al., 2008; Ching et al., 2017). Following differentiation, the CopGFP cells expressed statistically higher levels of *Dmp-1*, *Dspp*, and *Bsp*, compared to the VEGFR-2 shRNA cells. This demonstrate that the VEGFR-2 shRNA DPSCs were unable to differentiate into the osteo-odontogenic lineage *in vitro*. Taken together, this suggests that VEGFR-2 may play an important role in osteo-odontogenic potential of DPSCs.

To further investigate whether VEGF-A signaling via VEGFR-2 directly induces odontoblastic differentiation of DPSCs, the CopGFP and VEGFR-2 shRNA DPSCs were cultured in VEGF media and evaluated by ALP and Alizarin Red S staining. A few CopGFP cells only demonstrated positive ALP staining; however, none of VEGFR-2 shRNA cells showed a positive result. Moreover, neither CopGFP cells nor VEGFR-2 shRNA cells exhibited the positive Alizarin Red S staining. This indicates that VEGF-A only cannot induce a complete and mature osteo-odontogenic differentiation. Instead, VEGF-A might facilitate or "prime" DPSCs for the early stages of differentiation but it seems insufficient on its own to induce full osteo-odontogenic differentiation. These results are consistent with previous studies showing that exogenous VEGF enhances proliferation and early differentiation of DPSCs (D'Alimonte et al., 2011; Forgues et al., 2019). Nevertheless, our current study helps us differentiate the role of VEGFR-2 in the osteo-odontogenic ability of DPSCs, independent of VEGF-A.

To confirm our *in vitro* differentiation, the CopGFP and VEGFR-2 shRNA DPSCs mixed with hydroxyapatite/tricalciumphosphate (HAp/TCP) were subcutaneously transplanted in the dorsum of Rag-1 knockout mice for 5 weeks. This knockout mouse model is immunodeficient due to an absence of B- and T-lymphocytes (Menoret et al., 2013), and thus a good model to avoid transplant rejection. The CopGFP DPSC transplants exhibited elongated and polarized OLCs with vascularized pulp-like structures, corresponding to several previous studies of DPSC transplants with osteoconductive HAp/TCP scaffolds (Gronthos et al., 2000, 2002). In contrast, the VEGFR-2 shRNA DPSC transplants showed only the formation of collagenous and fibrous tissues. Consequently, the CopGFP DPSC transplantation confirms a biological role of VEGFR-2 for DPSC odontogenic potential.

In addition to endothelial cells, VEGF signaling has been shown to exhibit an important function in osteoblasts (Deckers et al., 2000). Previous *in vitro* experiments suggested that VEGFR-2 plays a role in osteoblast differentiation and survival (Alonso et al., 2008). Recently, the role of VEGF signaling and intracellular VEGF in osteoblast differentiation have been reported (Liu et al., 2012). BMP-induced VEGF expression is an important mechanism during osteoblast differentiation and bone formation (Zelzer and Olsen, 2005). Unlike the osteoblast differentiation, the role of VEGFR-2 in odontoblastic differentiation of DPSCs is still undetermined. Our data showed that VEGFR-2 shRNA DPSCs cultured in BMP-2 media were unable to give rise into the osteo-odontogenic lineage. One possibility is that VEGFR2 signaling is necessary for up-regulation of BMP-2 receptors (BRII and BRII) and downstream signaling molecules (Smads), thus priming odontoblast precursors to be responsive to BMP-2 (Qin et al., 2012). Additionally, silencing VEGFR-2 in DPSCs led to reductions in VEGF-A levels. Therefore, impaired odontoblast differentiation of VEGFR-2 null cells may be due to synergist or independent effects of VEGF-A.

However, our studies showed that adding exogenous VEGF to treat CopGFP and VEGFR-2 shRNA cells did not induce complete odontogenic differentiation. This is corresponding to a previous study showing that treatment with recombinant VEGF does not affect osteoblastic and adipocytic differentiation in normal bone marrow derived MSCs and VEGF deficient MSCs (Liu et al., 2012). Altogether, this suggests the effects of VEGFR-2 are mediated by intracellular mechanisms, possibly including transcriptional regulation of BMP signaling proteins rather than by paracrine VEGF signaling. In addition to intracrine mechanisms, it is possible that DPSC-derived VEGF also affects the formation of dentin/pulp complex *in vivo* through enhanced angiogenesis (Wang et al., 2007). Nonetheless, the specific mechanism(s) of how VEGFR-2 affects odontoblast differentiation of DPSCs need to be further investigated.

In conclusion, our data originally identifies VEGFR-2 signaling as an important molecular pathway for odontoblastic differentiation in DPSCs. These results provide key evidence of the importance of VEGFR-2 in tooth development and highlight the potential of VEGFR-2 modulation to enhance dentin regeneration and tissue engineering as a promising clinical application such as targeted drug therapy and delivery for regenerative dentistry.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Washington.

AUTHOR CONTRIBUTIONS

KJ and MR: writing and editing and study design. KJ, RC, and AH: experimentation. KJ, RC, and MR: data analysis. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.665886/full#supplementary-material

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Conflict 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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Indispensable Role of HIF-1α Signaling in Post-implantation Survival and Angio-/Vasculogenic Properties of SHED

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Objectives: Post-implantation survival and timely vascularization of stem-cell based constructs are critical factors in achieving successful outcomes in tissue regeneration approaches. Hypoxia inducible factor-1α (HIF-1α) is known to mediate adaptive functions to ischemic stress in many different cell types. The current study aimed to explore the role of HIF-1α in post-implantation survival and angio-/vasculogenesis of stem cells from human exfoliated deciduous teeth (SHED).

Methods: HIF-1α in SHED was suppressed using siRNA or chemical inhibitor (YC-1) and used in Matrigel plug assay conducted on severe combined immunodeficient mice. The plugs were retrieved on day 3 or 7 post-injection and analyzed for hypoxia status, ki67 expression, DNA fragmentation (TUNEL), cellularity, and vascularization by histology and immunohistochemistry for CD31, HIF-1α, pyruvate dehydrogenase kinase-1 (PDK1), hexokinase 2 (HK2), and glucose transporter 1 (Glut1). Cell viability of HIF-1α silenced SHED under different stress conditions (hypoxia, H_2O_2, and low glucose) *in vitro* was measured by CCK-8 assay. CM-H_2DCFDA and MitoSOX Red were used to detect cellular and mitochondrial reactive oxygen species (ROS) levels, respectively. PDK1, HK2, and Glut1 expression were measured by western blotting and immunofluorescence. Secretory protein levels of vascular endothelial growth factor (VEGF) and the respective paracrine effects on endothelial cell proliferation and migration were detected by ELISA, CCK-8 assay, and *trans*-well assay, respectively.

Results: Histological analysis of Matrigel plugs showed significantly reduced cell survival in HIF-1α silenced or chemically inhibited SHED groups, which could be attributed to diminished metabolic adaptations as shown by decreased PDK1, HK2, and Glut1 expression. HIF-1α inhibition in SHED also resulted in significantly low blood vessel formation as observed by a low number of perfused and non-perfused vessels of human or mouse CD31 origin. The viability of HIF-1α silenced SHED was significantly affected under hypoxia, H_2O_2, and low-glucose conditions *in vitro*, which was reflected in increased cytoplasmic and mitochondrial ROS levels. Significantly reduced levels of

VEGF in HIF-1α silenced SHED resulted in decreased paracrine angiogenic effects as shown by low proliferation and migration of endothelial cells.

Conclusion: HIF-1α plays an indispensable role in post-implantation survival and angio-/vasculogenic properties of SHED by maintaining ROS homeostasis, inducing metabolic adaptations, and VEGF secretion.

Keywords: HIF-1α, post-implantation survival, cell metabolism, redox balance, regenerative medicine, dental stem cells

INTRODUCTION

Stem-cell-based tissue engineering is a promising approach to restore damaged, injured, or missing tissues, in which stem cells differentiate into specific phenotypes and induce a regenerative microenvironment (Caplan, 2007). Stem cells from human exfoliated deciduous teeth (SHED) are regarded as one of the most striking cell sources because of their easy isolation, highly self-renew ability, and multiple differentiation capacities (Han et al., 2020b). There are, however, two critical challenges for stem-cell-based tissue engineering to overcome in order to achieve consistent preclinical and clinical trial outcomes. One major challenge is the low post-implantation cell survival, which can vary from as low as 10% up to 60% (Becquart et al., 2012; Stegen et al., 2016). Once the cells are introduced into the *in vivo* microenvironment, they experience an immediate hostile condition where oxygen and nutrient supply are scarce and oxidative stress is high, which can lead to significant cell death (Becquart et al., 2012). The second challenge is to achieve timely vascularization of the engineering construct in order to secure a constant supply of nutrients and oxygen, and ensure the removal of cell metabolic waste, which is vital for the long-term survival and functionality of the regenerating tissues.

Cells have developed physiological adaptive mechanisms for hypoxic/ischemic stress, which are mainly mediated through hypoxia-inducible factors (HIFs). HIF-1α, a master transcription factor in hypoxia, is specifically stabilized under low oxygen conditions as oxygen acts as a cofactor for prolyl hydroxylases that initiate degradation of HIF-1α. In hypoxia, HIF-1α interacts with ubiquitously expressed HIF-1β, and activates the expression of target genes containing hypoxia response elements (Rashid et al., 2019). Cells produce reactive oxygen species (ROS) as a byproduct of the electron transport chain, which is essential in cellular energy production; however, in hypoxia, severe increase of ROS will lead to cell death (Zhang et al., 2019). Once the cellular redox homeostasis is disrupted, oxidative stress in the cell may lead to DNA/RNA damage, contributing to cell death (Zhang et al., 2020; Chen et al., 2021). HIF-1α signaling pathway is found to be tightly interconnected with the ROS pathway. Hypoxia-inducible ROS could contribute to HIF-1α accumulation, and increased HIF-1α could prevent ROS overproduction (Wheaton and Chandel, 2011). The major target of HIF-1α on decreasing endogenous ROS is through pyruvate conversion to acetyl-CoA by pyruvate dehydrogenase kinase 1 (PDK1), which activates the tricarboxylic acid (TCA) cycle enzymes (Kim et al., 2006).

Cell survival and cellular metabolism are also closely related since tissue repair is an energy-demanding process that includes cell proliferation, self-renewal, and cell differentiation. Metabolic pathways, however, are very sensitive to cellular cues, such as oxygen tension and, in general, triggered by hypoxia. It is reported that HIF-1α has the function of regulating glucose metabolites by switching mitochondrial respiration to cytosolic glycolysis in cancer cells (Lu et al., 2002). Accordingly, under hypoxia, HIF-1α stabilization and formation of HIF-1α/β complex activate genes encoding a series of glycolytic enzymes and glucose transporters (Zhu et al., 2014).

In addition to metabolic adaptations, HIF-1α expression is known to induce an angiogenic response through vascular endothelial growth factor (VEGF; Ahluwalia and Tarnawski, 2012). VEGF plays an essential role in activating cellular pathways that lead to the promotion of endothelial cell proliferation, migration and assembly into vascular structures (Kliche and Waltenberger, 2001). Therefore, upregulation of VEGF-VEGFR signaling pathways under hypoxia assists the recovery of cells or tissues affected by ischemia (Ramakrishnan et al., 2014).

Despite the reported functions of HIF-1α in relation to other cell types, its role and mechanisms on post-implantation cell survival and angio-/vasculogenic properties of dental stem cells under stress conditions are still not clear. In the current study, we aimed to investigate the significance of HIF-1α expression in SHED in cell survival and angio-/vasculogenesis using an *in vivo* Matrigel plug assay. We found that genetic silencing or chemical inhibition of HIF-1α in SHED reduces the cell survival and angio-/vasculogenic response significantly. Furthermore, we revealed that HIF-1α regulates the expression of target proteins that mediate adaptive metabolic mechanisms in both *in vivo* and *in vitro* survival and angiogenesis in SHED.

MATERIALS AND METHODS

Cell Culture

SHED were purchased from AllCells (Alameda, CA, United States) and cultured in α-Modified Eagle's Medium (α-MEM) supplemented with 10% FBS and 1% penicillin/streptomycin. Mesenchymal origin and multipotent differentiation capacity of the cells were evaluated and published in our previous study (Xu et al., 2017). Human umbilical vein endothelial cells (HUVECs) were purchased from ScienCell

(Carlsbad, CA, United States) and cultured in endothelial growth medium-2 (EGM-2, Lonza, Walkersville, MD, United States). All cell cultures were kept in a 37°C and 5% CO_2 incubator. Passage 4–7 of SHED and 3–6 of HUVECs were used in all the downstream experiments. Hypoxia condition (1% O_2) *in vitro* cultures was achieved using the hypoxia incubator (Thermo Scientific, MA, United States).

HIF-1α Knockdown by siRNA and Chemical Inhibition by YC-1

Silencing of HIF-1α expression in SHED was achieved by transfection of premade siRNA (siHIF-α:4390824, negative control: 4390843, Thermo Scientific) with Lipofectamine^{TM} 3000 (Thermo Scientific) according to the manufacturer's instructions. Briefly, the cells were seeded in 6-well plates for 70 ∼ 90% density. After overnight incubation, lipofectamine^{TM} 3000 reagent – siRNA complexes were prepared in Opti-MEM^{TM} Reduced Serum Medium (Thermo Scientific). Subsequently, the fresh culture medium was changed and siRNA-lipid complexes were added into the cells. siRNA carrying a no significant sequence was used as a negative control. Transfection efficiency was assessed by western blotting (WB) and immunofluorescence (IF) after 48 h under hypoxia. For identification of different groups, we termed the siRNA-control group as "siControl" and the siRNA-HIF-1α group as "siHIF-1α." To confirm the function of HIF-1α, we also used HIF-1α inhibitor YC-1 (80 μM, Sigma, MO, United States), which acts by prevention of HIF-1α accumulation in response to hypoxia. Accordingly, we termed the YC-1-control and YC-1-treated groups as "CTR" and "YC-1" groups, respectively.

In vivo Matrigel Plug Assay

All experimental animal procedures were approved by the Committee on the Use of Live Animals in Teaching and Research (CULATR 4625-18) of the University of Hong Kong and performed following the Guide for the Care and Use of Laboratory Animals published by the United States National Research Council and regulations. 24 severe combined immunodeficient (SCID/CB17) mice (male, ∼6 weeks of age, 20 ∼ 40 g) were randomly allocated into eight groups: siControl-3 days, siHIF-1α-3 days, siControl-7 days, siHIF-1α-7 days, CTR-3 days, YC-1-3 days, CTR-7 days, and YC-1-7 days. Each group had 3 mice (6 plugs per group, $n = 6$). A subcutaneous injection procedure was performed as described before (Han et al., 2020a). Briefly, the cells (3×10^6/per plug, cells were treated with siRNA *in vitro* for 48 h) were trypsinized, centrifuged, and resuspended in 100 μL EGM-2 followed by mixing with 400 μL of Matrigel and injected slowly into the subcutaneous space of the lateral hind regions of mice bilaterally. In the chemical inhibition group, YC-1 dissolved in DMSO was mixed with cell/Matrigel solution to achieve a final concentration of 80 μM before injection. Matrigel plugs were retrieved after 3 days (3 D) and 7 days (7 D) of injection, fixed with 4% paraformaldehyde (PFA) for 24 h and paraffin-embedded in an orientation that allowed obtaining sections with intact skin and muscle layer. Sections of 5 μm thickness were cut and examined for histology and immunohistochemistry (IHC).

Histology and Immunohistochemistry

Histological staining (H&E), IHC, and quantification procedures were performed as described previously (Han et al., 2020a). For H&E staining, sections were immersed in hematoxylin for 3 min and eosin for 1 min after alcohol gradient dewaxing. After mounting, images were taken with an inverted microscope (Nikon Eclipse LV100N POL, Tokyo, Japan) at 20× and 50× magnifications. Vessel-like structures without red blood cells were counted as non-perfused vessels and structures with red blood cells in the lumen were counted as perfused vessels. For IHC staining, following primary antibodies were used: hypoxyprobe omni kit (Hypoxyprobe, MA, United States), ki67 (ab92742, Abcam), isotype control (ab37415, Abcam), human CD31 (ab32457, Abcam), mouse CD31 (ab124432, Abcam), HIF-1α (ab51608, Abcam), PDK1 (sc-293160, Santa Cruz, TX, United States), hexokinase 2 (HK2, sc-374091, Santa Cruz), and glucose transporter 1 (Glut1, ab150299, Abcam). Mouse and rabbit specific HRP/DAB IHC detection kit (Abcam) was used according to the protocol. Briefly, following the deparaffinization process, sections were incubated with hydrogen peroxide block for 10 min, antigen retrieval pretreatment for 6 min, protein block for 5 min, and different primary antibodies overnight. On the following day, sections were incubated with anti-mouse and rabbit secondary antibody for 10 min, streptavidin peroxidase for 10 min, and DAB solution for 10 s – 1 min. Lastly, sections were counterstained with hematoxylin for 1 min. After capturing images by NIS-Elements AR 3.1 software (Nikon, Tokyo, Japan) at 20× and 50× magnification, percentages of positive areas/cell numbers (HIF-1α, PDK1, HK2, Glut1, and human CD31) were calculated on 5 randomly selected regions under 50 × magnification using NIS-Elements AR 3.1 software (Nikon, Tokyo, Japan). *In situ* direct DNA fragmentation assay (TUNEL Assay Kit, Abcam) was performed following the kit protocol to visualize the DNA damage in tissue. Fluorescent microscope (Nikon, Tokyo, Japan) was used to capture the TUNEL-positive cells.

Conditioned Media

siControl and siHIF-1α SHED were cultured in standard culture medium until 80% confluence and changed to serum-free medium. Conditioned media were collected after 24 h in normoxia and hypoxia condition, followed by centrifugation at 1,500 rpm and filtration to remove cell debris.

CCK-8 Assay

SHED in Stress Condition CCK-8 Assay

To analyze *in vitro* stress resistance, siControl and siHIF-1α SHED were cultured for 24–48 h in the presence of either hypoxia, hydrogen peroxide (H_2O_2, 200 μM), glucose-deprived medium, or hypoxia + H_2O_2 + glucose-deprived medium condition. *In vitro* cell viability was detected by CCK-8 assay kit (Abcam).

HUVECs in Conditioned Media CCK-8 Assay

To examine the paracrine effects of siHIF-1α SHED on the proliferation of endothelial cells, HUVECs were seeded on 96-well plates at a density of 6,000 cells per well. After 24 h, EGM-2 (positive control), α-MEM (negative control), conditioned media from siControl or siHIF-1α SHED in normoxia or hypoxia were added to separate wells. After culturing for 48 h, cell proliferation was determined using the CCK-8 assay kit. Briefly, fresh medium was changed and 10 μl CCK-8 reagent was added per well. After incubating for 3 h at 37°C, the absorbance was measured at 460 nm by SpectraMax® M2 microplate reader (Molecular Devices, CA, United States).

Western Blotting and Immunofluorescence

Western blotting and IF were performed to evaluate the specific protein expression as described previously (Han et al., 2020a). In addition to the above-mentioned primary antibodies for IHC staining, HIF-1α (610958, BD Biosciences, New Jersey, United States) for WB was also used. Secondary antibodies included anti-rabbit or anti-mouse HRP-linked antibody (Cell Signaling Technology, MA, United States), Alexa Fluor 488®-conjugated goat anti-mouse antibody (Abcam), Alexa Fluor 594®-conjugated goat anti-rabbit antibody (Abcam). The protein samples of siControl and siHIF-1α SHED were collected after culturing under normoxia and hypoxia for 24 h. The results of WB were quantified by Image J and normalized by Glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Cells seeded on coverslips in 24-well plates were fixed by 4% PFA after incubated in normoxia and hypoxia for IF staining. After incubated with primary antibody overnight, secondary antibody for 1 h and DAPI for 5 min, images were captured by fluorescence microscope (Nikon, Tokyo, Japan) at 20 × magnification.

Detection of Cellular ROS Levels

CM-H_2DCFDA (Thermo Scientific) and MitoSOX Red (Thermo Scientific) staining were used for detecting cytoplasmic and mitochondrial ROS levels, respectively. Cells were seeded in 24-well plates and cultured in normoxia or hypoxia for 24 h. Subsequently, cells were incubated with 10 μM CM-H_2DCFDA for 30 min or 5 μM MitoSOX Red for 10 min at 37°C, and fluorescence was captured by immunofluorescence microscope (Nikon, Tokyo, Japan) at 20 × magnification. Immunofluorescence density was quantified by Image J software (U. S. National Institutes of Health, MD, United States) and normalized by the siControl normoxia group.

Cell Migration Assay

Trans-well assay was performed to study the paracrine effects of siHIF-1α SHED on endothelial cells as described before (Han et al., 2020a). Briefly, HUVECs were trypsinized, resuspended with serum-free medium and seeded on the upper chamber of inserts at a density of 1×10^4/200 μL, whereas 600 μL of conditioned media from siControl or siHIF-1α SHED were added to the lower compartment of each well. After 24 h, HUVECs on the lower surface of the membrane were fixed and stained with 0.1% (w/v) crystal violet (Sigma) for 15 min. Migrated HUVEC numbers were quantified.

ELISA

VEGF secretion levels were measured in the culture supernatant of siHIF-1α SHED and siControl SHED using the human VEGF DuoSet Elisa Kit (R&D systems, MN, United States) as described before (Han et al., 2020a). Supernatants were collected from siControl and siHIF-1α SHED after culturing in normoxic and hypoxic conditions for 24 h. Protein levels were normalized to that of siControl normoxia group.

Statistical Analysis

All the experiments were conducted in triplicate and repeated at least 3 times. Results were presented as mean ± standard deviation (SD). Statistical analysis was carried out using Student's *t*-test for assays done between two groups, and one-way ANOVA with a Tukey's *post hoc* test in multiple comparisons. Two-way ANOVA with Sidak Test in multiple comparisons was used for two independent variables on a dependent variable. All the statistical testing and creation of graphs were performed using Prism 8.0 software (GraphPad Software, Inc., San Diego, CA, United States). Statistical significance was considered at $p < 0.05$.

RESULTS

Silencing of HIF-1α Impairs the Post-Implantation Cell Viability in Matrigel Plugs

The hypoxic status of the implanted Matrigel plugs was investigated by IHC for Hypoxyprobe™ (pimonidazole hydrochloride), which is a 2-nitroimidazole that is activated specifically in hypoxic cells. SHED-seeded Matrigel plugs showed significantly higher number of hypoxic cells in the central region of the construct as evident by positive IHC staining (**Figure 1A**). Cell proliferation and apoptosis in hypoxia *in vivo* were evaluated by IHC for Ki67, a proliferative marker (**Figure 1B**) and DNA fragments (TUNEL) assay (**Figure 1C**), respectively. Higher number of cells with ki67 expression and DNA fragments were found in the center of the Matrigel compared with that of the periphery, which means the cell viability under hypoxia is a result of countervailing effects between cell apoptosis and cell proliferation. Accordingly, as shown by H&E staining and quantification of cellularity after 7 days (**Figure 1D**), both the central and peripheral areas of the siHIF-1α plug contained significantly less number of survived cells compared with that of the siControl group.

Silencing or Chemical Inhibition of HIF-1α Down-Regulated the Proteins Important in Metabolic Adaptations *in vivo*

It has been reported that in severely hypoxic conditions, HIF-1α-mediated induction of genes could regulate metabolic shift from

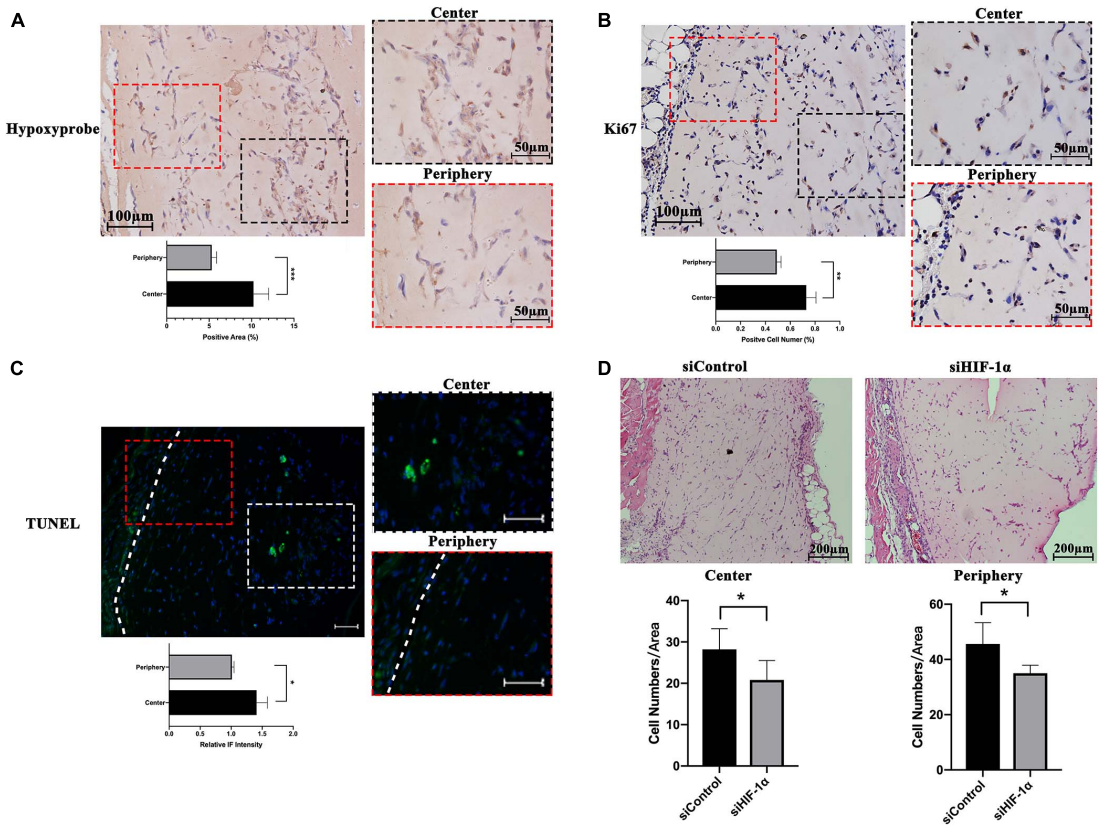

FIGURE 1 | Hypoxic status and cell survival within SHED-seeded Matrigel plugs *in vivo*. Immunohistochemistry for **(A)** Hypoxyprobe and **(B)** ki67, and **(C)** immunofluorescence for TUNEL assay in the center and the periphery of the Matrigel plug **(D)**. Cellularity at the center and the periphery of H&E stained Matrigel plugs of siControl and siHIF-1α SHED after 7 days of implantation. White dotted line – the boundary between Matrigel plug and mouse tissue. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$. Scale bar: 10 × = 200 μm, 20 × = 100 μm, 50 × = 50 μm, and IF = 50 μm.

oxidative phosphorylation to glucose oxidation by preventing the entry of pyruvate into the tricarboxylic cycle. To investigate the role of HIF-1α signaling in maintaining early post-implantation cell survival, we immunohistochemically stained the tissue sections for the expression of proteins important in the metabolic shift to glycolysis as well as regulating cellular ROS homeostasis. As shown in **Figure 2A** (3 D) and **Figure 2B** (7 D), the siHIF-1α group demonstrated less PDK1 ($p < 0.01$), HK2 ($p < 0.0001$), and Glut1 expression ($p < 0.01$) compared with the control groups. Similarly, as shown in **Figure 2C** (3 D) and **Figure 2D** (7 D), chemical inhibition of HIF-1α by YC-1 significantly suppressed the expression of PDK1, HK2, and Glut1 at both 3 D and 7 D of transplantation. When compared with 7 D samples, we observed that the expression of these proteins were higher in 3 D plugs, which indicated a more critical role at the early stages of post-implantation.

Silencing or Inhibition of HIF-1α Impairs the Angio-/Vasculogenic Capacity of SHED *in vivo*

Matrigel plugs with HIF-1α silenced SHED showed delayed vessel formation and perfusion compared with that of normal SHED plugs. In control groups, either siControl or CTR, vessel-like structures (**Figures 3A,C**, black arrows) were seen after 3 days while perfused vessels (white arrows) were observed after 7 days of implantation. Similar structures, however, could not be seen either in siHIF-1α or YC-1 group. The quantification of the number of non-perfused vessels and perfused vessels at 7 day samples showed that the silencing or chemically inhibition of HIF-1α in SHED significantly reduced the vascularization of the Matrigel plugs.

Immunohistochemistry staining for human CD31 revealed that the siControl and CTR groups contained more positive cells and vessel-like structures (**Figures 3A,C**, red arrows) compared with siHIF-1α or YC-1 group, indicating endothelial differentiation of HIF-1α intact SHED. The quantified percentages of human CD31 positive area at 7 days (**Figures 3B,D**) showed both siHIF-1α and YC-1 groups failed to induce a prominent vasculogenic response. Similarly, IHC for mouse CD31 disclosed that in 7 day samples, the siControl and CTR groups contained mouse CD31 positive vessel-like structures (**Figures 3A,C**, blue arrows). The black dotted line showed the boundary between Matrigel plug and mouse tissue. Mouse CD31 positive vessels were scarcely found in both siHIF-1α and YC-1 groups, which could be attributed to the

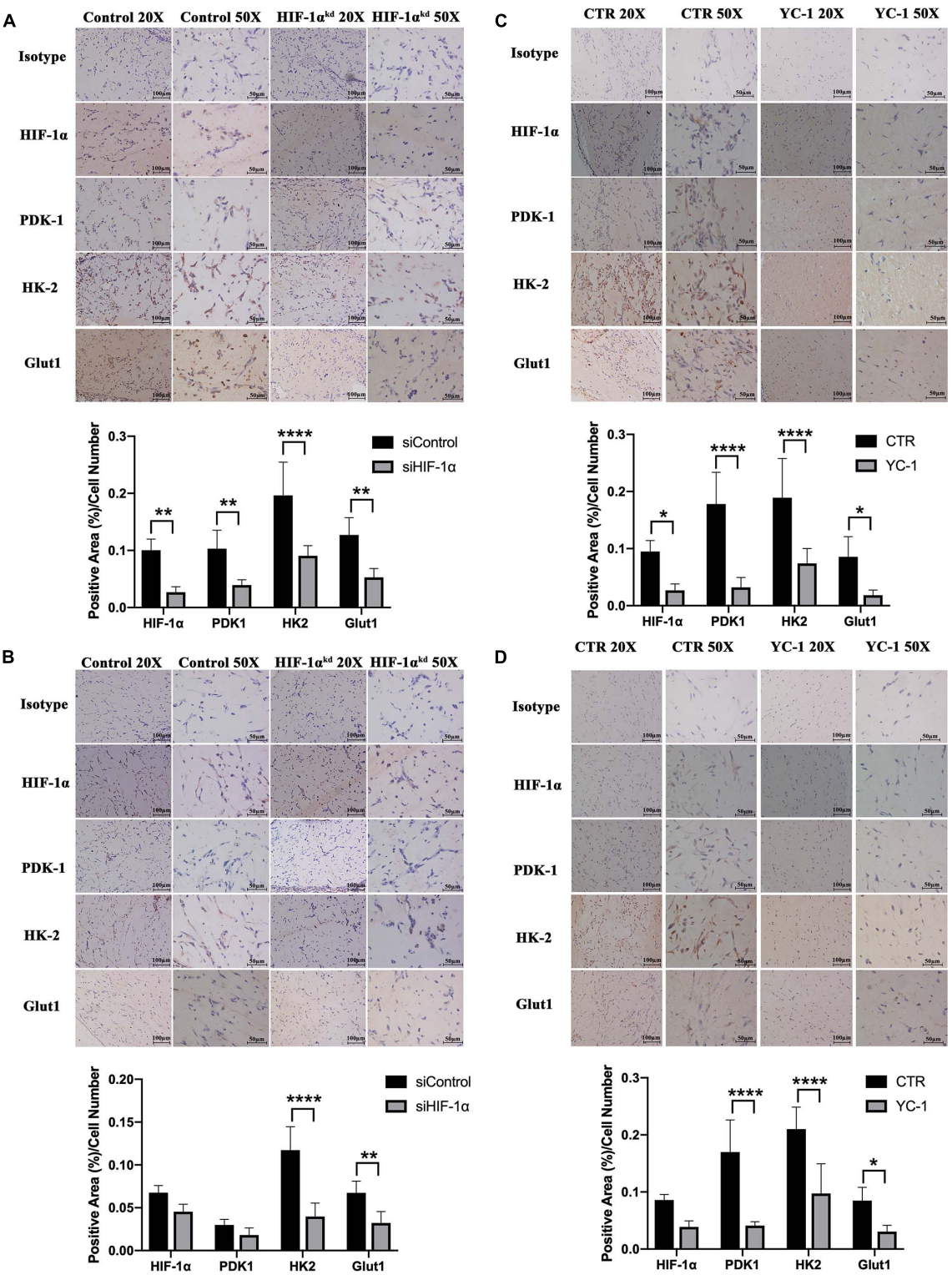

FIGURE 2 | Silencing or chemical inhibition of HIF-1α down-regulated the proteins important in metabolic adaptations *in vivo*. Immunohistochemistry for HIF-1α, PDK-1, HK-2, and Glut-1 in siControl and si-HIF-1α SHED containing Matrigel plugs at **(A)** 3 days and **(B)** 7 days, and CTR and YC-1 SHED containing groups at **(C)** 3 days and **(D)** 7 days of transplantation. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$. Scale bar: 20 × = 100 μm, 50 × = 50 μm.

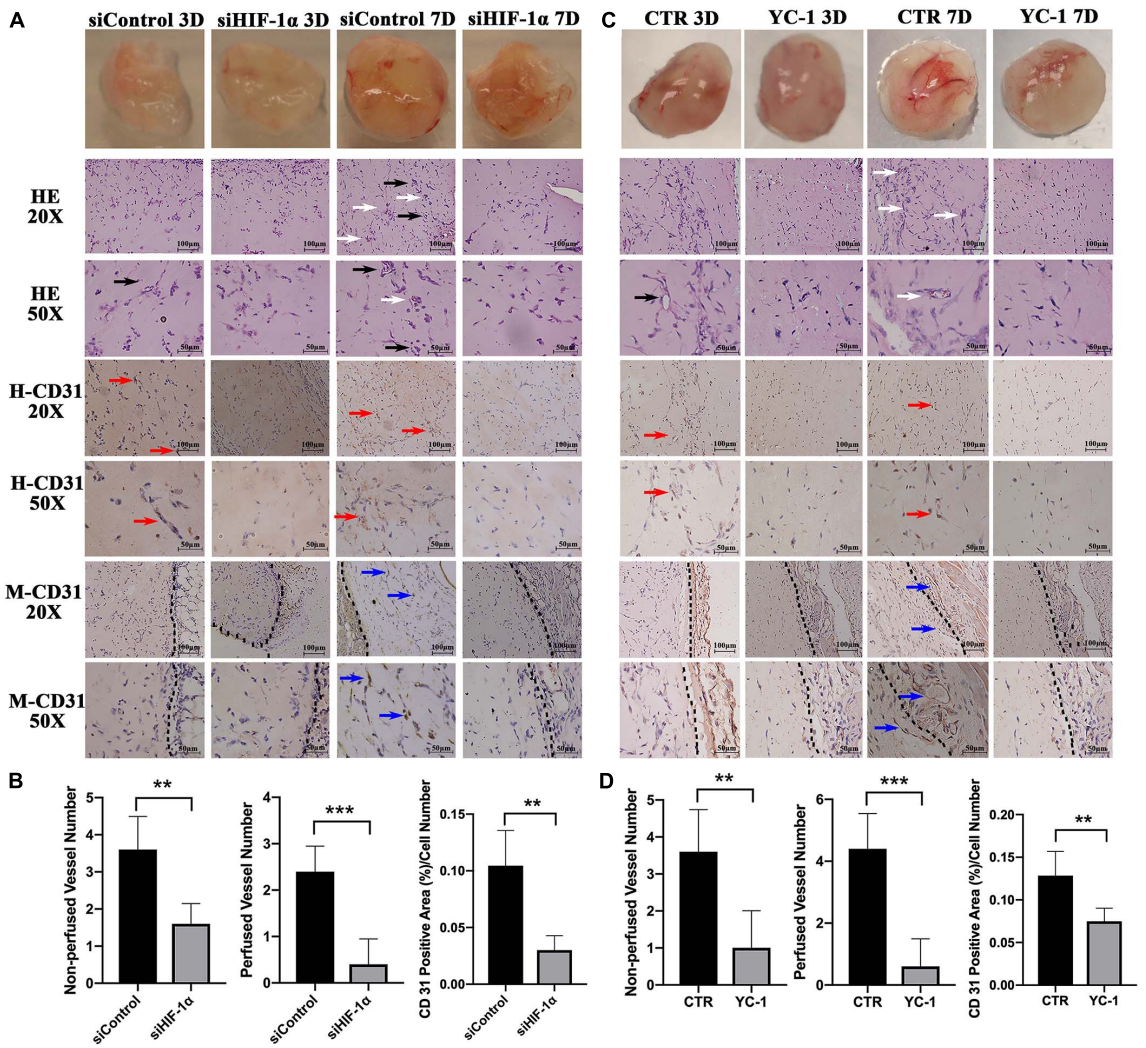

FIGURE 3 | Silencing or chemical inhibition of HIF-1α expression impairs the angio-/vasculogenic capacity of SHED. Macroscopic photos, H&E staining, immunohistochemical staining for human CD31 and mouse CD31 of **(A)** siControl SHED and siHIF-1α SHED **(C)** CTR SHED and YC-1 SHED Matrigel plugs 3 days and 7 days after implantation. Quantified graphs of non-perfused and perfused vessel numbers and percentages of human CD31 positive area of **(B)** siControl SHED and siHIF-1α SHED **(D)** CTR SHED and YC-1 SHED Matrigel plugs at 7 days after implantation. Black arrows – non-perfused vessel-like structures, White arrows – perfused vessels, Red arrows – human CD31 positive vessel-like structures, Blue arrows – mouse CD31 positive vessel-like structures, and Black dotted line – the boundary between Matrigel plug and mouse tissue. $^{**}p < 0.01$, and $^{***}p < 0.001$. Scale bar: 20 × = 100 μm, 50 × = 50 μm.

indispensable role of HIF-1α expression in the induction of host blood vessel ingrowth into the implant.

Silencing of HIF-1α in SHED Affects ROS Homeostasis and Cell Viability Under Stress *in vitro*

Having observed a significantly reduced cellularity in HIF-1α silenced SHED in Matrigel plugs *in vivo*, we aimed to examine the role of HIF-1α signaling in cell survival under different stress conditions *in vitro*. CCK-8 assay results (**Figure 4A**) revealed that there is no significant difference in cell viability between siControl and siHIF-1α SHED in normal culture conditions. In hypoxia condition, cell survival was reduced in both groups compared with that of normoxia while siHIF-1α SHED showed a significant reduction than siControl after 48 h of culture. Furthermore, HIF-1α seems to play a more important role in maintaining cell viability under oxidative stress and glucose-deprived conditions as shown by significantly reduced OD values in siHIF-1α SHED compared with that of siControl group at both 24 and 48 h.

Additionally, cytoplasmic and mitochondrial ROS levels in SHED were evaluated by using CM-H_2DCFDA (green, **Figure 4B**) and MitoSOX (Red, **Figure 4B**), respectively. The results showed increased cytoplasmic ROS levels in cells exposed to hypoxia while the levels were significantly higher in HIF-1α silenced SHED ($p < 0.05$). Similar results were observed by mitochondrial superoxide indicator, which showed higher

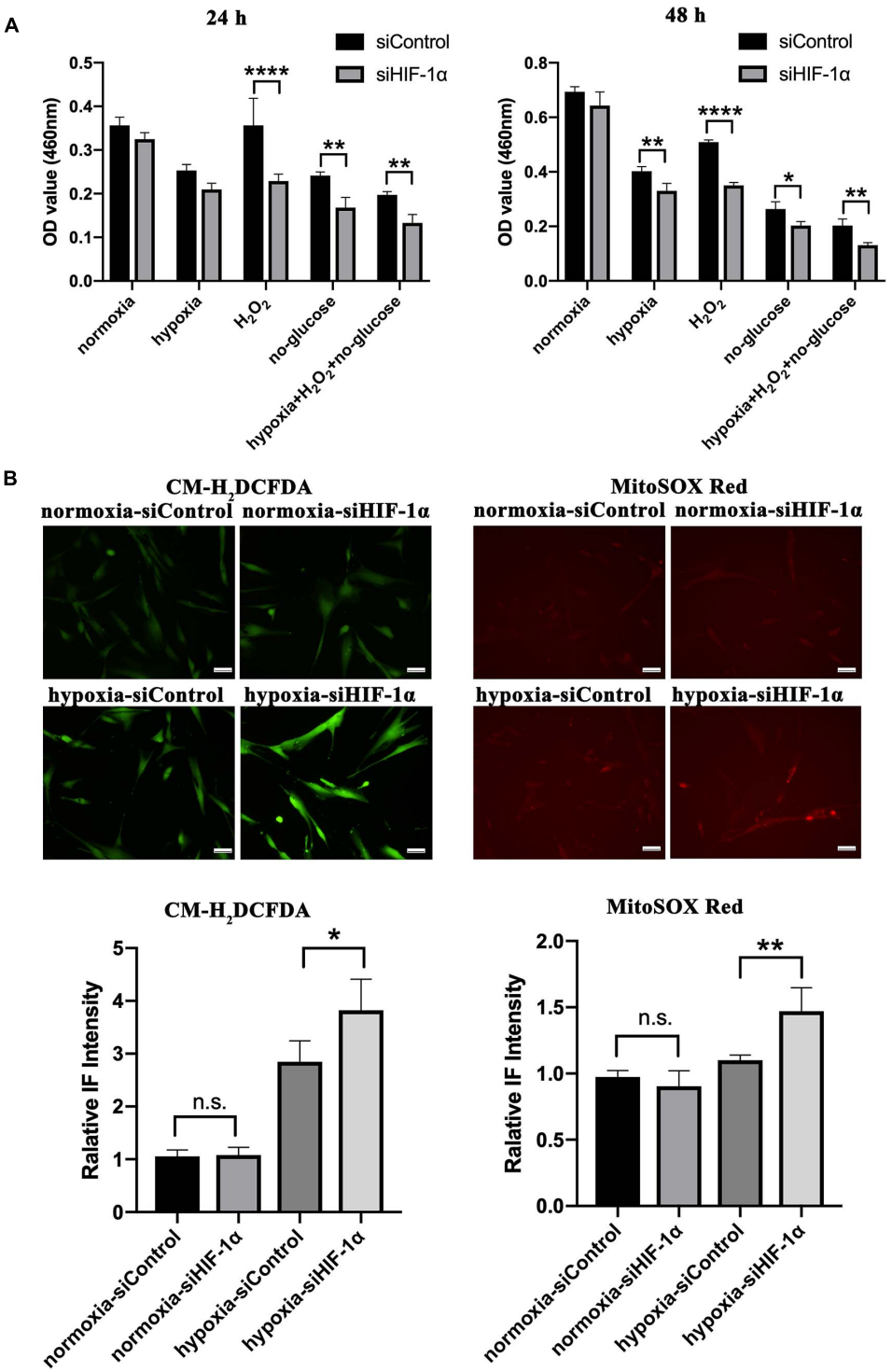

FIGURE 4 | Silencing HIF-1α in SHED limits their cell viability and ROS homeostasis under stress *in vitro.* **(A)** CCK-8 assay of siControl and siHIF-1α SHED in normoxia, hypoxia, H_2O_2, glucose deprived medium, and hypoxia + H_2O_2 + glucose-deprived medium. **(B)** Immunofluorescence for CM-H_2DCFDA (green) and MitoSOX (red) showed the cytoplasmic and mitochondrial ROS levels in SHED, respectively. *$p < 0.05$, **$p < 0.01$, and ****$p < 0.0001$. Scale bar = 50 μm.

oxidative stress in the mitochondria of siHIF-1α SHED in hypoxia ($p < 0.01$) compared with that of siControl SHED.

Silencing of HIF-1α Suppressed the Expression of Proteins Important in Metabolic Adaptations *in vitro*

Similar to *in vivo* assays, we also examined the levels of PDK1, HK2, and Glut1 proteins *in vitro* cell cultures by WB (**Figure 5A**) and IF (**Figure 5B**). There was not much difference in PDK1, HK2, and Glut1 protein expressions between the two cell groups in normoxia. In contrast, the three proteins were significantly activated in siControl SHED under hypoxia, while markedly impaired in HIF-1α silenced SHED. These results confirmed the role of HIF-1α in regulating the expression of genes responsible for metabolic adaptations under oxidative stress in SHED.

Silencing of HIF-1α in SHED Reduced VEGF Secretion and Paracrine Effects on HUVECs

The *in vivo* Matrigel plug assay results demonstrated significantly less mouse and human CD31 positive vessels and perfused vessels in siHIF-1α and YC-1 SHED groups, which indicated a crucial role of HIF-1α in mediating autocrine and paracrine angiogenic effects. Therefore, we examined the levels of VEGF protein, the most important angiogenic factor, in the conditioned media collected from siControl and siHIF-1α SHED by ELISA. The results (**Figure 6A**) revealed that siHIF-1α SHED had secreted significantly lower VEGF levels than siControl SHED in hypoxia ($p < 0.0001$). When we used the conditioned media to culture HUVECs, CCK-8 assay results (**Figure 6B**) showed that HUVECs cultured in conditioned medium from siControl SHED under hypoxia had the highest growth among the four conditioned media, which were all significantly higher than that of cultured in α-MEM. In contrast, conditioned medium collected from siHIF-1α SHED under hypoxia failed to show increased proliferation of HUVECs than siControl SHED conditioned medium in normoxia.

Trans-well assay was performed to evaluate the function of HIF-1α expression in SHED on inducing migration of HUVECs. The representative images and quantification of migrated cells (**Figure 6C**) revealed that conditioned medium from siControl SHED could significantly enhance the migration of HUVECs while conditioned medium from HIF-1α silenced SHED failed to do so. Collectively, the results confirmed that HIF-1α expression plays a crucial role in mediating paracrine angiogenic effects of SHED under hypoxia.

DISCUSSION

The harsh microenvironment, which could be due to hypoxia, low nutrients, and oxidative stress, is the main reason for low post-implantation stem cell survival. Physiologically, the HIF-1α signaling pathway is reported to be activated to counter hypoxic conditions. In the current study, we uncovered dual functions of HIF-1α in dental stem cells as adaptive responses to the microenvironmental stress. Firstly, HIF-1α is essential to activate the downstream genes responsible for switching from oxidative phosphorylation to glycolysis in order to maintain redox balance at the early stage of cell adaptation. Secondly, HIF-1α dependent VEGF secretion plays an essential role in inducing the angiogenic response by promoting the proliferation and recruitment of host vascular endothelial cells and differentiation of dental stem cells into endothelial cells.

In the early stage of cell implantation, there is almost no vessel invasion from the host since it is an extremely slow process (Stegen et al., 2016). Therefore, it is inevitable that the stem cell niche gets devoid of oxygen and nutrients. In normal oxygen levels, ROS, mainly produced by mitochondria, regulates cell proliferation and angiogenesis. In hypoxia, however, the excessively increased ROS production can destroy cell membranes (Sun et al., 2019), damage stem cells' self-renewal capacity, and induce cell apoptosis (Zhou et al., 2014). Stem cells are always more vulnerable to the destructive impacts of drastic increase in ROS than other differentiated cells. In our study, we observed that there was a significant hypoxic environment particularly in the center of Matrigel plugs and markedly decreased cell numbers consistent with the low oxygen tension *in vivo*. This finding indicated the crucial role of HIF-1α in maintaining the viability of SHED under hypoxia.

Reactive oxygen species and HIF-1α signaling have been known to be involved in various diseases, such as tumors and ischemia injury. It was reported that PDK1 is a direct target of HIF-1α and plays an essential role in mitochondrial ROS production, maintenance of ATP levels, and adaptation to hypoxia (Kim et al., 2006). PDK1 inhibits mitochondrial respiration and ROS production by restraining pyruvate metabolism via the TCA cycle. Similarly, in our results, we observed that the HIF-1α silenced cells in hypoxia failed to activate PDK1, which is reflected in higher cellular and mitochondrial ROS levels observed in these cells that is also associated with lower cell survival. Moreover, it has been recently found that HIF-1α can be translocated to the mitochondria in response to hypoxia or H_2O_2 directly through a PDK1 independent manner and prevent oxidative stress-related apoptosis (Li et al., 2019).

In order to maintain a sufficient energy supply while maintaining a low oxidative stress, cells in hypoxia are forced to switch from oxidative phosphorylation to less efficient glycolytic pathway, which requires more glucose uptake into the cells (Polet and Feron, 2013). HIF-1α is considered a primary oxygen sensor and a critical transcriptional regulator of numerous genes involved in the glycolysis, the process that consumes glucose (Moldogazieva et al., 2020). In this process, HIF-1α promotes the expression of Glut1, which is highly abundant in membranes of mammalian cells and facilitates glucose transport (Denko, 2008; Yao et al., 2018). Similarly, in our results, Glut1 was highly activated in SHED under hypoxia. Once HIF-1α was silenced or inhibited, Glut1 expression was affected in hypoxia simultaneously. On the other hand, intake of more glucose alone is far from enough for the cell survival due to the inhibitory effects of downstream products of the glycolytic

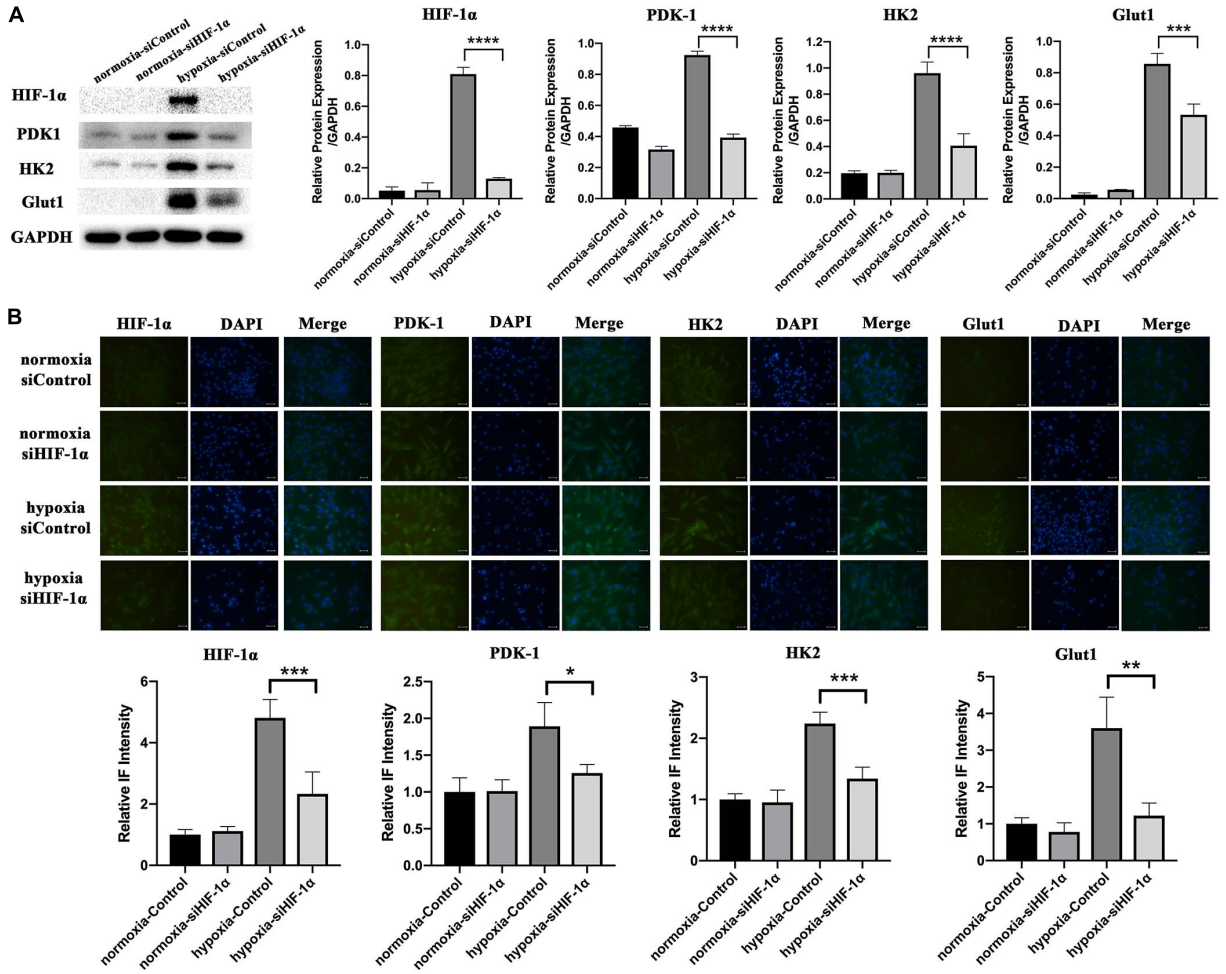

FIGURE 5 | Silencing of HIF-1α suppressed the expression of proteins important in metabolic adaptations *in vitro*. **(A)** Protein levels of HIF-1α, PDK1, HK2, and Glut1 in siControl and siHIF-1α SHED in normoxia and hypoxia as shown by western blotting. **(B)** Immunofluorescence and relative intensity quantification for HIF-1α, PDK1, HK2, and Glut1 in siControl and siHIF-1α SHED in normoxia and hypoxia $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Scale bar = 100 μm.

process, such as glucose-6-phosphate (G-6-P). As the first rate-limiting enzyme, hexokinase (HK) catalyzes the conversion of glucose to G-6-P (Lis et al., 2016). Among all four isoforms of HK (HK1, HK2, HK3, and HK4), HK2 was found to be highly expressed in patients with carcinomas (DeWaal et al., 2018) and considered the most efficient isoform in aerobic glycolysis (Gong et al., 2012). After interacting and binding to one specific protein in the outer membrane of mitochondria, HK2 acts in not only enhancing the glycolysis process but also facilitating ATP production through activation of ATP synthesis-related enzymes (Xu and Herschman, 2019). Once HK2 is deleted, the glycolytic flux and the proliferation of cancer cells have been inhibited (DeWaal et al., 2018) with increased susceptibility to apoptosis induced by hypoxia (Wolf et al., 2011). Moreover, it has been demonstrated that HIF-1α is also involved in several additional signaling pathways that lead to accelerated glycolytic levels, such as PI3K/Akt/mTOR, Raf/MAPK, and AMPK (DeBerardinis et al., 2008).

Our previous study demonstrated that HIF-1α stabilization in SHED under normoxia up-regulated the VEGF expression, which resulted in increased endothelial differentiation via autocrine effects and elevated angiogenesis via paracrine signaling both *in vitro* and *in vivo* (Han et al., 2020a). Accordingly, it has been shown that VEGF expression was significantly down-regulated when HIF-1α was knocked down in bone marrow stem cells and cancer cells (Chen et al., 2016; Zhang et al., 2018), leading to poorly vascularized constructs (Stegen et al., 2016). HIF-1α/VEGF signaling pathway activation promoted the survival, proliferation, and migration of endothelial cells, which are vital steps in blood vessel formation (Yang et al., 2016; Rattner et al., 2019). The results of the current study revealed that the reduced VEGF secretion following HIF-1α silencing in SHED led to diminished proliferation and migration of HUVECs as well as vascularization in accordance with the previously reported findings in relation to other types of cells. Moreover, upregulation

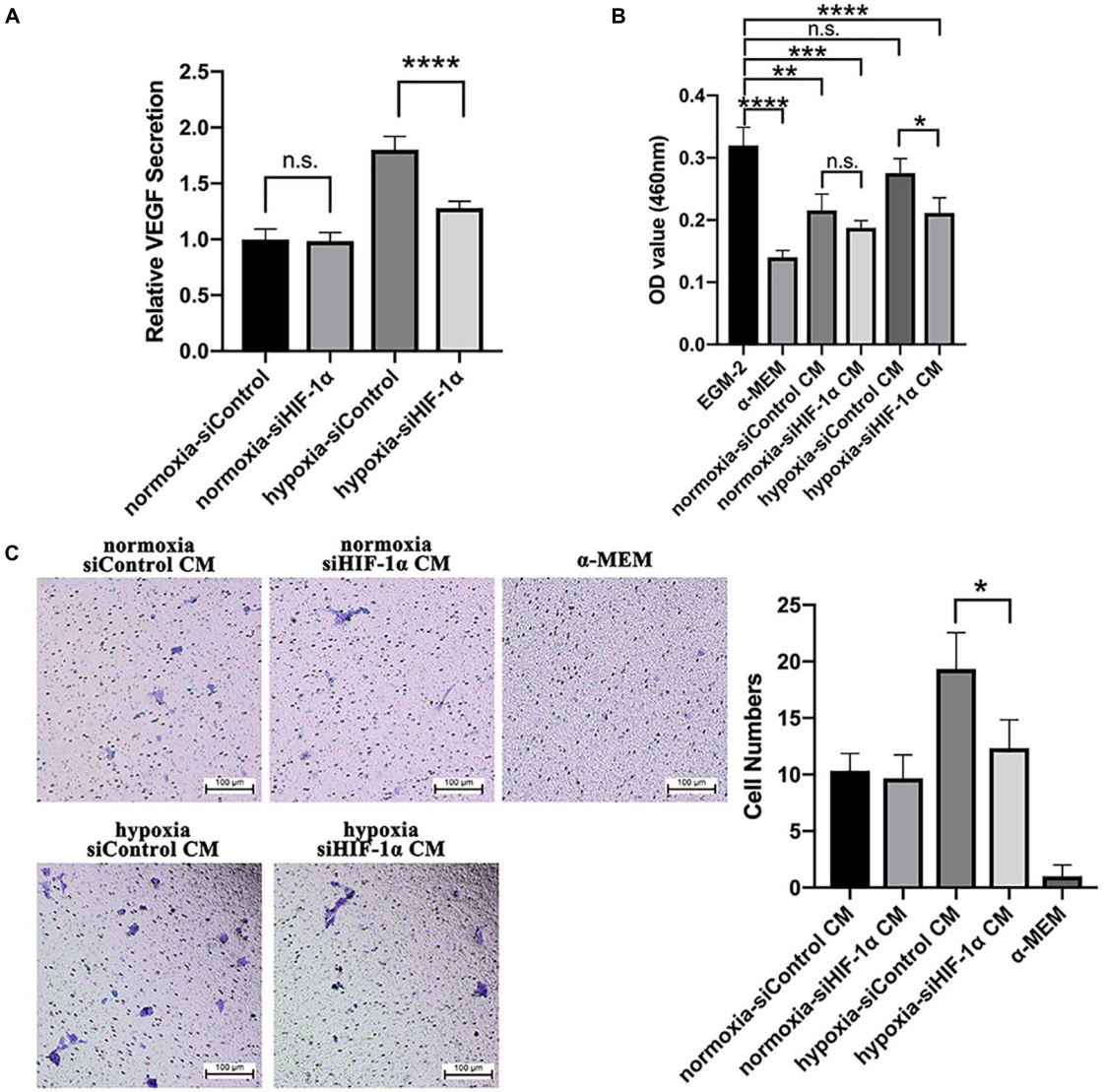

FIGURE 6 | Silencing of HIF-1α in SHED reduced VEGF secretion and paracrine effects on HUVECs. **(A)** VEGF levels in different conditioned media as detected by ELISA and normalized to that of siControl under normoxia. **(B)** CCK-8 assay of HUVECs cultured in different conditioned media, EGM-2 (positive control), α-MEM (negative control). **(C)** *Trans*-well assay of HUVEC migration under different conditioned media, α-MEM (negative control). $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$. Scale bar = 100 μm.

of VEGF via HIF-1α activation under hypoxia can also protect cardiomyocytes against ischemia injury (Dai et al., 2007) and repair ischemia/reperfusion injury in lung tissue *in vivo* (Fan et al., 2019).

Taken together, our data indicate that HIF-1α serves as a central regulator of redox homeostasis and glucose metabolism in maintaining cell survival at the early stages of hypoxia. In securing long-term cell survival, HIF-1α also plays an essential role in inducing an angio-/vasculogenic response via VEGF-induced differentiation, proliferation and migration of endothelial cells. However, the exact contribution of each of these factors and the involvement of other signaling pathways are yet to be unraveled. In addition, growth factors other than VEGF might also be involved in the angio-/vasculogenic process. Further investigations need to be done to elucidate the complex crosstalk between redox homeostasis, reprogrammed metabolism, cell survival, and angio-/vasculogenesis.

In conclusion, our results demonstrated that endogenous HIF-1α signaling is crucial to dental stem cell-based tissue regeneration. HIF-1α induced ROS homeostasis and glycolysis-related gene expression are involved in the early stage of post-implantation survival of SHED, while VEGF is primarily responsible for the angio-/vasculogenic response. Based on these pivotal roles, HIF-1α could be used as a highly promising target for improving post-implantation cell survival

and developing new treatment approaches in stem cell-based tissue engineering.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on the Use of Live Animals in Teaching and Research, The University of Hong Kong.

AUTHOR CONTRIBUTIONS

YH and WD conceived and designed the project. YH, QC, and LZ participated in designing the experiments, analyzing data, and assembling figures. YH and WD contributed to drafting, editing, and revising the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Parathyroid Hormone 1 Receptor Signaling in Dental Mesenchymal Stem Cells: Basic and Clinical Implications

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Parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP) are two peptides that regulate mineral ion homeostasis, skeletal development, and bone turnover by activating parathyroid hormone 1 receptor (PTH1R). PTH1R signaling is of profound clinical interest for its potential to stimulate bone formation and regeneration. Recent pre-clinical animal studies and clinical trials have investigated the effects of PTH and PTHrP analogs in the orofacial region. Dental mesenchymal stem cells (MSCs) are targets of PTH1R signaling and have long been known as major factors in tissue repair and regeneration. Previous studies have begun to reveal important roles for PTH1R signaling in modulating the proliferation and differentiation of MSCs in the orofacial region. A better understanding of the molecular networks and underlying mechanisms for modulating MSCs in dental diseases will pave the way for the therapeutic applications of PTH and PTHrP in the future. Here we review recent studies involving dental MSCs, focusing on relationships with PTH1R. We also summarize recent basic and clinical observations of PTH and PTHrP treatment to help understand their use in MSCs-based dental and bone regeneration.

Keywords: parathyroid hormone, parathyroid hormone-related peptide, regeneration, tooth development, periodontal ligament

BRIEF OVERVIEW OF PARATHYROID HORMONE 1 RECEPTOR, PARATHYROID HORMONE, AND PARATHYROID HORMONE-RELATED PROTEIN

Parathyroid Hormone 1 Receptor

PTH1R is one of the class B G-protein-coupled receptor (GPCR) family members with a seven-transmembrane structure (Sutkeviciute et al., 2019). PTH1R signaling plays a pivotal role in the regulation of multiple physiological functions including mineral ion homeostasis and skeletal development, as well as bone metabolism (Cheloha et al., 2015). It is widely expressed during

early development but has its highest expression in bone and kidney (Urena et al., 1993). Upon binding with either of its two ligands, PTH and PTHrP, PTH1R is able to activate the $G\alpha_s$/adenylyl cyclase/protein kinase A (PKA) pathway, $G\alpha_q$/phospholipase C/protein kinase C (PKC) pathway, $G\alpha_{12/13}$-phospholipase D/RhoA pathway as well as mitogen-activated protein kinase (MAPK) signaling cascade (Kondo et al., 2002; Singh et al., 2005; Syme et al., 2005; Gesty-Palmer et al., 2006; Sneddon and Friedman, 2007).

Parathyroid Hormone

PTH is an 84 amino-acid (AA) endocrine hormone secreted by the parathyroid glands and serves as the mediator of extracellular calcium and phosphate levels and skeletal homeostasis (Wein and Kronenberg, 2018).

Parathyroid Hormone 1 Receptor Signaling in Mineral Ion Metabolism

The metabolism of calcium and inorganic phosphate is tightly controlled by the orchestration of several key organs: parathyroid gland, intestine, kidney, and bone. PTH is secreted from parathyroid glands and plays a critical role in controlling hormonal and cellular responses that regulate mineral ion homeostasis. PTH restores serum calcium levels by three distinct actions. In the kidneys, PTH stimulates the expression of 25-hydroxyvitamin D-1α-hydroxylase, which leads to increased $1,25(OH)_2D$ synthesis (Bergwitz and Jüppner, 2010). It also promotes Ca^{2+} reabsorption in the distal tubules in the kidney (Boros et al., 2009). In bone, PTH has been identified as a crucial mediator of bone-formation and bone-resorption to release calcium and phosphate from the matrix into the bloodstream (Jilka, 2007; Yang et al., 2007). In the intestine, PTH-induced 1,25(OH)2D increases dietary absorption of calcium. Regarding phosphate maintenance, PTH functions to inhibit Napi2a and Napi2c expressions in the luminal brush border membrane of proximal tubules cells in the kidney, thereby inhibiting phosphate reabsorption (Picard et al., 2010). Moreover, it induces fibroblast growth factor 23 (FGF23) production in osteoblasts and osteocytes (Rhee et al., 2011b; Meir et al., 2014; Fan et al., 2015). FGF23 functions to suppress phosphate reabsorption and reduce calcitriol synthesis (Shimada et al., 2004; Ohnishi et al., 2009). Therefore, systemic mineral ion balance largely depends on PTH, $1,25(OH)_2D$, and FGF23 acting in cooperation.

The Role of Parathyroid Hormone in Bone Remodeling

PTH plays a key role in bone remodeling by targeting skeletal stem cells, bone marrow stromal cells, osteoprogenitors, osteoclasts, bone lining cells, osteocytes, osteoclast, and T lymphocytes/macrophages (Rhee et al., 2011a; Wein and Kronenberg, 2018). The anabolic effect of PTH on the skeleton is well-established in osteoporosis (Neer et al., 2001; Black et al., 2008). Intermittent PTH (iPTH) administration stimulates new bone formation by directing mesenchymal stem cell fate and activating bone lining cells, as well as promoting the activity and differentiation of osteoblasts (Kim et al., 2012; Fan et al., 2017). It can also suppress the apoptosis of mature osteoblasts and osteocytes as well as decrease sclerostin expression (Jilka et al., 1999; Keller and Kneissel, 2005; Jilka, 2007; Delgado-Calle et al., 2017). On the other hand, sustained elevations of PTH levels by hyperparathyroidism or continuous PTH administration results in loss of bone mass due to excessive bone resorption through the production of receptor activator for nuclear factor-κB ligand (Rankl) in PTH-targeted cells (Bilezikian et al., 2018; Delgado-Calle et al., 2018).

Parathyroid Hormone-Related Protein

PTHrP can exert its physiological functions by acting as a paracrine, autocrine, or intracrine mediator in multiple target tissues. It has a similar N-terminal amino acid sequence to PTH (1–34) and binds the same receptor, PTH1R (Sutkeviciute et al., 2019). The first 13 residues of PTHrP show the highest degree of primary sequence homology with PTH, 8 of which are identical (Suva et al., 1987). This region is important for almost all the agonist effects of PTHrP and PTH. The PTHrP 14–36 region has little or no sequence homology with PTH but is needed for binding to PTH1R and activating subsequent signaling cascades (Juppner et al., 1991). Additionally, the 36–139 AA region of PTHrP contains unique functional domains. For instance, PTHrP residues 35–84 are responsible for placental calcium transport (Abbas et al., 1989), while the 107–139 region can promote osteoblast proliferation and function while inhibiting osteoclast activity (Fenton et al., 1993; Cornish et al., 1997, 1999; Alonso et al., 2008). PTHrP has context-dependent effects in multiple tissues, including the growth plate, bone, placenta, blood vessel, skin, and tooth (Hirai et al., 2015; Martin, 2016). It is primarily involved in embryonic skeleton development and postnatal bone formation, as well as in placental calcium mobilization during gestation and lactation (Neville et al., 2002; Kronenberg, 2006; McCauley and Martin, 2012). In the growth plate, PTHrP is secreted locally by chondrocytes, and has a pivotal function in endochondral bone formation during development (Lanske et al., 1996; Vortkamp et al., 1996). A population of skeletal stem cell is identified in PTHrP-positive chondrocytes within the resting zone of a growth plate (Mizuhashi et al., 2018). PTHrP interacts with Indian hedgehog (Ihh) secreted from the hypertrophic zone and delays the differentiation toward hypertrophic chondrocytes (Vortkamp et al., 1996). Deletion of the gene encoding PTHrP (*Pthlh*) causes chondrocyte hypertrophy on the anterior rib cartilage, thus leading to early lethality in mutants due to respiratory failure (Karaplis et al., 1994). Overexpression of human PTHrP protein in collagen type II-expressing cells rescued the chondrocyte hypertrophy phenotype, suggesting that PTHrP synthesized by and secreted from chondrocytes functions to inhibit chondrocyte hypertrophy in the growth plate (Weir et al., 1996). Furthermore, PTHrP-haplo-insufficient mice display low bone mass owing to decreased recruitment of precursor cells concomitant with accelerated osteoblast apoptosis (Amizuka et al., 1996). In osteoblasts, specific *Pthlh* deletion leads to osteoporosis and impaired bone formation (Miao et al., 2005). In osteocytes, PTHrP acts in a paracrine/autocrine manner to induce bone formation and to modulate adult cortical bone strength (Ansari et al., 2018). These

data indicate that PTHrP may duplicate the anabolic effects of iPTH in osteoblastic cells.

In addition to the many past studies that focus on the role of PTH1R signaling in mineral ion metabolism and skeletal homeostasis (Fan et al., 2015; Carrillo-López et al., 2019; Fu et al., 2020; Martin et al., 2021), interest has recently turned to its function in craniofacial development and remodeling. The major elements of the oral cavity, including teeth, periodontal tissues, and the jaw bones (maxilla and mandible), are intimately connected. Teeth develop in jaw bones and are closely connected to alveolar bone through periodontal tissues. Thus, researchers have embarked on efforts to understand the regulatory network of signaling pathways in teeth and adjacent tissue. As a result, PTH1R signaling has been found to exert profound influence in the orofacial region (Frazier-Bowers et al., 2014). PTH1R signaling is involved in tooth eruption, tooth root formation, alveolar bone regeneration, and periodontium repair (Aggarwal and Zavras, 2012; Ono et al., 2016; Nagata et al., 2020; Zhang et al., 2020). Dental mesenchymal stem cells (MSCs) are key targets for PTH1R signaling in these physiological activities. This emphasizes the need to understand both MSCs and the underlying mechanisms that regulate them. Here, we review the recent literature for regulatory networks involved with PTH1R signaling in mediating MSCs with a particular focus on orofacial MSCs. Furthermore, there is growing knowledge of the therapeutic application of PTH and PTHrP analogs in dental implant surgery, orthodontics, periodontitis, and orofacial regeneration medicine. This review discusses these advances and provides a summary of the key functions of PTH1R signaling on MSCs in dental tissue homeostasis and repair.

PARATHYROID HORMONE 1 RECEPTOR SIGNALING AND BONE MARROW-DERIVED MESENCHYMAL STEM CELLS

MSCs can be isolated from many tissues, including bone marrow, circulating blood, placenta, cord blood, synovial tissue, pancreas, skeletal muscle, adipose tissue, and oral and maxillofacial tissue (Vaananen, 2005). The self-renewal capacity and potential plasticity of MSCs make them indispensable for organ development and tissue repair (Bianco et al., 2013). Beginning with Friedenstein's elegant experiments, much work has been focused on bone marrow mesenchymal stem cells (BMMSCs) (Friedenstein et al., 1974a,b). PTH1R signaling is an important regulator of the ontogeny of bone marrow, its stroma and the BMMSCs residing in it (Kuznetsov et al., 2004). For instance, PTH acts directly on cultured bone marrow-derived stromal cells to increase their proliferation and differentiation (Nishida et al., 1994). *In vivo* lineage tracing experiments showed that intermittent administration of PTH (1–34) upregulated Nestin-positive BMMSCs (Mendez-Ferrer et al., 2010). Similarly, the number of cells expressing Sox9 significantly increased upon PTH (1–34) treatment. Moreover, these cells differentiated into osteoblasts more quickly than those in vehicle-treated mice (Balani et al., 2017). Also, PTH has been shown to target a subset of leptin receptor (LepR) expressing cells to increase the expression of runt-related transcription factor 2 (Runx2), a transcription factor that is required for osteogenesis, and promotes the differentiation of these stem cells (Ducy et al., 1997; Yang et al., 2017). More recently, we and others have described the function of PTH1R signaling on mesenchymal cell fate decision. PTHrP-haplo-insufficient mice have low bone mass and increased marrow adiposity (Amizuka et al., 1996). Ablation of PTH1R in Prx1-positive MSCs results in decreased bone formation, increased bone marrow adipose tissue, and accelerated bone resorption (Fan et al., 2017). This is likely due to the downstream cascade of PTH1R signaling through $G\alpha_s$ since loss of $G\alpha_s$ in osteoprogenitors results in a similar phenotype (Sinha et al., 2014). Additionally, iPTH treatment shifted the differentiation of LepR-positive progenitor cells from an adipo-lineage toward an osteo-lineage, accompanied by higher expression of osteogenic markers and reduced adipocyte markers (Yang M. et al., 2019). These data emphasize the central role of PTH1R signaling in guiding BMMSCs toward an osteoblast lineage and away from adipogenesis.

PTH1R, in conjunction with different downstream pathways, acts in BMMSCs to drive various biological activities. The mechanisms and the therapeutic potential of this activity are of great interest. Studies suggest that PTH (1–34) enhances the migration and adhesion of BMMSCs through Rictor/mTORC2 signaling *in vitro* (Lv et al., 2020). Additionally, PTH has been shown to influence and expand the bone marrow stem cell niche (Huber et al., 2014). iPTH treatment was found to enhance stem cell homing via an SDF-1α/CXCR4 axis (Huber et al., 2014). Further study focusing on the therapeutic application of PTH showed that iPTH improved i.v. MSC therapy to promote bone loss healing by inducing the migration of MSCs to defective sites (Sheyn et al., 2016). Moreover, PTH can be combined with a scaffold to act as a biomaterial to induce bone regeneration by enhancing osteogenesis of BMMSCs via Notch signaling (Zou et al., 2021).

PARATHYROID HORMONE 1 RECEPTOR SIGNALING REGULATES DENTAL MESENCHYMAL STEM CELLS

There is accumulating evidence for a link between PTH1R signaling and MSCs. In addition to bone marrow, orofacial tissues are one of the major sources of MSCs. There are a variety of stem cell populations that can be identified in teeth and their supporting structures, including dental pulp stem cells (DPSCs), stem cells from human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs), stem cells from apical papilla (SCAPs), orofacial bone/bone-marrow-derived MSCs (OMSCs), tooth germ progenitor cells (TGPCs), and gingival MSCs (GMSCs) (Zheng et al., 2019). These stem cells express distinct surface markers and have multi-lineage differentiation capacities with region-specific characteristics. **Figure 1** and **Table 1** illustrate the regulatory function of PTH1R signaling in dental MSCs.

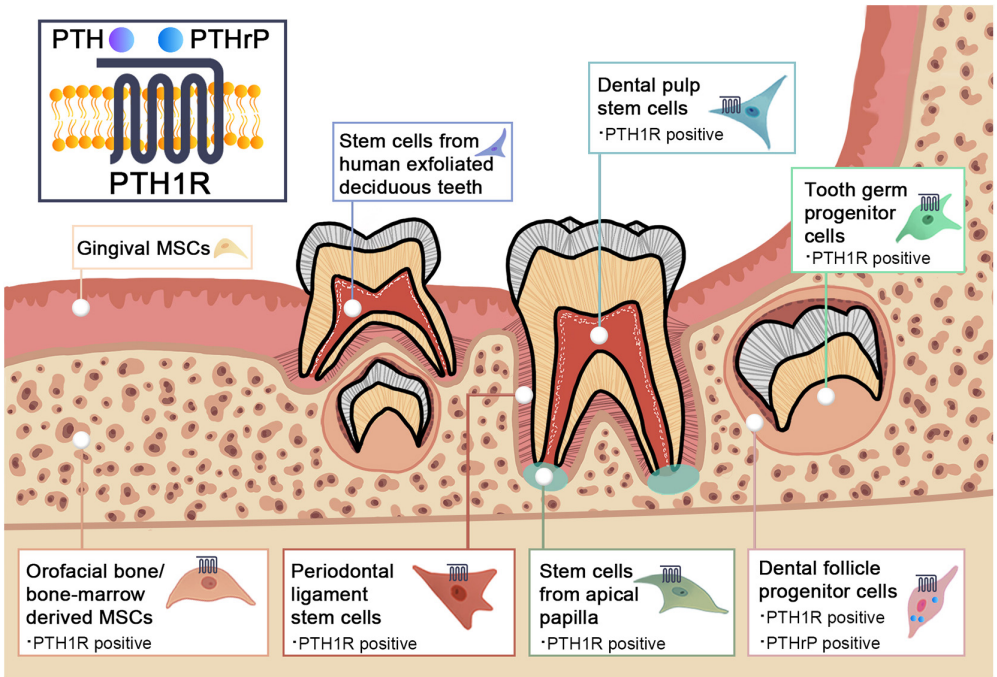

FIGURE 1 | Schematic graph illustrating the PTH1R signaling in dental mesenchymal stem cell populations.

Dental Pulp Stem Cells

Dental pulp stem cells (DPSCs) are the first dental MSCs to be identified. They are highly proliferative with multilineage differentiation potential including osteogenic and chondrogenic capability (Gronthos et al., 2000). DPSCs, derived from dental pulp tissues by enzymatic digestion, are able to form a dentin-pulp-like complex when transplanted into immunocompromised mice (Gronthos et al., 2002). They participate in dental repair processes and play an important role in stem cell-based therapy for endodontic regeneration (Shi et al., 2020). Several studies have revealed the applicability of DPSCs in regeneration of the dentin-pulp complex and the repair of peripheral nerve injury (Lambrichts et al., 2017).

Recent studies have implicated PTH1R signaling in mediating DPSCs function. However, PTH treatment did not alter the proliferation rate of DPSCs. Rather, the expression levels of osteo/odontogenic related genes were significantly upregulated along with an increase in the formation of mineralized nodules and calcium content. This suggests that PTH has a positive effect on DPSCs differentiation (Ge et al., 2020). MAPKs are involved in DPSCs proliferation and differentiation, so researchers have explored the roles of extracellular signal-regulated kinase (ERK) and P38 MAPK pathways in PTH-treated DPSCs (Wang B.L. et al., 2019; Yu et al., 2019; Ge et al., 2020). PTHrP also enhances odontogenic differentiation of dental pulp cells by stimulating phosphorylation levels of protein kinase B (PKB/AKT), ERK, and c-Jun N-terminal kinase (JNK), all essential molecules of the ERK and P38 MAPK pathways (Kim et al., 2020). Immunohistochemistry analysis revealed that PTHrP is only present in a few fibroblasts and cells of the odontoblastic zone in normal pulps, whereas the distribution of PTHrP markedly expands in inflamed pulps (Marigo et al., 2010). PTHrP-positive cells were found in the vascular zone, pulp stroma, and the odontoblastic and sub-odontoblastic zones. This suggests that PTHrP may contribute to angiogenesis during inflammation (Marigo et al., 2010). However, further studies are needed to elucidate the underlying mechanisms of PTH1R signaling in regulating DPSCs.

Dental Follicle Progenitor Cells

These MSCs are found in the dental follicle (DF), a mesenchymal condensation structure surrounding the developing tooth germ prior to eruption. DFPCs are progenitor cells of periodontal tissues with the ability to form PDL, cementum, and alveolar bone during tooth development (Morsczeck et al., 2005). Like DPSCs, DFPCs are multipotent cells with the capability to differentiate into osteoblasts, chondrocytes, cementoblasts, and adipocytes, as well as neuronal cells (Yao et al., 2008; Zhou et al., 2019). DFPCs are responsible for the development of cementum, periodontal ligament, and alveolar bone during tooth eruption and tooth root morphogenesis (Zhou et al., 2019). Strong evidence indicates that PTH1R signaling plays a crucial role during tooth eruption (Izumida et al., 2020). Genetic studies have revealed that familial primary failure of tooth eruption (PFE) is a PTH1R-associated hereditary disease (Frazier-Bowers et al., 2014). It is causally linked to heterozygous mutations of PTH1R (Frazier-Bowers et al., 2014). More studies are focusing on characterization of the biological role of PTH1R in the dental follicle during tooth eruption. These studies suggest that mesenchymal progenitor cell populations reside in the

DF, including cells expressing Osterix (Osx), PTHrP, and Gli1 (Liu et al., 2015; Ono et al., 2016; Takahashi et al., 2019). This information was used to generate conditional knockout of PTH1R based on these markers in order to explore its mechanism in DF progenitors. Ablation of PTH1R in Osx^+ progenitors resulted in failed tooth eruption with significantly truncated roots that lack periodontal ligaments and ankylosis of the dental root (Ono et al., 2016). PTH1R-deficient progenitors also showed impaired proliferation ability and accelerated differentiation into cementoblasts. This was accompanied by upregulation of the bone/cementum matrix protein osteopontin (Opn) and nuclear factor I/C (Nfic), leading to an unusual formation of cellular cementum on the root surface (Ono et al., 2016). The ability of Nfic to enhance proliferation and differentiation of odontoblasts was verified *in vitro*. Also, Nfic deficiency led to truncation of molar roots (Steele-Perkins et al., 2003; Lee et al., 2009). The detailed mechanism of how PTH1R signaling regulates Nfic requires further exploration. Interestingly, knocking out histone deacetylace-4 (HDAC4) partially recapitulated the phenotypes in *OsxCre;PTH1R*$^{fl/fl}$ mice such as short root and thicker cementum, indicating that HDAC4 might be a key mediator downstream of PTH1R signaling in DFPCs (Ono et al., 2016).

PTHrP, one of the ligands of PTH1R, is a required paracrine/autocrine cytokine in regulating DFPCs (Nagata et al., 2020). Earlier research identified PTHrP as a secretory factor in epithelial components, functioning as a paracrine molecule mediating epithelial-mesenchymal interactions during development (Kitahara et al., 2002). PTHrP is present in the enamel organ (Suda et al., 2003) and is responsible for the formation of the eruption pathway (Philbrick et al., 1998). This observation has inspired various studies that focused on the role of PTHrP in mediating osteoclastogenesis (Wise et al., 2000). An experiment was conducted in which PTHrP activity was inhibited with antiserum and conditional media in PTHrP-treated dental follicle cells to induce bone resorption in fetal-rat long bone. This work by Nakchbandi et al. (2000) showed that PTHrP was required for osteoclast formation and differentiation. Coculture experiments with DFPCs and epithelial stellate reticulum cells indicated that PTHrP secreted by epithelial cells could stimulate DFPCs-induced osteoclast formation, contributing to bone resorption on the coronal aspect of an erupting tooth (Nakchbandi et al., 2000). In addition, PTHrP has effects beyond its paracrine functions. A recent study using lineage tracing experiments discovered the presence of PTHrP in a group of DFPCs where it acted as an essential autocrine ligand modulating the physiological cell fates of DFPCs through PTH1R (Takahashi et al., 2019). PTH1R deficiency in $PTHrP^+$ DFPCs leads to failure of tooth eruption accompanied by loss of periodontal attachment and abnormal cellular cementum formation. This is mainly due to a shifting of the cell fate to non-physiological cementoblast-like cells in association with higher expression of bone/cementum matrix protein and a crucial transcription factor Mef2c (Takahashi et al., 2019). Additionally, PTHrP simulated osteogenesis of human DFPCs cells independently of Ihh in an autocrine manner (Klingelhoffer et al., 2016). Furthermore, PTHrP has been observed to have intracrine actions as well (Fiaschi-Taesch and Stewart, 2003). It is reported that a dental follicle progenitor cell line with higher endogenous expression of PTHrP had increased alkaline phosphatase (ALP) activity and the ability to induce mineralization by activating the bone morphogenetic protein (BMP) signaling pathway (Pieles et al., 2020). The intracrine mode of PTHrP was discovered with the observation that intranuclear endogenously expressed PTHrP and its function are not affected by external PTHrP or PTH1R inhibitors (Pieles et al., 2020).

In addition to PTH and PTHrP, other molecules can mediate DFPCs through PTH1R signaling. Recent work indicates that PTH1R expression correlated with that of chromodomain helicase DNA-binding protein 7 (CHD7), a chromatin remodeling enzyme. Upregulation of CHD7 significantly accelerated osteogenic differentiation of human dental follicle cells while knockdown of CHD7 gave the reverse result. The latter was partially rescued by overexpression of PTH1R (Liu C. et al., 2020). Others have noted that PTH1R signaling potentially interacts with several key signaling pathways such as Wnt/β-catenin, Hedgehog (Hh), and TGF-β/BMP in DFPCs during tooth eruption (Nagata et al., 2020), yet the underlying mechanisms remain to be determined.

Orofacial Bone/Bone Marrow-Derived Mesenchymal Stem Cells

The common sources of BMMSCs are the femur and tibia, but MSCs have also been isolated from alveolar bone marrow (Matsubara et al., 2005; Miura et al., 2006). These are termed orofacial bone/bone marrow-derived MSCs (OMSCs) (Yamaza et al., 2011). They share some features with long bone or iliac bone-derived BMMSCs. However, OMSCs exhibit distinct characteristics in terms of higher proliferation, higher expression of ALP, and more calcium accumulation *ex vivo* (Akintoye et al., 2006). OMSCs have shown promising potential for bone regeneration, especially in the orofacial region owing to the proximate embryonic origin and microenvironment (Lee et al., 2019).

Zhou et al. (2014) recently discovered that a small proportion of bone marrow cells express LepR, a marker highly enriched in BMMSCs. These cells play a major role in bone formation, bone repair upon injury, and adipo-differentiation in adult bone marrow (Zhou et al., 2014). Research with $LepR^+$ MSCs in the alveolar region verified that LepR labels a population of OMSCs which are quiescent physiologically under normal conditions but contribute to intramembranous bone formation when activated by external stimulation such as tooth extraction. Moreover, intermittent PTH (1–34) treatment increased the number of $LepR^+$ cells in the alveolar ridge and promoted osteogenic differentiation of $LepR^+$ cells. In contrast, mice that lack PTH1R in $LepR^+$ cells showed impaired socket repair after tooth extraction, highlighting the crucial role of PTH1R signaling in $LepR^+$ OMSCs during regeneration of jawbone defects (Zhang et al., 2020).

Prx1 is another marker for craniofacial mesenchyme and Prx1-positive progenitors are located at the base of molars and alveolar bone marrow surrounding incisors (Logan et al., 2002;

TABLE 1 | Features of dental tissue derived MSCs and the regulation role of PTH1R signaling.

	Origins	Surface markers		Lineage tracing animal models	Differentiation capabilities		Effect of PTH1R signaling
		+	−		*In vitro*	*In vivo*	
Dental pulp stem cells (DPSCs)	Dental pulp	CD13, CD29, CD44, CD73, CD90, CD105, CD106, CD146, CD166, CD271, STRO-1, STRO-3	CD3, CD8, CD14, CD15, CD19, CD33, CD34, CD45, CD71, CD117, CD133	Wnt1, Gli1, α-SMA, Prx1	Osteogenic, chondrogenic, adipogenic, chondrogenic, neurogenic, hepatogenic, cardiogenic, endothelial cells, pancreatic cells	Form dentin-pulp like complex Regenerate pulp Repair peripheral nerve injury	Enhanced osteo/odontogenic differentiation Stimulated phosphorylation of PKB/AKT, ERK, JNK
Dental follicle progenitor cells (DFPCs)	Dental follicle	CD29, CD44, CD73, CD90, CD105, CD146, Notch1, STRO-1, Nestin	CD14, CD31, CD34, CD45, CD117	Osx, PTHrP, Gli1, Prx1	Osteogenic, adipogenic, chondrogenic, cementogenic, neurogenic	Regenerate periodontal tissue Root regeneration	Regulated the differentiation of Osx^+ and $PTHrP^+$ progenitors during tooth root formation PTHrP in epithelium stimulated DFPCs-induced osteoclast formation
Orofacial bone/bone-marrow-derived MSCs (OMSCs)	Orofacial bone/bone-marrow	CD29, CD44, CD73, CD90, CD105, CD106, SSEA-4, Oct-4	CD14, CD19, CD31, CD34, CD45	Wnt1, LepR, Prx1	Osteogenic, adipogenic, chondrogenic	Form bony tissue	Increased osteogenesis of $Prx1^+$ progenitors during eruption Enhanced proliferation and osteogenesis of $LepR^+$ progenitors Downregulated p16ink4a and specific senescent-associated secretory phenotype in aged OMSCs
Periodontal ligament stem cells (PDLSCs)	Periodontal ligament	CD29, CD44, CD73, CD90, CD105, CD146, CD166, STRO-1, STRO-4	CD14, CD31, CD34, CD40, CD45, CD54, CD79a, CD80, CD86	Lrig1, Wnt1, Axin2, Gli1	Adipogenic, chondrogenic, osteogenic, neurogenic, cementogenic, cardiogenic, pancreatic cells, ectoderm lineage cells	Form cementum-PDL like structure Regenerate periodontal tissue	Regulated PDL formation, construction of collagen fibers, and Periostin expression Induced osteogenesis of $STRO1^+$ PDLSCs Assisted SDF-1α in recruiting PDLSCs to periodontal defects
Stem cells from apical papilla (SCAPs)	Apical papilla	CD24, CD29, CD73, CD90, CD105, CD106, CD146, CD166, STRO-1	CD14, CD18, CD34, CD45, CD150	Wnt1, Gli1	Odontogenic, osteogenic, adipogenic, chondrogenic, neurogenic	Form root-periodontal like complex	Enhanced osteo/odontogenic differentiation Stimulated phosphorylation of ERK, JNK
Tooth germ progenitor cells (TGPCs)	Third molar tooth germ	CD29, CD44, CD73, CD90, CD105, CD166	CD14, CD34, CD45, CD133, CD117	N/A	Odontogenic, osteogenic, adipogenic, neurogenic, endothelial cells, epithelial cells	Contribute to neuro protection	PTH (1–34) enhanced osteogenic ability at early developmental stage PTH (53–84) stimulate osteogenic ability at late stage
Stem cells from human exfoliated deciduous teeth (SHEDs)	Human exfoliated deciduous teeth	CD13, CD29, CD44, CD90, CD105, CD146, STRO-1	CD3, CD14, CD19, CD34, CD106, HLA-DR	N/A	Osteogenic, odontogenic, adipogenic, neurogenic, endothelial-genic	Form bony tissue Regenerate pulp Regenerate periodontal tissue Promote facial nerve repair	N/A
Gingival mesenchymal stem cells (GMSCs)	Gingiva	STRO-1, CD29, CD44, CD73, CD90, CD105, CD146	CD11b, CD34, CD45, HLA-DR	N/A	Osteogenic, chondrogenic, adipogenic, keratogenic	Form bony tissue	N/A

Cui et al., 2020a). Mice with conditional knockout of PTH1R in $Prx1^+$ cells had arrested tooth eruption, decreased alveolar bone formation and downregulated osteogenesis-related genes. Consistent with *in vivo* analyses, PTH1R-deficient OMSCs showed decreased ALP and alizarin red (ARS) staining concomitant with reduced expression of osteogenic related markers Osx, Runx2, Osteocalcin (Ocn), and Dentin matrix protein 1 (Dmp1). There was also reduced expression of downstream factors of the BMP/TGF-β pathway, including Tgfbr1, Tgfb1, and Bmp1. These data suggest that PTH1R signaling mediates tooth eruption by regulating osteogenic differentiation of OMSCs in alveolar bone, which contributes to the motive force during eruption (Cui et al., 2020a).

PTH1R signaling also plays a crucial role in aging and senility related changes and diseases (Cui et al., 2020b). Intermittent PTH (1–34) treatment may function to lower the number of membrane TGF-β receptors that then suppress the expression of p-Smad3 (Crane and Cao, 2014), a molecule can accelerate secretion of senescence biomarker p16ink4a. The suppression of p-Smad3 leads to downregulation of p16ink4a and alleviation of the specific senescent-associated secretory phenotype (SASP) in OMSCs. This creates a microenvironment with decreased senescent cell burden. These factors provide a favorable treatment path for aging/senility related diseases (Cui et al., 2020b).

Periodontal Ligament Stem Cells

PDLSCs were identified in the periodontal ligament (PDL), the soft tissue that links cementum of roots to the alveolar bone (Fleischmannova et al., 2010). PDLSCs contribute to periodontal tissue formation *in vivo* and generate a cementum/PDL-like structure *in vitro* (Seo et al., 2004). PDLSCs exhibit multi-lineage differentiation potential. They can give rise to adipocytes, chondrocytes, osteoblasts, and cementoblast-like cells under certain stimuli (Seo et al., 2004). Research showed that PDLSCs, together with scaffolds, promote PDL, cementum, and alveolar bone formation at periodontal injury sites with bone defects (Iwasaki et al., 2019). The evidence indicates that PDLSCs contribute to periodontal tissue repair and support the great potential of PDLSCs for promoting tissue regeneration and reconstructing the connection between the alveolar socket and tooth root in periodontitis (Hernández-Monjaraz et al., 2018).

A variety of studies using animal models have demonstrated the importance of PTH1R signaling in regulating stem cells that reside in the PDL. Cui et al. (2020a) recently revealed that PDL cells from a mouse incisor originated from $Prx1^+$ progenitors. Lack of PTH1R expression resulted in a narrowed PDL with irregularly aligned collagen fibers, downregulated Periostin expression, and aberrant formation of bone-like tissue in PDL (Cui et al., 2020a). In the same vein, conditional ablation of PTH1R in Osx-lineage cells resulted in a thinner, less organized PDL in *OsxCre;PTH1R*$^{fl/+}$ mice and complete loss of the PDL with ankylosed root in homozygous knockout mice (Ono et al., 2016). These studies provide a deeper insight into PTH1R signaling in PDL development during tooth root formation and the eruption process.

Stem Cells From Apical Papilla

The apical papilla is a transient zone located at the apices of immature permanent teeth and is related to root formation (Gan et al., 2020). SCAPs have higher proliferative ability than DPSCs and can give rise to odontoblastic/osteoblastic cells, adipocytes, and neural progenitor-like cells under certain stimulation (Sonoyama et al., 2008; Songsaad et al., 2020). Several discoveries have shed light on the function of SCAPs in root formation and tooth eruption. SCAPs have the ability to induce continued root maturation and have been used to treat interrupted root formation caused by periradicular periodontitis or abscess (Huang et al., 2008). Moreover, a recent study suggests the promising application of SCAPs in pulp regeneration and bioengineered tooth root (bio-root) engineering (Gan et al., 2020).

Pang et al. (2020) administered intermittent PTH (1–34) treatment to SCAPs and noted an increase in ALP activity and expression of odonto/osteogenic markers, including Ocn, Opn, Osx, Runx2, Col1αI, and dentin sialophosphoprotein (DSPP). Yet the proliferation of SCAPs was unchanged upon PTH administration. It was shown that iPTH administration upregulated p-JNK in SCAPs during the first 15 min, and induced p-P38 in 30 min while downregulating p-ERK. This is consistent with another finding that dephosphorylating ERK induced osteogenesis differentiation and bone formation (Huang et al., 2007). It is important to note that p-ERK was increased in PTH-treated DPSCs in conjunction with osteo-differentiation enhancement, suggesting that the nature of the crosstalk between PTH1R signaling and JNK, P38 MAPK pathways could be different in specific stem cell lineages (Ge et al., 2020).

Tooth Germ Progenitor Cells

Tooth germ is comprised of enamel organ, dental papilla, and dental follicle. Through the sequential and mutual epithelial-mesenchymal interactions, tooth germ gradually developed into enamel, dentin, pulp, and surrounding supportive tissues. A novel population of MSCs referred to as TGPCs was discovered in the late bell stage tooth germ of the third molar. Since tooth germ is at an early developmental stage with both mesenchymal and epithelial original tissues, TGPCs were able to differentiate into odontogenic, osteogenic, adipogenic, and neurogenic cells, as well as endothelial-, epithelial-like cells (Yalvaç et al., 2010; Taşlı et al., 2013, 2014; Doğan et al., 2015). Moreover, they have a neural protection function by increasing antioxidant enzymes and reducing neuronal death or apoptosis (Yalvaç et al., 2013). TGPCs have stable stem cell properties and present more immature features compared with other dental MSCs. Resulted from the wide range of indications for third molar extractions, TGPCs are easily accessed with minimal invasiveness, implying promising therapeutic applications of TGPCs in tooth regeneration in the future (Yalvaç et al., 2010).

Previous research reported the effects of distinct fragments of PTH on tooth germ development at different stages. Administration of PTH (1–34) to tooth germ from mouse embryos resulted in upregulated ALP activity at an early developmental stage while downregulated ALP occurred at a late

stage. In contrast, the action of another PTH fragment, PTH (53–84) exerted opposite effects (Tsuboi and Togari, 1998). Further investigation is required to understand the temporospatial effect of distinct PTH fragments in mediating tooth germ development.

Stem Cells From Human Exfoliated Deciduous Teeth

SHEDs were first isolated from the pulp tissue of the crowns of exfoliated deciduous teeth (Miura et al., 2003). SHEDs are characterized as immature DPSCs expressing embryonic stem cell markers. They show multidirectional differentiation potential and higher proliferation capacity when compared with adult MSCs such as DPSCs and PDLSCs (Miura et al., 2003). *In vivo* transplantation of SHEDs demonstrated that they could induce bone formation and promote facial nerve regeneration (Pereira et al., 2019). Implanted SHEDs could survive in mouse brain and express neural markers (Miura et al., 2003). They are also promising candidates for dental pulp tissue engineering (Rosa et al., 2016). The capacity of odontogenic differentiation makes them an alternative source for stem cell-mediated bio-root regeneration (Yang X. et al., 2019). Combined with treated dentin matrix, SHEDs can successfully regenerate periodontal tissues, including PDL, blood vessels, and alveolar bone (Yang X. et al., 2019). Obtained from primary teeth, SHEDs are easily accessed by a minimally invasive procedure. Thus, SHEDs have drawn great and long-lasting attention since they were first discovered (Miura et al., 2003). Recent research reported that FGF2 enhanced the angiogenesis, osteogenesis, and proliferation of SHEDs (Novais et al., 2019). FGF signaling is involved in directing osteo/odontogenic differentiation of SHEDs by regulating phosphate/pyrophosphate regulatory genes (Nowwarote et al., 2018). Another highlight of SHEDs is their participation in physiological root resorption during the eruption process of permanent teeth. SHEDs can promote osteoclastogenesis, which is driven by tumor necrosis factor-α (TNF-α) through NF-κB signaling (Wang C. et al., 2019). It remains uncertain whether PTH1R signaling has a function in regulating SHEDs. Considering the resemblance and tight connections between SHEDs and DPSCs, it is worth exploring the underlying regulation mechanisms related to PTH1R signaling in various biological properties and tissue engineering applications of SHEDs.

Gingival Mesenchymal Stem Cells

Gingiva, a crucial component of tooth adjacent soft tissue, is another source of dental MSCs. GMSCs exhibit self-renewal and multilineage differentiation capacity (Tang et al., 2011). GMSCs lack tumorigenicity and can give rise to osteoblasts, chondroblasts, adipocytes, and keratinocytes (Santamaría et al., 2017; Murugan Girija et al., 2018). Several *in vivo* studies confirmed the ability of GMSCs in bone defects regeneration (Al-Qadhi et al., 2021). The osteogenic capability of GMSCs can be stimulated by proinflammatory cytokines and stress response proteins during inflammation (Tomasello et al., 2017). It is reported that GMSCs could suppress osteoclastogenesis and ultimately bone resorption via the CD39-adenosine pathway, suggesting a therapeutic application of GMSCs for rheumatoid arthritis and other related diseases (Luo et al., 2019). Thus, GMSCs can be a promising candidate for the repair and regeneration of inflammation- related bone loss, such as periodontitis and periapical infectious diseases. The advantage of GMSCs in treating inflammatory diseases can be attributed to their ability in regulating immune responses. It has been reported that exosomes from TNF-α treated GMSCs induced M2 macrophage polarization, therefore suppressing inflammation and periodontal bone loss (Nakao et al., 2021). Another study demonstrated the function of GMSCs in treating graft-vs.-host disease (GVHD) through mediating the conversion of Tregs to Th1 and/or Th17-like cells (Ni et al., 2019). Moreover, evidence shows that GMSCs display a more stable morphology and retain MSC features at higher passages compared to BMMSCs (Tomar et al., 2010). STRO-1 may be a useful marker to evaluate the stem cell properties of GMSCs since its expression will gradually decrease as the passage number increases. There is a paucity of data relative to PTH1R signaling in mediating GMSCs. Further study will help to understand the mechanisms for modulating these MSCs and widen the potential use of these MSCs in therapeutic applications.

Limitations and Future Directions

A combination of studies has revealed the involvement of PTH1R signaling in regulating various types of dental MSCs. Progress has been made in understanding the downstream molecules and pathway networks, but there are several aspects that have not been fully elucidated. First, there are overlapping regulatory mechanisms active in different dental MSCs. PTH1R signaling promotes osteo/odontogenic differentiation of both DPSCs and SCAPs through MAPK pathways. Increased Runx2, Osx, and Ocn expression were found in both OMSCs and SCAPs upon PTH1R activation. Whether there are stem cell-specific targeting factors or pathways remains to be determined. Second, administrating PTH or PTHrP to dental MSCs may interact with multiple signaling pathways, including Wnt, Hh, and TGF-β. For instance, it has been reported that PTH stimulates bone formation in osteoblasts and osteocytes, partially through canonical Wnt signaling (Tobimatsu et al., 2006; Suzuki et al., 2008; Robling et al., 2011; Bellido et al., 2013). Details of the crosstalk among these signaling cascades in dental MSCs are of interest and need to be analyzed in future studies. Third, current studies mainly focus on PTH1R signaling in mediating the properties of dental MSCs in normal physiological states. Whether there are diverse regulatory mechanisms involved under pathological conditions remains a subject of further research.

Furthermore, *Cre* transgenic mouse models revealed that diverse lineages of dental MSCs orchestrate and then contribute to craniofacial development under regulation by PTH1R signaling. However, there are still several limitations regarding the utilization of conditional knock-out mouse models. First, there is lack of specific *Cre* mouse models targeting MSCs residing in dental tissues. Many of the studies involving the orofacial region were inspired by research focusing on long bone, including the choice of transgenic *Cre* animal models. For instance, conditional knockout of PTH1R is not

restricted to craniofacial area using *Osx-Cre*, *PTHrP-Cre,* or *Prx1-Cre*. Whole skeleton *Cre* recombinase knockout may affect mineral ion homeostasis or whole-body metabolism, which subsequently influences craniofacial development. Therefore, specific mouse models targeting dental MSCs are required for further investigation. Second, some *Cre* mouse models induce defects in the craniofacial region during the embryonic stage. For example, *Osx-Cre* mice display slight growth delay and intramembranous bone hypomineralization (Wang et al., 2015a) and this may cause overlapping phenotypes and interference in the craniofacial region. Therefore, it is critical to select Cre-positive littermates as controls when analyzing the effect of targeted deletion of a floxed gene. Third, mouse models that depend on Cre recombinase result in embryonic deletion of target genes. All of the daughter cells inherit the same inactivated gene, which may lead to an overestimation in the function of gene or cell population. Complete loss of the proximal mandibular arch was observed in *Nestin-Cre*;*Fgf8*$^{fl/fl}$ mice (Trumpp et al., 1999). Although this phenotype proved the significance of Fgf8 in the development of the mandible, it held back further investigations on the tissue specific function of Fgf8 (Trumpp et al., 1999). Moreover, some embryonic knockouts may raise the mortality in mutant mice (Lei et al., 2016; Ono et al., 2016). Therefore, Cre^{ER} system is a more desirable strategy since it enables the choice of the timepoint for triggering the knockout. To date, many of the PTH1R conditional ablation models focused on the craniofacial developmental stage. Inducible Cre mouse models will enable an understanding of the function of PTH1R in adulthood. For example, although the function of PTH1R in DFPCs has been comprehensively characterized during root development, the postnatal role of PTH1R of PDLSCs in PDL homeostasis and injury repair remains to be determined. When applying a Cre^{ER} strategy, it is important to ascertain the heterogenicity of different subsets in specific start points and dosage of the drug delivery when inducing the Cre recombinase. The development of single-cell technology may favor dissection of the subsets and their lineage allocation (Nagata et al., 2020). It may also lead to discovery of new marker genes specific to dental MSCs, which provides clues for generating novel inducible transgenic lines to directly target specific cell types.

THERAPEUTIC APPLICATION OF PARATHYROID HORMONE/PARATHYROID HORMONE-RELATED PROTEIN IN OROFACIAL REGION

Multiple stem cell populations reside in teeth and their supporting tissues. Many that are regulated by PTH1R signaling have been identified, indicating the potential to use PTH/PTHrP to facilitate stem-cell-based oral tissue engineering and regeneration. The positive effects of PTH and PTHrP on vertebral and appendicular bone mineral density have been well-characterized. Teriparatide is a recombinant form of human PTH (1–34) and was the first anabolic drug approved by the U.S. Food and Drug Administration (FDA) to treat osteoporosis (Us Food and Drug Administration, 2002). A second anabolic agent, abaloparatide (ABL), is the 1–34 analog of PTHrP and was approved in the U.S. and Canada (Health Canada, 2017; Us Food and Drug Administration, 2017). Teriparatide and ABL both act through PTH1R on bone cells to stimulate intracellular cAMP and subsequent gene expression that favors bone formation (Yang et al., 2007). Both can increase bone mineral density while reducing the probability of fracture (Rosen, 2004; Miller et al., 2016; Ramchand and Seeman, 2020). Recent studies of ABL suggested that it has greater potential to widen the bone anabolic window and alleviate side effects compared to teriparatide (Makino et al., 2021). ABL contains modifications in AA residue insertions to maximize its anabolic capacity (Tella et al., 2017). It can be further differentiated from teriparatide based on its affinity and selectivity for PTH1R R^G (Dean et al., 2008). This property allows more transient cAMP signaling to increase osteogenesis, thus exerting more prominent anabolic potential (Hattersley et al., 2016). Moreover, endogenous PTH has a catabolic function in bone, while the incidence of hypercalcemia and increased $1,25(OH)_2D_3$ is lower in treatment with ABL when compared to teriparatide (Miller et al., 2016). Low resorptive actions makes ABL a new generation of anabolic drugs with a promising future (Ardura et al., 2019).

Several studies have subsequently investigated the effects of PTH and PTHrP on oral and maxillofacial bone regeneration (Rowshan et al., 2010; Valderrama et al., 2010). Indeed, alveolar bone is the major target of iPTH treatment in periodontitis, osteonecrosis of the jaw, and jawbone defects (Bashutski et al., 2010; Kuroshima et al., 2013; Sim et al., 2020). In addition, iPTH has been used to enhance bone-implant osseointegration and bone remodeling in orthodontic treatment (Nimeri et al., 2013; Jiang et al., 2018). The function of PTHrP has also been explored in dental implant treatment. In this section, we will briefly summarize the recent applications of PTH and PTHrP in the orofacial region.

Lesion of Periodontal Tissue

A variety of studies using both animal models and clinical trials have suggested a promising outcome for PTH treatment in periodontitis. PTH-treated periodontitis rodents had reduced alveolar bone resorption and milder infiltration of inflammatory cells at the marginal gingiva (Barros et al., 2003; Chen et al., 2017). PTH applied to rats with partially removed PDL and cementum attenuated the extension of the bone defect, increased total callus bone, and accelerated the formation of cementum-like tissue at the healing site (Vasconcelos et al., 2014). Moreover, local injection of PTH, or PTH in combination with neutral self-assembling peptide hydrogel, could improve clinical outcomes of chronic periodontitis (Tokunaga et al., 2011; Yoshida et al., 2019). A pre-clinical study conducted on type 1 diabetic rats with periodontitis demonstrated that iPTH treatment downregulates sclerostin, the Wnt signaling inhibitor, and stimulates osteoid formation and mineralization (Kim et al., 2018). Importantly, the most notable clinical example is a double-blind, placebo controlled, randomized trial involving the application of PTH to 40 patients undergoing periodontal surgery. In this study,

patients receiving teriparatide showed higher gain in bone height, improved periodontal attachment, and better outcome of a periodontal probing examination (Bashutski et al., 2010).

The therapeutic potential of PTH (1–34) for periodontitis may depend on PDLSCs. Evidence shows that PTH (1–34) treatment, together with osteogenic induction, conducted on STRO-1^+ human PDLSCs led to increased expression of Runx2 and Osx, along with upregulated mineralization ability. It is worth noting that PTH1R expression increased in hPDLSCs upon PTH treatment, implying that a positive feedback loop was established (Wang X. et al., 2016). Stromal cell-derived factor-1α (SDF-1α) is a key factor in stem cell recruitment and homing in many diseased organs requiring regeneration (Ceradini et al., 2004). Yet its therapeutic potential is limited by CD26/dipeptidyl peptidase-IV (DPP-IV) which leads to N-terminal cleavage at the position-2 proline of SDF-1α (Christopherson et al., 2004). PTH is a DPP-IV inhibitor and therefore can assist SDF-1α in recruiting PDLSCs to the injured site (Huber et al., 2014). Furthermore, since SDF-1α and PTH can both promote proliferation and osteogenic differentiation of PDLSCs, PTH/SDF-1α co-therapy becomes a promising strategy for periodontitis (Du et al., 2016). An *in vivo* study confirmed that PTH/SDF-1α co-therapy could induce chemotaxis of $CD90^+CD34^-$ stromal cells and stimulate their migration toward periodontal defects, concomitant with increased expression of Runx2, ALP, and Col1αI in the newly formed bone area. The result is accelerated bone regeneration and better organization of the periodontal ligament interface (Wang F. et al., 2016).

Bisphosphonate-Related Osteonecrosis of the Jaws

Bisphosphonates (BPs), the first line drugs used to treat osteoporosis, act by suppressing osteoclasts to achieve anti-resorptive activity (Black and Rosen, 2016). Bisphosphonate-related osteonecrosis of the jaws (BRONJ), a rare but severe complication, is characterized by non-healing jaw defects in patients who have undergone BP treatment. The etiology remains unclear, but one hypothesis is that BPs inhibit bone turnover in skeletal homeostasis and healing processes (Grey, 2010; Rollason et al., 2016). Considering the anabolic and catabolic effects of PTH, off-label use of teriparatide has been applied to BRONJ treatment with promising outcomes in several clinical trials (Cheung and Seeman, 2010; Yoshiga et al., 2013; Kim et al., 2014; Jung et al., 2017; Sim et al., 2020). It is reported that teriparatide administration was associated with a higher resolution rate of necrosis lesions, reduced bone defects, and improved bone healing (Kim et al., 2014; Sim et al., 2020). Most trials conducted daily PTH injection. Whether weekly administration will have satisfactory therapeutic effects is unclear and remains under further investigation (Yoshiga et al., 2013; Ohbayashi et al., 2020). An animal model showed that in addition to BRONJ treatment, PTH also has potential to prevent the development of jaw necrotic lesions by maintaining osteocyte survival (Kuroshima et al., 2014a). The administration of teriparatide led to a higher ratio of RANKL-positive osteocytes (Liu J. et al., 2020). Another study that applied different doses of teriparatide suggested that its therapeutic effect may not be dose dependent (Yu and Su, 2020). Further investigation of the dosage, frequency, duration of treatment, and drug combination plan for PTH treatment in BRONJ is warranted.

Dental Implant

iPTH treatment is critical for osteogenesis at the peri-implant area and for osseointegration at the external and internal surfaces of implants in aged rats and in osteoporosis animal models induced by ovariectomy, glucocorticoid, or low protein diet (Dayer et al., 2010; Almagro et al., 2013; Oki et al., 2017; Park et al., 2017; Jiang et al., 2018). It is worth noting that treating jaw osteoporosis with PTH (1–34) before implant surgery restored bone quality, bone volume, and bone turnover, leading to favorable implant stability (Gomes-Ferreira et al., 2020). Furthermore, PTH (1–34) can be used to reverse the deleterious effects of cigarette smoke such as poor bone healing and low bone mass in the bone-implant interface (Lima et al., 2013). In cases where BRONJ was induced by dental implants, PTH also enhanced peri-implant bone formation (Park et al., 2020). However, a few studies suggest that iPTH does not improve healing of the augmented maxillary sinus in osteoporosis rabbit models or osteointegration in diabetic rats (Rybaczek et al., 2015; Dam et al., 2020). Local delivery systems have been implemented by binding PTH to polyethylene glycol (PEG)-based hydrogel or by directly depositing PTHrP on implants using a layer-by-layer (LBL) electro assembly technique. The therapeutic efficacy of both systems is promising (Valderrama et al., 2010; Tang et al., 2020). In addition, a pre-clinical trial that combined PTH with other factors such as bisphosphonates, menaquinone-4 (vitamin K2; MK), or simvastatin showed a cumulative advantage (Li et al., 2013, 2018; Tao et al., 2016). Moreover, there is evidence that withdrawal of PTH after the course of treatment reversed the effects and bone mineral density decreased rapidly. However, anti-resorptive therapy such as bisphosphonates applied after iPTH could restore the implant anchorage and maintain the curative effect (Hu et al., 2019). Mechanical loading is another factor that contributes to peri-implant bone mass, but whether the combination of loading and PTH has an additive effect requires further investigation (Fahlgren et al., 2013; Grosso et al., 2015; Shibamoto et al., 2018). In a clinical trial, Kuchler et al. (2011) conducted an open-label, placebo-controlled, randomized trial of 24 patients with edentulous mandibles to investigate the therapeutic effects of teriparatide administration on dental implant osseointegration. The results revealed that 20 μg of teriparatide administered once-per-day for 28 days increased bone to implant contact as well as bone formation in the periosteal, cortical, and medullary compartment (Kuchler et al., 2011).

Extraction Socket Healing

Osteoporosis delayed extraction socket healing in a pre-clinical study (Liu et al., 2019). iPTH therapy through subcutaneous or intra-oral injection were both effective in promoting healing in the tooth extraction socket by increasing new bone formation and connective tissue maturation (Kuroshima et al., 2013). In teriparatide treated osteoporosis rats, the extraction socket

exhibited increased expression levels of osteogenic markers including Wnt, Alp, and Ocn and reduced osteoclast numbers (de Oliveira et al., 2019). In comparison with bisphosphonates, PTH has a better therapeutic effect in both hard and soft tissue regeneration at the extraction site by enhancing osteogenesis and collagen deposition while suppressing inflammation (Kuroshima et al., 2014b). However, PTH did not have a significant effect on extraction wound healing in hyperglycemic rats (Xu et al., 2020). Another study also demonstrated that PTH did not benefit the healing process of the bone-implant interface in diabetic rats (Rybaczek et al., 2015). However, PTH treatment had a positive effect on periodontitis in a diabetic rodent model (Kim et al., 2018). These conflicting results indicate that whether diabetes exerts an influence on the effect of PTH requires further investigation.

Autograft/Allograft Bone Integration

The success of autograft or allograft treatment for massive craniofacial bone defects is limited by rapid resorption, poor integration, and scar formation (Burchardt, 1983; Cohn Yakubovich et al., 2017a). PTH can augment new bone formation, as well as reduce bone resorption and fibrotic tissue formation in grafts harvested from calvaria or iliac (dos Santos et al., 2016; Zandi et al., 2019), improving reconstruction of bone defects with autografts. Allografts face the challenge of integrating with the host bone. However, PTH is reported to stimulate osteoprogenitor differentiation and enhance bone formation and bone mineralization in calvaria or mandible defects (Sheyn et al., 2013; Pelled et al., 2020). It promotes neovascularization in the graft surroundings (Cohn Yakubovich et al., 2017a), resulting in less fibrosis and scar tissue formation as well as superior allograft integration.

Radiation-Induced Bone Injury

Radiotherapy for tumors in the orofacial area may cause radiation-induced bone injury such as decreased bone density and strength, or osteoradionecrosis in the jawbone. The main reasons for these issues are radiation-induced cell apoptosis and impaired regeneration ability (Smith et al., 2017). iPTH administration has been shown to rescue radiation-induced osteoblast apoptosis by enhancing DNA repair through the Wnt signaling pathway (Chandra et al., 2015). Moreover, PTH functions to maintain osteocyte count and enhance bone regeneration, thus alleviating radiation-induced bone loss (Chandra et al., 2014; Kang et al., 2017). The bone union quality was improved with increased trabecular number, thickness, and connectivity upon PTH treatment, indicating that PTH can rescue irradiated microstructural deterioration (Chandra et al., 2013; Gallagher et al., 2013).

Orthodontic Treatment

Studies in rodents have suggested a function for PTH in accelerating orthodontic tooth movement (Nimeri et al., 2013). Research shows that iPTH accelerated tooth movement in osteoporosis rats and rabbits receiving mandibular ramus osteotomy (Salazar et al., 2011; Li et al., 2019). In terms of retention, iPTH could promote periodontium regeneration to reduce the relapsing distance, therefore maintaining the stability of orthodontic treatment (Li et al., 2020). Its ability to enhance periodontal tissue repair following orthodontic movement may be associated with suppression of high mobility group box protein 1 (HMGB1) (Wolf et al., 2013). In addition, PTH mediated cementum formation possible by regulating Dmp1 expression via the cAMP/PKA pathway in cementoblasts, offering a promising method to prevent root resorption during orthodontic treatment (Wang et al., 2015b; Li et al., 2021).

Temporomandibular Joint Related Diseases

There has been progress made in exploring the effect of iPTH administration in the temporomandibular joint (TMJ). iPTH increases cell proliferation and differentiation in mandibular condylar cartilage (MCC), increasing the length of the condyle head (Mh et al., 2017). However, PTH could also exert negative effects on MCC by inducing early mineralization, leading to cartilage degeneration, and condyle surface irregularities (Dutra et al., 2017). We have recently generated an age-related TMJ osteoarthritis (TMJ OA) mouse model and injected them with PTH (1–34) for 4 weeks. The results suggest that iPTH ameliorated the degenerative changes in TMJ condyles and increased subchondral bone turnover by accelerating the differentiation of stem cells residing in the subchondral bone (Cui et al., 2020b). A recent study using a canine total meniscal meniscectomy model suggested PTH (1–34) with a BMMSCs-loaded scaffold stimulated meniscus regeneration and alleviated cartilage damage. This implies that PTH could promote the regenerative and chondroprotective function of a BMMSCs scaffold (Zhao et al., 2020). These data yield insight into the efficacy of combining PTH and stem cells in TMJ tissue engineering in the future.

Fracture Healing and Distraction Osteogenesis

PTH (1–34) augments MSCs injection to treat fractures by enhancing MSCs homing and differentiation (Cohn Yakubovich et al., 2017b). It also enhances angiogenesis in bone callus (Jiang et al., 2019). Conversely, lack of endogenous PTH led to reduced vessel formation and downregulation of the PKA/pAKT/HIF1α/VEGF pathway during fracture healing (Ding et al., 2018). Distraction osteogenesis is a surgical technique for lengthening the bone, but rapid distraction may lead to poor bone healing (Ye et al., 2017). PTH functions to promote new bone formation and improve bone microarchitecture in the distracted callus (Ye et al., 2017). PTH also has positive effects in distraction of an irradiated mandible by reversing poor vascularity induced by radiation (Kang et al., 2013).

Clinical Concerned Problems and Future Directions

Large numbers of pre-clinical animal studies and clinical trials have confirmed the therapeutic effect of PTH in the orofacial region. However, there are some limitations associated with PTH administration. Evidence from a single pre-clinical study using a rat model suggested long-term use and overdoses of teriparatide

increased the risk of osteosarcoma (Vahle et al., 2002). The duration of teriparatide treatment is limited to 2 years to avoid this possibility (Hodsman et al., 2005). The contraindications for PTH injection include patients sensitive to osteosarcoma, patients with Paget's disease, pre-existing hypercalcemia, and history of other skeletal disorders (Hodsman et al., 2005). Moreover, drug combination therapy and sequential therapy need further exploration since the therapeutic effect of PTH decreases rapidly after withdrawal (Black et al., 2005). It is reported that anti-RANKL antibodies and bisphosphonates may be therapeutic options for the discontinuation of iPTH treatment (Omiya et al., 2018; Hu et al., 2019).

PTH administration methods consist of systemic injection, local injection, dissolving in an injectable gel to form a local slow-release system and others (Soma et al., 2000). Several new methods have been developed to provide more convenient and effective ways to apply PTH or PTHrP as well as to reduce side effects. Among the various delivery methods proposed are an implantable and biodegradable pulsatile device (Dang et al., 2017), bio-membrane fabrication (Yin et al., 2020), and a nanoemulsion system (Altaani et al., 2020). Most of the current research has not progressed beyond the pre-clinical phase, thus detailed guidance for dosage, frequency, duration, and clinical indication of PTH or PTHrP administration in orofacial region is still required. So far, ABL-associated pre-clinical research and clinical trials targeting the orofacial region are limited. Furthermore, the role of other engineered amino-terminally modified variants of PTH, such as long-acting PTH (Shimizu et al., 2016) has not been analyzed in orofacial tissue. Further investigation is required to completely explore the theoretical basis of PTH/PTHrP analogs and its application in orofacial related diseases.

CONCLUSION

A large and diverse number of studies have shed light on the role of PTH1R signaling in dental MSCs, emphasizing the important regulatory role of PTH1R signaling during development and pointing out the therapeutic potential of PTH and PTHrP analogs for dental tissue regeneration. The stem cell properties of a diverse family of dental MSCs have been well characterized in the past two decades. However, the underlying mechanisms for PTH1R signaling to mediate stem cell behavior are still uncertain. Many of the regulatory mechanisms have been demonstrated by *in vitro* experiments that suggest PTH1R signaling promotes proliferation and differentiation of multiple dental progenitors. The recent genetic tools of lineage tracing and conditional knock-out mouse models have helped to identify the specific population of MSCs regulated by PTH1R. Remarkably, pre-clinical studies and clinical trials have already revealed promising outcomes when administrating PTH or PTHrP in various dental-related diseases, such as periodontitis, osteonecrosis of the jaw, and jawbone defects. It is conceivable that activating PTH1R signaling has an effect on tissue resident MSCs during the process of repair. Yet a better understanding is necessary for a holistic view of the *in vivo* regulatory mechanisms of PTH1R in specialized cell types. In the development of stem-cell-based regeneration medicine, therapy based on PTH1R signaling plus dental MSCs holds great promise.

AUTHOR CONTRIBUTIONS

PiL, BL, PeL, RB, CC, and YF collected the literature and drafted the manuscript. ZZ, XZ, and YF supervised the procedures and approved the manuscript. All authors gave their final approval and agreed to be accountable for all aspects of the work.

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Translational and Clinical Applications of Dental Stem Cell-Derived Exosomes

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Mesenchymal stem cells (MSCs) are promising seed cells in tissue repair and regeneration due to their featured properties of self-renewal and multipotency. However, a growing body of evidence has demonstrated that MSCs exert biological functions mainly through secreting exosomes. Exosomes, which contain RNA, proteins, lipids, and metabolites, are new players in regulating many fundamental processes and play important roles in regenerative medicine. Exosomes not only mimic the effects of their parent cells but also possess many advantages such as high drug loading capacity, low immunogenicity, excellent biocompatibility, and low side effects. Currently, a total of 6 different dental stem cells (DSCs) including dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs), stem cells from apical papilla (SCAPs) and gingival mesenchymal stem cells (GMSCs) have been isolated and identified. DSC-derived exosomes (DSC-Exos) are actively involved in intercellular communication, anti-inflammation, osteogenesis, angiogenesis, immunomodulation, nurturing neurons, and promoting tumor cell apoptosis. In this review, we will critically review the emerging role and clinical application potential of DSC-Exos.

Keywords: dental stem cells, exosomes, regenerative medicine, tissue repair, clinical application

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INTRODUCTION

Mesenchymal stem cells (MSCs) are multipotent progenitor cells that can be isolated from different tissues including but not limited to, bone marrow (Friedenstein et al., 1966), adipose tissue (Gruber et al., 2010), placenta (In 't Anker et al., 2004), umbilical cord (Secco et al., 2008), hair follicle (Bajpai, Mistriotis, and Andreadis 2012), palatine tonsil (Ryu et al., 2012), amniotic fluid (In 't Anker et al., 2003), fetal blood and liver (Campagnoli et al., 2001). Accumulative evidence has demonstrated that MSCs are capable of self-renewal, multipotent differentiation (Minguell et al., 2001; Munmun and Witt-Enderby 2021), regulating immune and inflammatory responses (Uccelli et al., 2008), and suppressing apoptosis and oxidative stress (Tsubokawa et al., 2010). More importantly, numerous pre-clinical studies have shown that MSCs hold promise in treating a wide range of diseases, including cancer, liver disease, cartilage repair, heart failure, stroke, neurological disorders, diabetes mellitus, autoimmune diseases, Duchenne muscular dystrophy, ocular surface diseases (Lodi et al., 2011; Lu et al., 2021). Over the past 10 years, more than 1000 MSC-based clinical trials have been

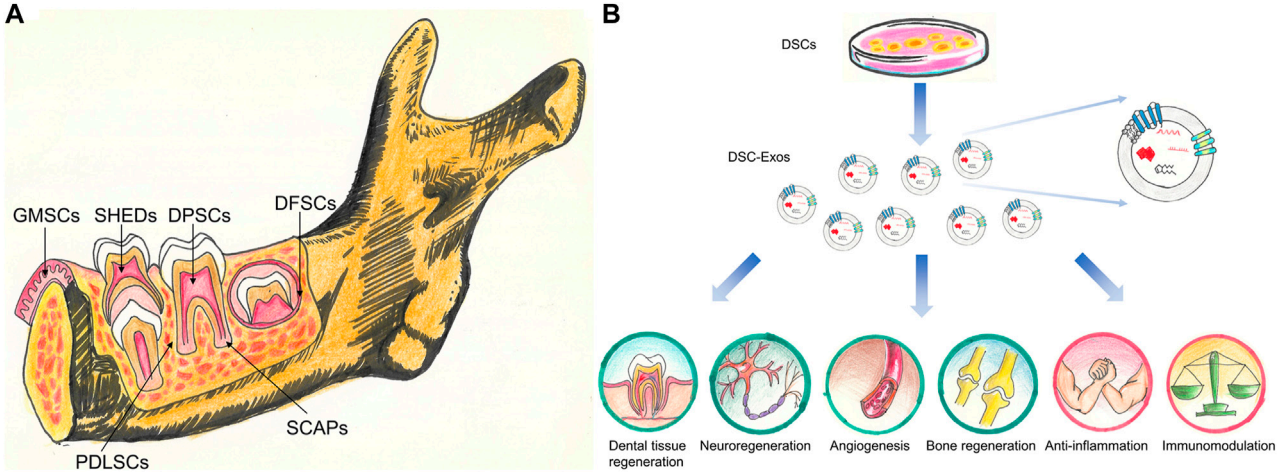

FIGURE 1 | (A) Stem cell populations derived from different dental tissues. DPSCs, stem cells from dental pulp; SHEDs, stem cells from human exfoliated deciduous teeth; PDLSCs, stem cells from PDL; DFSCs, stem cells from dental follicle; SCAPs, stem cells from the apical papilla; GMSCs, stem cells from gingival tissues. **(B)** The potential clinical application of DSC-Exos. DSC-Exos hold great promise for dental tissue regeneration, neuroregeneration, angiogenesis, bone regeneration, anti-inflammation and immunomodulation.

conducted with recruitment of approximately 50,000 patients due to the safety of autogenous stem cells (Pittenger et al., 2019). Interestingly, accumulative evidence has suggested that the therapeutic effects of transplanted MSCs largely depend on the secretome of MSCs rather than the MSCs themselves (Phinney and Pittenger 2017).

Distinct from other types of cell therapy, MSC-based therapy achieves the therapeutic effects not only through direct cell-cell contacts but also by releasing secretome-derived bioactive factors (Levy et al., 2020). Recently, the MSC secreted extracellular vesicles (MSC-EVs), which include exosomes, microvesicles, and apoptotic bodies, have been suggested as a viable cell-free therapeutic alternative for MSCs (Jarrige et al., 2021). Compared to the cellular therapies, the MSC-EVs-based therapy offer many advantages such as high drug loading capacity, high specificity, low immunogenicity, excellent biocompatibility, high stability, lack of cytotoxicity, competitive price, and efficient intercellular communication. Therefore, MSC-EVs-based therapy, especially using exosomes, has emerged as a promising therapeutic tool for tissue repair and regeneration. In this review, we critically focus on the potential value of exosomes derived from dental stem cells (DSC-Exos) for treating oral and systemic diseases.

FEATURED PROPERTIES OF DENTAL STEM CELLS AND EXOSOMES

Dental Stem Cells (DSCs)

Currently, a total of six different dental stem cells (DSCs) including dental pulp stem cells (DPSCs), stem cells from exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), dental follicle progenitor cells (DFPCs), stem cells from apical papilla (SCAPs) and gingival mesenchymal stem cells (GMSCs) have been isolated and identified (Zhang et al., 2009; Sedgley and Botero 2012) (**Figure 1A**).

DPSCs, the first characterized DSCs, were isolated from the human dental pulp in 2000 (Gronthos et al., 2000). The MSC-like properties of DPSCs enable them to differentiate into multiple cell lineages such as neural-like cells, osteoblasts, adipocytes, and chondrocytes, and form mineralized tissue, blood vessels, and nerve tissues *in vivo* (Gronthos et al., 2000; Gronthos et al., 2002; Victor and Reiter 2017). Besides, DPSCs have also been demonstrated to regenerate dentin and functional dental pulp with vasculature and nerves (Iohara et al., 2014).

SHEDs, obtained from human exfoliated deciduous teeth by Miura et al. (2003), is a population of highly proliferative cells capable of differentiating into odontoblasts and endothelial cells (Miura et al., 2003). After being injected into human root canals, the transplanted SHEDs could differentiate into functional odontoblasts and form dentin-like tissues (Rosa et al., 2013). Interestingly, SHEDs remained viable following transplantation into the mouse brain and expressed neural markers (Miura et al., 2003).

Similarly, PDLSCs are named according to their tissue of origin, and isolated from the periodontal ligament which is a soft connective tissue between the teeth and alveolar bone. PDLSCs are promising seed cells for the restoration of periodontal tissues and are capable of differentiating into adipocytes, chondrocytes, osteoblasts, cardiac myocytes and neural cells (Gay et al., 2007; Tomokiyo et al., 2019). PDLSCs interact tightly with the periodontitis niche in a positive feedback loop. The injured PDLSCs may aggravate the disrupted periodontal tissue homeostasis, while bacterial infections and subsequent host immune responses affect the functional properties of resident PDLSCs by shaping the periodontal microenvironment (Zhang Y. et al., 2021).

TABLE 1 | The common and different properties for each type of DSCs.

Cell type	Advantage	Weakness	Similarity
DPSCs	Formation of dentin–pulp-like complex, Source for reparative dentin	Essential to be extracted from healthy adult teeth, differential growth rates, cell morphologies, and sizes	Fibroblast-like morphology
SHEDs	Formation of dentin-like tissue or pulp-like tissue, The most proliferative DSCs	Unable to form dentin-pulp-like complex	Common markers: $CD13^+$, $CD29^+$, $CD73^+$, $CD90^+$, CD105+, CD106+, CD146+, Stro-1+, $CD34^-$, $CD45^-$, CD11b-, $CD14^-$, $CD19^-$, CD79a-, and HLA-DR-High clonogenic potential
PDLSCs	Formation of PDL-cementum-like construction	Lack odontogenic potential	Multilineage differentiation capacity
DFPCs	Formation of alveolar bone, Formation of PDL-cementum-like construction	Lack odontogenic potential	
SCAPs	Maintenance of root maturation, Formation of dentin-pulp-like complex	Not easily obtainable	
GMSCs	Easy to isolate, Long-term stability	Lack odontogenic potential	

DFPCs were first isolated from the dental follicle surrounding the impacted third molar tooth germ and identified by Morsczeck et al. (2005). DFPCs not only have better immunomodulatory and anti-apoptotic effects on the immune system than DPSCs and SHED, but also exhibit greater osteogenic properties than SHED and DPSCs as the osteogenic-related markers such as Runx2 and DSPP are highly expressed in DFPCs (Zhu et al., 2018). In addition, DFPCs were able to differentiate into cementoblasts *in vivo* (Handa et al., 2002). Therefore, DFSCs are a promising alternative source for dental hard tissue regeneration.

SCAPs are isolated from the apical papilla of immature permanent teeth, and play a critical role in tooth root development and dentin regeneration (Sonoyama et al., 2008). The high telomerase activity of SCAPs makes them a better choice of dentin regeneration compared to DPSCs (Sonoyama et al., 2006). In addition, SCAPs are able to form cementum/PDL-like complex *in vivo* (Han et al., 2010). Moreover, SCAPs have been shown to be low immunogenicity and possess immunomodulatory functions, which make them an attractive and promising therapeutic tool for tissue regeneration (Ding et al., 2010).

GMSCs were first isolated from healthy gingival tissues and characterized by Zhang et al., in 2009 (Zhang et al., 2009). GMSCs can differentiate into adipocytes, chondrocytes, and endothelial cells, and have shown great promise for nerve regeneration. Besides, GMSCs exerted anti-proliferative and pro-apoptotic effects on oral cancer cells both *in vitro* and *in vivo* (Ji et al., 2016).

To the best of our knowledge, it is still extremely difficult to distinguish various sources of DSCs on a molecular level. All different types of DSCs are derived from migrating neural crest cells, which are originated from the embryonic ectoderm germ layer (Pisciotta et al., 2020). The fibroblast-like DSCs share similar surface marker expression profiles, and have high clonogenic potential and multipotent differentiation capacity. A recent study suggested that calreticulin might be a promising biomarker for distinguishing DPSCs and GMSCs. We have summarized the similarity, advantages, and weaknesses of each type of DSCs in **Table 1**.

Exosomes

Exosomes, with a size range of 40–160 nm (average 100 nm), are originated from the endosomal system by inward budding of the endosomal membrane (Xu F. et al., 2020; Yoon et al., 2020). Ultracentrifugation, size-based isolation techniques, immunoaffinity capture-based techniques, exosome precipitation, and microfluidic-based isolation techniques are utilized to isolate exosomes (Doyle and Wang 2019). They are found in abundance in body fluids including breast milk, saliva, blood, and urine (Vlassov et al., 2012). This type of extracellular vesicles was initially thought of as waste products (Wolf 1967). However, accumulative evidence has demonstrated that exosomes carry many bioactive molecules including nucleic acids, proteins, lipids, metabolites (Zhang Y. et al., 2020; Kalluri and LeBleu 2020). These encapsulated materials are transported to neighboring or distant cells selectively, which contribute to cell-cell communication, signal transduction, immune response modulation, antigen presentation, and epigenetic reprogramming of recipient cells (Janockova et al., 2021). The biological functions of exosomes are heavily dependent on physiological/pathological conditions of originating tissues or cells at the time of exosome secretion, and the surface receptors of the recipient cells (Alcayaga-Miranda et al., 2016). As shown in **Figure 1B**, the DSC-derived exosomes might represent an ideal therapeutic tool for tissue repair and regeneration as well as treating other systemic diseases.

TRANSLATIONAL AND CLINICAL APPLICATIONS OF DENTAL STEM CELL-DERIVED EXOSOMES

DPSC-Exos

The high osteo/odonto-induction capability and easy availability of DPSCs-derived exosomes (DPSC-Exos) make them highly attractive in regenerative medicine (Imanishi et al., 2021). In addition, aged DPSCs still have active cellular metabolism and secrete functional exosomes which

can penetrate the blood-brain barrier, indicating DPSC-Exos might be an effective drug carrier for the treatment of various diseases, especially for neurological disorders like Parkinson's disease (PD) (Haney et al., 2015; Iezzi et al., 2019). For instance, DPSC-Exos were deemed as a suitable carrier to deliver tumor suppressor miR-34a to inhibit the proliferation of breast cancer cells (Vakhshiteh et al., 2021). In terms of odontogenic differentiation, Huang et al. revealed that DPSC-Exos attached to biomaterials by binding to matrix proteins like fibronectin and type I collagen (Huang et al., 2016). Besides, DPSC-Exos, especially those isolated from DPSCs cultured under an odontogenic differentiation environment, increased the expression of genes indispensable for odontogenic differentiation of naïve DPSCs *in vitro* and promoted the regeneration of dental pulp-like tissues *in vivo* (Huang et al., 2016; Swanson et al., 2020). Similarly, compared to exosomes isolated from DPSCs cultured undergrowth state, DPSC-Exos obtained under odontogenic conditions exhibited better performance for trigging odontogenic differentiation of DPSCs by activating the TGF-β1/smads signaling pathway (Huang et al., 2016; Hu et al., 2019). Exosomes derived from both mineralizing DPSCs and an immortalized murine odontoblast cell line (MDPC-23) were superior to traditional glass-ionomer cement for forming the reparative dentin bridge (Swanson et al., 2020). It seems that lipopolysaccharide (LPS) treatment can significantly alter the biological functions of DPSCs-Exos. Li et al. demonstrated that LPS-preconditioned DPSC derived exosomes (LPS-DPSC-Exos) promoted the proliferation, migration, and odontogenic differentiation of Schwann cells (Li et al., 2021). Similarly, LPS-DPSC-Exos were shown to promote angiogenesis by facilitating the proliferation, migration, and tube formation abilities of human umbilical vein endothelial cells (HUVECs) *in vitro* through changing the microRNA (miRNA) expression profile and increasing the levels of kinase-insert domain-containing receptor and vascular endothelial growth factor (Huang et al., 2021). DPSC-Exos also exhibits strong anti-inflammatory and immunomodulatory effects. For instance, DPSC-Exos facilitated alveolar bone reconstruction and periodontal epithelium healing in a mouse model of periodontitis *via* delivering exosomal miR-1246 (Shen et al., 2020). DPSC-Exos exerted immunomodulatory effects by suppressing the differentiation of CD4+T cells into T helper 17 cells (Th17) and facilitated the transformation of CD4+T cells into regulatory T cells (Tregs), leading to the increased level of anti-inflammatory cytokines (Ji et al., 2019). In addition to oral diseases, DPSC-Exos based therapy is promising for treating systemic diseases. For example, exosomes derived from DPSC might be more suitable in the treatment of neurodegenerative diseases than MSCs from mesodermal tissues such as bone marrow or adipose tissues (Wang et al., 2019). Exosomes secreted from miR-140-5p overexpressing DPSCs promoted the expression of genes related to chondrogenic differentiation and exerted anti-apoptotic effects both *in vitro* and *in vivo*, which represented a potentially novel therapeutic strategy for osteoarthritis (Lin et al., 2021).

SHED-Exos

The applications of SHED-derived exosomes (SHED-Exos) can be classified into the following three categories: promotion of osteogenesis, neurotrophic property and anti-inflammatory function. In terms of osteogenesis, SHED-Exos promoted osteogenic differentiation of PDLSCs by upregulating key genes and signaling pathways related to osteogenesis (Wang A. et al., 2020). Similarly, SHED-Exos was shown to have the potency for mobilizing naïve BMMSCs, resulting in enhancing bone regeneration (Luo et al., 2021). Wei et al. revealed that SHED-Exo promoted osteogenesis and suppressed adipogenesis of bone marrow mesenchymal stem cells (BMMSCs) by decreasing lipid droplets and the expression of the adipogenic marker PPARγ (Wei et al., 2020). SHED-Exos promoted neovascularization of HUVECs and osteogenic differentiation of BMMSCs, and this regulatory effect could be counteracted by adding AMPK inhibitor, indicating that the AMPK signaling pathway might involve in mediating the pro-angiogenic effects and pro-bone regeneration activities of SHED-Exos (Wu et al., 2019). Concerning neurotrophic property, exosomes isolated from SHEDs grown on the laminin-coated three-dimensional alginate micro-carriers protected dopaminergic neurons from 6-hydroxy-dopamine induced apoptosis, whereas exosomes from SHEDs grown under standard culture conditions had no such effects (Jarmalaviciute et al., 2015). Li et al. injected SHED-Exos into the traumatic brain injury (TBI) rat model and observed that SHED-Exos contributed to rat motor functional restoration and cortical lesion reduction by shifting microglia polarization (Li et al., 2017). Narbute et al. showed that SHED-Exos significantly improved the gait impairments and contralateral rotations in the unilateral 6-hydroxydopamine (6-OHDA) rat model of PD (Narbute et al., 2019). Collectively, SHED-Exos are promising therapeutic tools for neurological disorders like PD. In terms of anti-inflammatory effect, SHED-Exos significantly suppressed the carrageenan-induced acute inflammation *in vivo* (Pivoraite et al., 2015). Similarly, Luo et al. showed that SHED-Exos markedly reduced the inflammation in chondrocytes derived from the temporomandibular joint through delivering miR-100-5p (Luo et al., 2019).

PDLSC-Exos

The major regulatory functions of PDLSCs-derived exosomes (PDLSC-Exos) include angiogenesis, anti-inflammation, and osteogenesis. Inflammation led to increasing exosome secretion in PDLSCs, and exosomes derived from inflamed PDLSC promoted angiogenesis of HUVECs by upregulating the expression of vascular specific marker CD31 and VEGFA (Zhang Z. et al., 2020). The exosomes isolated from PDLSCs that were exposed to the LPS-induced periodontitis environment demonstrated good anti-inflammatory ability by modulating the balance of T helper cell 17 (Th17)/regulatory T cell (Treg) through the miR-155-5p/SIRT1 pathway (Zheng et al., 2019). In terms of osteogenesis, PDLSC-Exos possess the capacity for inducing osteogenic differentiation of BMMSCs *via* regulating AMPK signaling, MAPK signaling, and insulin signaling pathways (Liu et al., 2020). P2X7R overexpressing PDLSCs-derived conditional medium and exosomes markedly

TABLE 2 | The potential clinical application of DSC-Exos.

Origin	Administration	Recipients	Application	Refs	The potentially most suitable application of specific DSC-Exos
DPSCs	*In vitro*	Breast cancer cells	Cancer	Vakhshiteh et al. (2021)	Pulp and dentin regeneration
—	*In vitro*	$CD4^+$ T cells	Immunomodulation	Ji et al. (2019)	
—	*In vitro*	Schwann cells	Pulp regeneration	Li et al. (2021)	
—	*In vitro*	HUVECs	Angiogenesis	Huang et al. (2021)	
—	*In vivo*	Athymic nude mice	Pulp regeneration	Huang et al. (2016)	
—	*In vivo*	Rat pulpotomy model	Dentin regeneration	Swanson et al. (2020)	
—	*In vivo*	Periodontitis model	Periodontitis	Shen et al. (2020)	
—	*In vivo*	Osteoarthritis model	Osteoarthritis	Lin et al. (2021)	
SHEDs	*In vitro*	PDLSCs	Osteogenic differentiation	Wang et al. (2020b)	Parkinson's disease
—	*In vitro*	BMMSCs	Migration promotion	Luo et al. (2021)	Neuroregeneration
—	*In vitro*	Neurons	Parkinson's disease	Jarmalaviciute et al. (2015)	
—	*In vitro*	Chondrocytes	Osteoarthritis	Luo et al. (2019)	
—	*In vivo*	Periodontal defect	Bone regeneration	Wu et al. (2019)	
—	*In vivo*	BMMSCS	Bone regeneration	Wei et al. (2020)	
—	*In vivo*	Traumatic brain injury model	Traumatic brain injury	Li et al. (2017)	
—	*In vivo*	Parkinson's disease model	Parkinson's disease	Narbute et al. (2019)	
—	*In vivo*	Mouse paw edema	Anti-inflammation	Pivoraite et al. (2015)	
PDLSCs	*In vitro*	$CD4^+$ T cells	Immunomodulation	Zheng et al. (2019)	Periodontitis induced bone loss
—	*In vitro*	BMMSCs	Osteogenic differentiation	Liu et al. (2020)	
—	*In vitro*	PDLSCs	Osteogenic differentiation	Xu et al. (2020b)	
—	*In vitro*	HUVECs	Angiogenesis	Zhang et al. (2020a)	
SCAPs	*In vivo*	Immunodeficient mice	Dentinogenesis	Zhuang et al. (2020)	Not enough evidences for evaluation
—	*In vivo*	Critical-size defects	Soft tissue regeneration	Liu et al. (2021)	
GMSCs	*In vitro*	Pre-osteoblast	Osteogenic differentiation	Jiang and Xu (2020)	Taste bud regeneration and would healing
—	*In vitro*	Macrophages	Anti-inflammation	Zhang et al. (2021b)	
—	*In vivo*	Schwann and DRG cells	Nerve repair	Rao et al. (2019)	
—	*In vivo*	Skin defect model	Would healing	Shi et al. (2017)	
—	*In vivo*	Periodontitis model	Bone regeneration	Nakao et al. (2021)	
—	*In vivo*	Tongue defect model	Taste bud regeneration	Zhang et al. (2019)	
DFPCs	NA	NA	Not evaluated yet	NA	NA

improved the osteogenic capacity of PDLSCs in the inflammatory microenvironment by delivering miR-3679-5p, miR-6515-5p, and miR-6747-5p (Xu X. Y. et al., 2020). Interestingly, the circRNA and lncRNA expression profile was significantly altered in PDLSCs-exos during the osteogenic differentiation of PDLSCs, indicating that the exosomal non-coding RNAs might play a critical role in regulating PDLSCs osteogenesis (Xie et al., 2021). Collectively, PDLSC-Exos is beneficial to the maintenance of periodontal homeostasis by promoting proliferation, angiogenesis, and osteogenesis as well as regulating the inflammatory responses.

SCAP-Exos

SCAP-derived exosomes (SCAP-Exos) also show great potential for oral tissue regeneration. Zhuang et al. demonstrated that SCAP-Exos promoted the dentinogenesis of BMMSCs both *in vitro* and *in vivo*, indicating that SCAP-Exos might represent a potential therapeutic tool for dentine-pulp complex regeneration (Zhuang et al., 2020). Through injecting into the palatal gingival complex critical-size defects (CSD) of mice, SCAP-Exos significantly improved angiogenesis and soft tissue regeneration (Liu et al., 2021). In terms of mechanism, SCAP-Exos promoted filopodium formation, migration, and cytoskeletal reorganization of endothelial cells *via* delivering exosomal Cdc42. Wang et al. compared the piRNA expression profiles between SCAP-Exos and BMMSC-Exos (Wang M. et al., 2020). The differentially expressed piRNAs were found to be closely associated with many important biological functions such as catalytic activity, metabolic processes, cellular processes, binding, and biological regulation, suggesting that piRNAs might play a crucial role in regulating the molecular activities of exosomes.

GMSC-Exos

Although currently few studies are available regarding the potential therapeutic applications of GMSC-Exos, they hold a great promise for tissue regeneration. The pre-osteoblasts MC3T3-E1 treated with GMSC-Exos were found deep Alizarin red staining, increased ALP activity, and upregulated expression of osteogenic genes, suggesting that GMSC-Exos facilitated the osteogenic differentiation of MC3T3-E1 (Jiang and Xu 2020). The exosomes isolated from tumor necrosis factor-alpha (TNF-α) preconditioned GMSC-Exos induced the polarization of anti-inflammatory M2 macrophage by improving the secretion of GMSC-Exos and increased the exosomal expression of CD73 (Nakao et al., 2021). In a high-lipid microenvironment, GMSC-Exos suppressed lipid accumulation, transformed pro-inflammatory macrophages into anti-inflammatory phenotype,

and decrease the secretion and expression of inflammatory factors including IL-6, IL-1β, TNF-α, and cluster of differentiation 86 (Zhang Z. et al., 2021). Besides, GMSC-Exos presented anti-osteoclastogenic activity and suppressed inflammatory bone loss by delivering miR-1260b (Nakao et al., 2021). Rao et al. combined GMSC-Exos with biodegradable chitin conduits and injected the composite into the rat sciatic nerve defect model. The results showed that GMSC-Exos enhanced the proliferation of Schwann cells and the growth of the dorsal root ganglion neuron axon as well as promoting the formation of nerve fibers and myelin, which subsequently contributed to restoring motor skills, nerve conduction function, and muscle movement (Rao et al., 2019). GMSC-Exos also promoted healing of diabetic skin defects by facilitating re-epithelialization, collagen remodeling, angiogenesis, and nerve growth in a diabetic rat skin defect model (Shi et al., 2017). Combining GMSC-Exos with small intestinal submucosa-extracellular matrix promoted taste bud regeneration and tongue lingual papillae recovery in a rat tongue defect model (Zhang et al., 2019). We have summarized the currently available evidence regarding the potential clinical application of DSC-Exos (**Table 2**).

FUTURE PERSPECTIVES AND CHALLENGES

DSC-Exos hold great promise in tissue repair and regeneration as well as treating other diseases. However, to the best of our knowledge, there is still not enough evidence for evaluating and comparing the molecular differences, biological functions, and therapeutic applications among different types of DSC-Exos as well as between DSC-Exos and other MSCs-Exos. Compared to exosomes derived from other MSCs, DSC-Exos might possess their own advantages. For instance, DPSC-Exos have stronger immunomodulatory, anti-necrotic, and anti-apoptotic effects than BMMSC-Exos (Venugopal et al., 2018; Ji et al., 2019). The currently available evidence demonstrates that DSC-Exos might share similar limitations and weaknesses. Compared to the exosomes from BMMSCs and adipose tissue-derived MSCs, the isolation of a sufficient amount of DSCs-Exos is still an obstacle for hindering their therapeutic applications (Stanko et al., 2018). More importantly, compared to autologous BMMSC from bone marrow aspirate, DSCs are mainly collected from non-renewable exfoliated deciduous teeth, third molars, and teeth extracted by orthodontic treatment and irreversible periodontitis, or discarded tissues after dental surgery. Therefore, it is challenging to obtain DSC-Exos in time when they are needed. Only under the condition that the unnecessary tissues like exfoliated deciduous teeth and third molar were stored previously by a stable and long-term approach, the applications of DSC-Exos might be popularized on a large scale. In addition, there are a lack of standardized and accepted approaches for storage, transport and large-scale production of DSCs-Exos, which has significantly affected their clinical applications. Mover, determining the most appropriate dose of exosomes under different pathological conditions, avoiding the off-target effects, and ensuring sufficient biological safety of DSC-Exos need to be urgently addressed in the coming pre-clinical studies and/or clinical trials.

CONCLUSIONS

DSCs mainly exert their therapeutic effects by the secretion of exosomes *via* a paracrine mechanism. Compared to DSCs, DSC-Exos possess unique advantages such as high drug loading capacity, high specificity, low immunogenicity, excellent biocompatibility, easily obtainable, low side effects, and nanoscale size. In addition, DSC-Exos have been shown to regulate many important biological processes including intercellular communication, anti-inflammation, osteogenesis, angiogenesis, immunomodulation, nurturing neurons, and promoting tumor cell apoptosis. Although there are still many barriers to translation into clinical practice, DSC-Exos is emerging as a promising and practical therapeutic approach for tissue repair and regeneration.

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Conceptualization and funding acquisition, XZ and LC; writing-original draft and editing, ZM, HC, YY, ZH, WS, XZ, and LC; All authors have read and agreed to the submitted version of the manuscript.

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miR-6807-5p Inhibited the Odontogenic Differentiation of Human Dental Pulp Stem Cells Through Directly Targeting METTL7A

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Background: Tooth tissue regeneration mediated by mesenchymal stem cells (MSCs) has become the most ideal treatment. Although the known regulatory mechanism and some achievements have been discovered, directional differentiation cannot effectively induce regeneration of tooth tissue. In this study, we intended to explore the function and mechanism of miR-6807-5p and its target gene METTL7A in odontogenic differentiation.

Methods: In this study, human dental pulp stem cells (DPSCs) were used. Alkaline phosphatase (ALP), Alizarin red staining (ARS), and calcium ion quantification were used to detect the odontogenic differentiation of miR-6807-5p and METTL7A. Real-time RT-PCR, western blot, dual-luciferase reporter assay, and pull-down assay with biotinylated miRNA were used to confirm that METTL7A was the downstream gene of miR-6807-5p. Protein mass spectrometry and co-immunoprecipitation (Co-IP) were used to detect that SNRNP200 was the co-binding protein of METTL7A.

Results: After mineralized induction, the odontogenic differentiation was enhanced in the miR-6807-5p-knockdown group and weakened in the miR-6807-5p-overexpressed group compared with the control group. METTL7A was the downstream target of miR-6807-5p. After mineralized induction, the odontogenic differentiation was weakened in the METTL7A-knockdown group and enhanced in the METTL7A-overexpressed group compared with the control group. SNRNP200 was the co-binding protein of METTL7A. The knockdown of SNRNP200 inhibited the odontogenic differentiation of DPSCs.

Conclusion: This study verified that miR-6807-5p inhibited the odontogenic differentiation of DPSCs. The binding site of miR-6807-5p was the 3′UTR region of METTL7A, which was silenced by miR-6807-5p. METTL7A promoted the odontogenic differentiation of DPSCs. SNRNP200, a co-binding protein of METTL7A, promoted the odontogenic differentiation of DPSCs.

Keywords: miR-6807-5p, human dental pulp stem cells (DPSCs), METTL7A, odontogenic differentiation, SNRNP200, mineralized induction

INTRODUCTION

Tooth loss is a common and frequently occurring disease in humans. The function of mastication, swallowing, aesthetics, and pronunciation are seriously affected. At present, the common repair methods are mainly non-physiological repair methods, including fixed dentures, removable dentures, and implant dentures, which can restore the shape and some functions of the teeth. But they still have various problems. So the ideal repair method is physiological tooth regeneration, including periodontal ligament regeneration, pulp–dentin complex regeneration, bioengineered tooth root (bio-root) regeneration, and whole-tooth regeneration (Oshima and Tsuji, 2015; Gao et al., 2016; Wu et al., 2019). For bio-root regeneration, the key factors which affect its effect include seed cells, scaffold materials, growth factors, etc. (Ramanathan et al., 2018). Studies verified that dental pulp stem cells (DPSCs) are good seed cells for regeneration of bio-root. Compared with periodontal ligament stem cells (PDLSCs) and bone marrow stem cells (BMSCs), DPSCs have a stronger proliferation ability, mineralization ability, and anti-aging ability (Yu et al., 2007; Huang et al., 2009; Iezzi et al., 2019; Ma et al., 2019). Although some research achievements have been made, the known regulatory mechanisms cannot effectively induce the odontogenic differentiation of mesenchymal stem cells (MSCs). It inhibited the application for bio-root regeneration. Therefore, it is very important to further clarify the regulation mechanism of odontogenic differentiation in DPSCs.

Epigenetics refers to the heritability change of differential gene expression under the condition of the same DNA sequence, including DNA methylation, histone modification, RNA methylation, and non-coding RNA (ncRNA) (Mortada and Mortada, 2018; Sui et al., 2020). Among them, the modification of non-coding RNA performs an significant role in the odontogenic differentiation of MSCs (Ju et al., 2019; Wang et al., 2019; Zheng et al., 2019). miRNA is a small non-coding RNA with a length of about 22 nucleotides. The function of miRNA was to silence the downstream target gene by binding to its 3′UTR region (Strubberg and Madison, 2017). Previous investigations show that miRNA plays an important role in the odontogenic differentiation of MSCs (Bao et al., 2020; Liu et al., 2020). Our studies have found that there is a significant difference of some miRNA between dental tissue-derived MSCs including DPSCs and non-dental tissue-derived MSCs. And the expression of miR-6807-5p in dental tissue-derived MSCs was significantly higher than that in non-dental tissue-derived MSCs. An existing study indicates that miR-6807-5p mainly involved in the inflammatory process by downregulating drug metabolism cytochrome P450 (Kugler et al., 2020). In addition, miR-6807-5p was also one of the biomarkers of gastric cancer (Iwasaki et al., 2019). However, the role of miR-6807-5p in odontogenic differentiation of DPSCs has not been elucidated. In this study, we will explore the function and mechanism of miR-6807-5p in the odontogenic differentiation of DPSCs.

RNA methylation is a reverse modification at the RNA level, which is extremely important in epigenetic modification (Li et al., 2020). And m6A modification is the most abundant RNA internal modification in eukaryotic cells (Chen et al., 2021). m6A methylation is mediated by a multi-protein complex including METTL3, METTL14, and WTAP, and demethylase is mainly mediated by two m6A demethylated transferases FTO and ALKBH5 (Cao et al., 2016). A previous study shows that m6A modification plays an important role in the formation of the root of the tooth (Sheng et al., 2021). Methyltransferase like 7A (METTL7A) belongs to the human methyltransferase-like protein (METTL) family, which is characterized by the presence of the *S*-adenosylmethionine domain (Ignatova et al., 2019). It has been found that METTL7A promoted the viability and osteogenic differentiation of BMSCs by changing the methylation state of related genes (Lee et al., 2021). An existing study indicates that METTL7A is mainly involved in the formation of lipid droplets and mental diseases (Zhou et al., 2017). The differential expression of METTL7A leads to frontotemporal dementia (Taskesen et al., 2017). In addition, the abnormal expression of METTL7A may affect thyroid cancer, choriocarcinoma, and breast cancer (Zhou et al., 2017; Jun et al., 2020; Yang et al., 2021). By software prediction, METTL7A may be the downstream target gene of miR-6807-5p. Whether METTL7A is involved in miR-6807-5p regulation and influenced the odontogenic differentiation of DPSCs is unclear and needs to be further studied.

In this study, we intend to explore the function and mechanism of miR-6807-5p and METTL7A in the odontogenic differentiation of DPSCs. Our study found that miR-6807-5p inhibited the odontogenic differentiation of DPSCs through directly targeting METTL7A.

MATERIALS AND METHODS

Cell Cultures

The DPSCs were obtained from the third molars extracted from patients in Beijing Stomatological Hospital Affiliated to Capital Medical University. Informed consent of patients was obtained. The extracted third molars were immersed in sterile PBS and isolated under sterile conditions as in previous studies (Shi et al., 2019). Pulp tissue was removed from the broken tooth. The pulp tissue was cut up with scissors and then rinsed with penicillin–streptomycin. After digestion with collagenase type I (Invitrogen, Carlsbad, CA, United States) and dispase (Invitrogen) for 1 h at 37°C, the pulp tissue was inoculated into a dish. The fluid was changed 7 days later. Passages 3–5 of DPSCs were used in this study. Human embryonic kidney 293T cells (HEK293T, American Type Culture Collection, Manassas, VA, United States) were cultured in DMEM complete medium containing 10% FBS (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Invitrogen).

Construction of Plasmid and Transfection of Virus

The lentiviruses of Consh, METTL7Ash, miR-6807-5p inhibitor/mimic, and SNRNP200sh were purchased from the GenePharma Company (Suzhou, China). An HA tag was combined with the full-length sequence of human METTL7A

and constructed on the retroviral vector pQCXIN (Taihe Biotechnology Co., Ltd., Beijing, China). The HA-METTL7A plasmid and package vectors were transfected into 293T cells. After 24 h, 100 μl sodium butyrate was added. After 72 h, the retrovirus was collected. As in the previous study (Yang et al., 2020a), DPSCs were plated overnight. The DPSCs were transfected with the lentivirus for 12 h. And the transfected DPSCs were purified by puromycin for 3 days. The DPSCs were transfected with retrovirus for 12 h. And the transfected DPSCs were purified by G418 for 10 days.

RT-PCR and Real-Time RT-PCR

The TRIzol reagent (Invitrogen) was used to isolate all RNAs from DPSCs. The RT-PCR experiment was performed according to the instructions of the reverse-transcription kit (Vazyme #R333, Nanjing, China). And real-time RT-PCR was used to evaluate the gene expression based on the methods described in a previous study (Yang et al., 2020c). **Supplementary Table 1** shows the primers for specific genes.

Western Blot

As in the previous method (Yang et al., 2020b), the RIPA buffer was used to lyse the cells. After quantitative denaturation, SDS-PAGE and appropriate antibody were used to detect the expression of related proteins. The primary antibody is a monoclonal antibody: anti-METTL7A (Cat No. ab79207, Abcam, Cambridge, United Kingdom), anti-SNRNP200 (Cat No. ab241589, Abcam), anti-BSP (Cat No. ab52128, Abcam), anti-OCN (Cat No. ab133612, Abcam), anti-OSX (Cat No. ab209484, Abcam). The housekeeping protein was detected with monoclonal glyceraldehyde 3-phosphate dehydrogenase antibody (GAPDH Cat No. C1312, Applygen Technologies, Beijing, China) and histone H3 antibody (Cat No. SC-56616, Santa Cruz Biotechnology, Santa Cruz, CA, United States).

Alkaline Phosphatase Activity Assay and Alizarin Red Staining Detection

DPSCs were cultured in a mineralized-inducing medium. After 5 days of induction, ALP activity was detected by an ALP activity kit (Sigma-Aldrich, St. Louis, MO, United States). After 2 weeks of induction, the mineralization was detected by ARS and calcium ion quantification. These experiments followed the same protocols as the previous ones (Yan et al., 2019).

Co-immunoprecipitation

As in the previous experimental method (Yang et al., 2020a), we used the IP lysis buffer (Invitrogen) to lyse the cells. Cell lysates were incubated with a specific antibody for 4 h and then added with protein A/G Sepharose (Santa Cruz, Biotechnology, United States) or HA tag beads (Thermo Scientific Pierce, United States) and rotated overnight at 4°C. With the help of magnetic support, the beads were washed three times with PBS and were resuspended in an SDS-PAGE loading buffer for western blotting analysis using a corresponding antibody. The primary antibodies were as follows: anti-SNRNP200 (Cat No. ab241589, Abcam), anti-METTL7A (Cat No. ab79207, Abcam), anti-IgG (Cat No. bs-0297P, Bioss, Beijing, China).

Pull-Down Assay With Biotinylated miRNA

As in a previous experimental protocol (Wang et al., 2015), the DPSCs were transfected with biotinylated miR-6807-5p (50 nM). The transfected DPSCs were harvested after 48 h. The lysates were incubated with M-280 streptavidin magnetic beads (Sigma-Aldrich) at 4°C for 2 h. After that, part of them was added with TRIzol to extract RNA. And then the gene expression was detected by real-time RT-PCR. Another part of them was added with a loading buffer to extract protein. The possible co-binding protein was detected by protein mass spectrometry.

Dual-Luciferase Reporter Assay

Wild-type or mutant-type METTL7A 3′UTR-Luc reporter constructs and miR-6807-5p mimic or miR-NC were co-transfected into 293T cells. After 48 h, the 293T cells were harvested. Then, the dual-luciferase reporter assay kit was used to detect the activity (E1910, Promega, Madison, WI, United States).

Mass Spectrometry Analysis

The protein was separated by SDS-PAGE. And then it was stained with a Fast Silver Stain Kit (Beyotime Biotechnology, Shanghai, China). Finally, the gel band was analyzed by mass spectrometry.

Transplantation in Nude Mice

The animal study was authorized by the Animal Care Institution and carried out according to the animal experiment ordinance of the Beijing Stomatological Hospital Affiliated to Capital Medical University. Nude mice (8-week-old female, nu/nu) were purchased from the Institute of Animal Science Research of the Vital River Co., Ltd. AS in the previous study (Yang et al., 2020c), 2×10^6 cells of DPSCs-miR-6807-5p inhibitor and DPSCs-Consh were mixed with 20 mg HA/tricalcium phosphate (Engineering Research Center for biomaterials, Chengdu, China). Then, all of them were transplanted subcutaneously into the back of nude mice. After 8 weeks, samples were collected and then stained with HE. Image Pro Plus analysis was used to calculate the area of mineralized tissue.

Immunohistochemistry Staining

As in the previous study (Yang et al., 2020c), 5-μm tissue sections were decarbonized and rehydrated. The tissue sections were treated with the antigen retrieval method, followed by quenching the endogenous peroxidase activity for 10 min. The sections were incubated with the first antibody at 4°C overnight and finally stained. The main antibodies were as follows: anti-DSPP (Cat No. bs-10316R, Bioss), anti-DMP1 (Cat No. bs-12359R, Bioss), anti-BSP (Cat. No. ab52128, Abcam).

Statistical Analyses

SPSS version 22 statistical software was used for statistical calculation. Statistical significance was determined by Student's *t*-test or Tukey's *post hoc* tests used after one-way ANOVA, with $p \leq 0.05$ considered significant.

RESULTS

miR-6807-5p Inhibited the Odontogenic Differentiation of Dental Pulp Stem Cells

In a previous study, the DPSCs have been validated by flow cytometry analysis (Yi et al., 2017). In order to clarify the function of miR-6807-5p in DPSCs, we transfected DPSCs with the lentivirus of Consh and miR-6807-5p inhibitor. After the infected DPSCs were treated with puromycin for 3 days, the expression of miR-6807-5p in the miR-6807-5p inhibitor group was lower than that in the control group detected by real-time RT-PCR (**Figure 1A**). Then, the DPSCs were cultured in mineralized-inducing mediums. After 5 days of induction, it was found that the ALP activity in the miR-6807-5p inhibitor group was higher than that in the control group (**Figure 1B**). After 2 weeks of induction, the results of ARS and calcium ion quantification showed that the mineralization was significantly increased in the miR-6807-5p inhibitor compared with the control group (**Figures 1C,D**). After 1 week of induction, the expressions of BSP and OCN in the miR-6807-5p inhibitor group were significantly higher than those in the control group detected by western blot (**Figure 1E**). The western blot results showed that OSX was also significantly higher in the miR-6807-5p inhibitor group than in the control group (**Figure 1F**).

Then we transfected DPSCs with the lentivirus of Consh and miR-6807-5p mimic. After 3 days of puromycin screening, the expression of miR-6807-5p was significantly increased in the miR-6807-5p mimic group compared with the control group detected by real-time RT-PCR (**Figure 1G**). Then, the transfected DPSCs were cultured in mineralized-inducing mediums. After 5 days of induction, the ALP activity in the miR-6807-5p mimic group was significantly lower than that in the control group (**Figure 1H**). After 2 weeks of induction, ARS and calcium ion quantification results showed that the mineralization in the miR-6807-5p mimic group was significantly lower than that in the control group (**Figures 1I,J**). After 1 week of induction, the western blot results showed that the expressions of BSP and OCN in the miR-6807-5p mimic group were significantly lower than those in the control group (**Figure 1K**). The expression of OSX was also significantly lower in the miR-6807-5p mimic group than in the control group as detected by western blot (**Figure 1L**).

Moreover, the DPSCs transfected with the lentivirus of Consh and miR-6807-5p inhibitor were mixed with HA/TCP material and then transplanted into nude mice for 8 weeks. The results of HE staining and quantitative measurements of the mineralization tissue area showed that the amount of mineralization tissue in the miR-6807-5p inhibitor group was significantly increased compared with that in the control group (**Figures 2A,B**). The immunohistochemical staining results showed that the expressions of DSPP, DMP1, and BSP in the miR-6807-5p inhibitor group were significantly increased compared with the those in the control group (**Figures 2C,E,G**). The quantitative measurements of immunohistochemical staining results showed that the expressions of the DSPP, DMP1, and BSP in the miR-6807-5p inhibitor group were increased compared with those in the control group (**Figures 2D,F,H**).

METTL7A Was Identified as the Downstream Target Gene of miR-6807-5p

In order to further clarify the mechanism of miR-6807-5p in the odontogenic differentiation, we performed the pull-down assay with biotinylated miRNA and mass spectrometry analysis. The results showed that there were 10 possible co-binding proteins of miR-6807-5p including MYH9, PLEC, and MYO1C (**Supplementary Table 2**).

Then, the downstream target genes of miR-6807-5p were predicted by miRBase software. It was predicted that GREM1, TRMT10C, METTL1, and METTL7A may be the downstream target genes of miR-6807-5p. Then, these candidate genes were investigated by real-time RT-PCR. The real-time RT-PCR results showed that only METTL7A expression was increased in the miR-6807-5p inhibitor group compared with the control group (**Figure 3A** and **Supplementary Figure 1**). Western blot results confirmed that the expression of METTL7A was increased in the miR-6807-5p inhibitor group (**Figure 3B**). And further results showed that METTL7A was decreased in the miR-6807-5p mimic group compared with the control group detected by real-time RT-PCR and western blot (**Figures 3C,D**). Further, the binding sites of miR-6807-5p in the 3′UTR of METTL7A were predicted by miRBase software (**Figure 3E**). Then, the double-luciferase reporter assay was used and verified the binding site of miR-6807-5p in the 3′UTR of METTL7A (**Figure 3F**). Moreover, after pull-down with biotinylated miRNA, the real-time RT-PCR results showed that the amount of METTL7A in the Bio-miR-6807-5p group was enhanced compared with that in the control group (**Figure 3G**).

METTL7A Promoted the Odontogenic Differentiation of Dental Pulp Stem Cells

In order to detect the function of METTL7A in the process of odontogenic differentiation of DPSCs, we transfected the DPSCs with the lentivirus of Consh and METTL7Ash. After the transfected DPSCs were purified by puromycin for 3 days, real-time RT-PCR and western blot results showed that the expression of METTL7A in DPSCs-METTL7Ash was lower than that in DPSCs-Consh (**Figures 4A,B**). Then, the transfected DPSCs were cultured in mineralized-inducing mediums. After 5 days of induction, the ALP activity in the DPSCs-METTL7Ash group was lower than that in DPSCs-Consh group (**Figure 4C**). After 2 weeks of induction, ARS and calcium ion quantification results showed that the mineralization in the DPSCs-METTL7Ash group was lower than that in the DPSCs-Consh group (**Figures 4D,E**). After 1 week of induction, western blot results showed that the expressions of BSP and OCN in the DPSCs-METTL7Ash group were significantly lower than those in DPSCs-Consh group (**Figure 4F**). Western blot results also showed that the expression of OSX in the DPSCs-METTL7Ash group was lower than that in the DPSCs-Consh group (**Figure 4G**).

Then, we transfected DPSCs with the retrovirus of vector and HA-METTL7A. After the transfected DPSCs were purified by G418 for 10 days, the expression of METTL7A was higher in

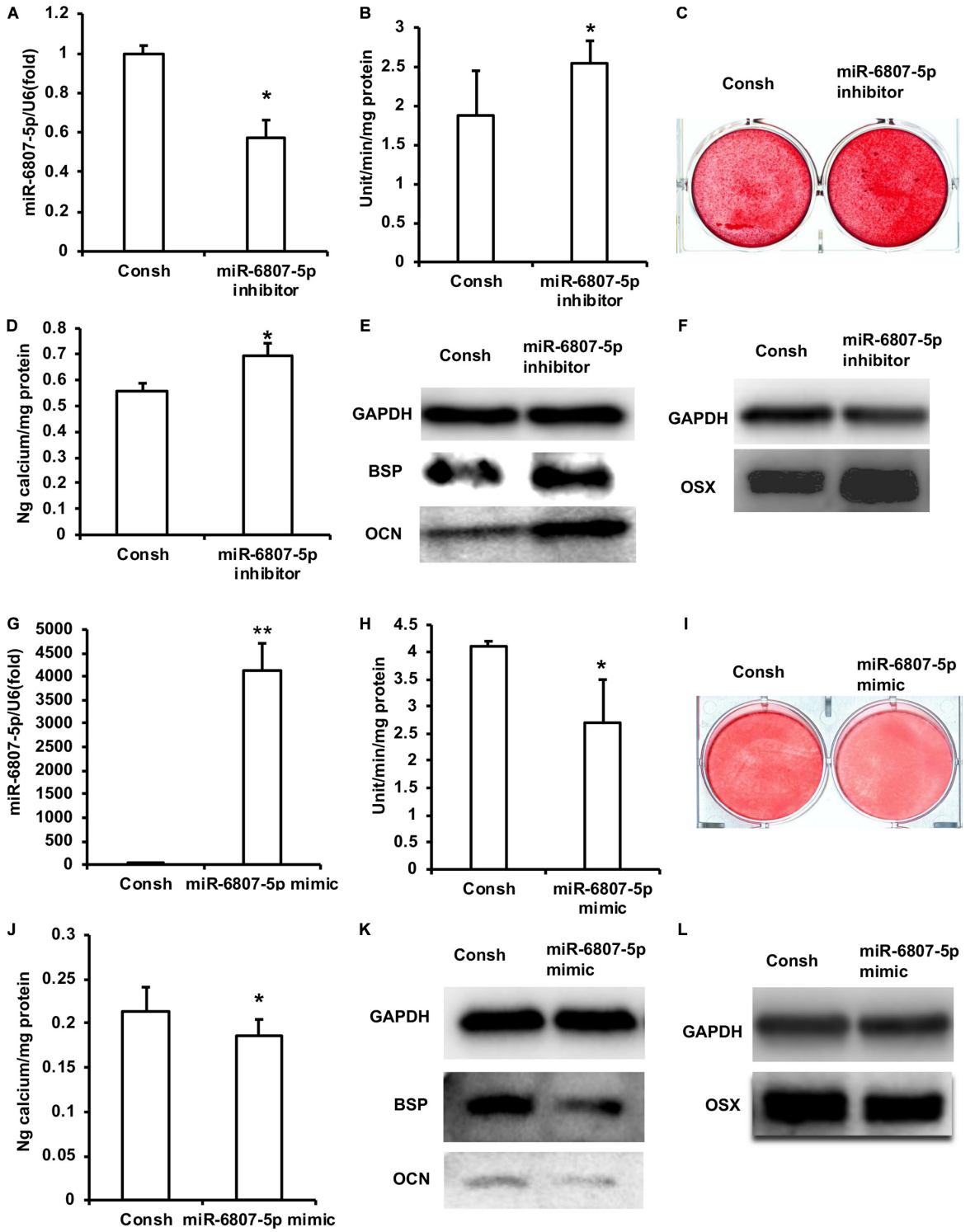

FIGURE 1 | miR-6807-5p inhibited the odontogenic differentiation of DPSCs. **(A)** The expression of miR-6807-5p was detected by real-time RT-PCR. **(B)** ALP activity after 5 days of mineralized induction. **(C,D)** ARS and Calcium ion quantification results after 2 weeks of mineralized induction. **(E)** After 1 week of mineralized induction, the expressions of BSP and OCN were detected by western blot. **(F)** The expression of OSX was detected by western blot. **(G)** The expression of miR-6807-5p was detected by real-time RT-PCR. **(H)** ALP activity after 5 days of induction. **(I,J)** ARS and calcium ion quantitative results after 2 weeks of induction. **(K)** After 1 week of induction, the expressions of BSP and OCN were detected by western blot. **(L)** The expression of OSX was detected by western blot. GAPDH was used as internal control. Student's *t*-test was performed to determine statistical significance. All error bars represent the standard deviation ($n = 3$). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

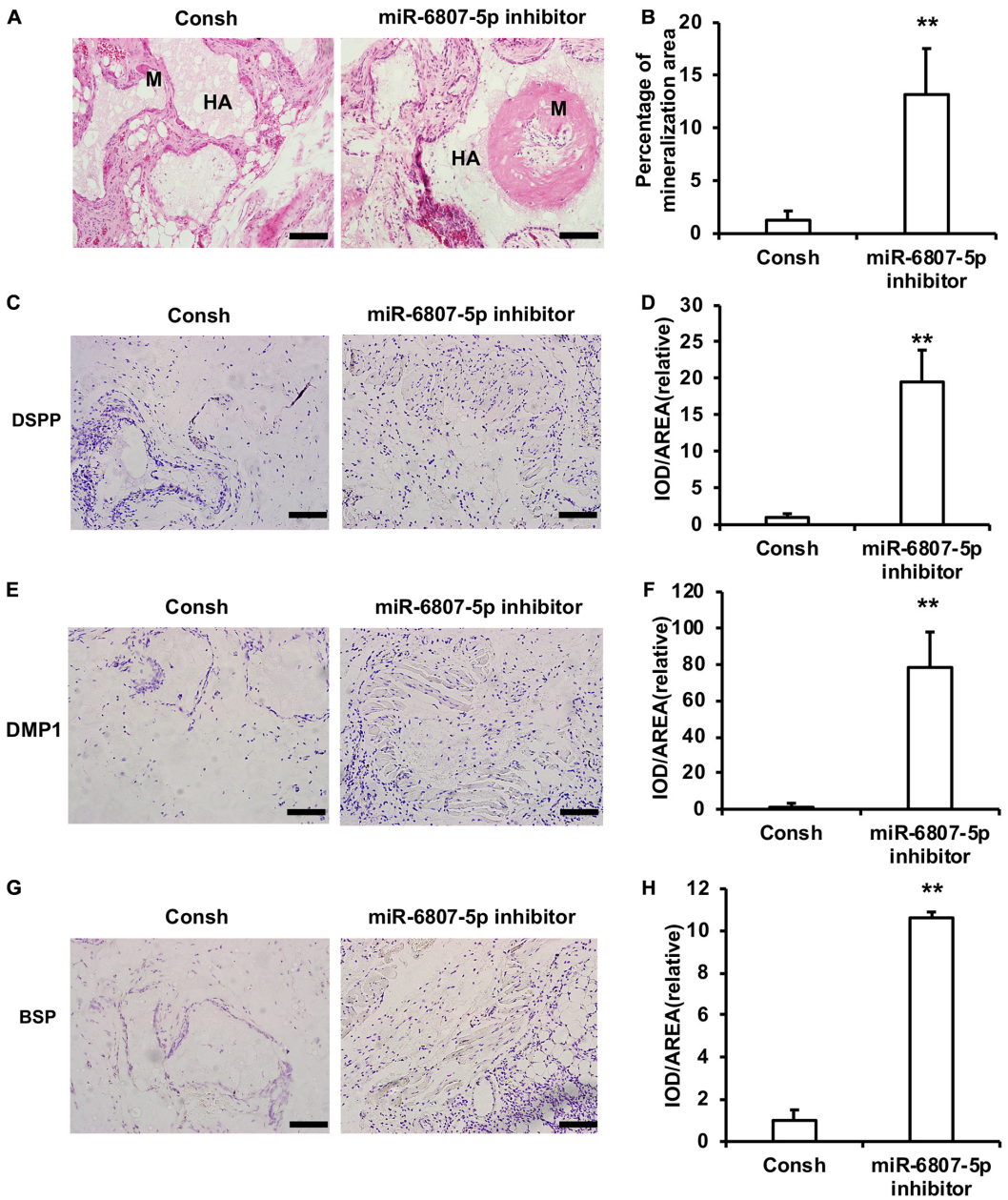

FIGURE 2 | miR-6807-5p inhibited the mineralization tissue formation mediated by DPSCs *in vivo*. **(A)** The result of HE staining showed the mineralized tissue. Scale bar: 100 μm. M: Mineralized tissue, HA: Hydroxyapatite tricalcium carrier. **(B)** The quantitative analysis of HE staining. **(C–H)** Immunohistochemical staining and quantitative analysis of DSPP **(C,D)**, DMP1 **(E,F)**, and BSP **(G,H)**. Student's *t*-test was performed to determine statistical significance. All error bars represent the standard deviation ($n = 5$). $^{**}p \leq 0.01$.

the DPSCs-HA-METTL7A than in the DPSCs-Vector detected by real-time RT-PCR and western blot (**Figures 4H,I**). Then, the transfected DPSCs were cultured in mineralized-inducing mediums. After 5 days of induction, the results of ALP activity showed that the mineralization in the DPSCs-HA-METTL7A was significantly higher than that in the DPSCs-Vector (**Figure 4J**). After 2 weeks of induction, ARS and calcium ion quantification results revealed that the overexpression of METTL7A promoted the mineralization of DPSCs compared with the control group (**Figures 4K,L**). After 1 week of induction, western blot results showed that the expressions of BSP and OCN in the DPSCs-HA-METTL7A group were significantly higher than those in the DPSCs-Vector group (**Figure 4M**). Western blot results also showed that the expression of OSX in the HA-METTL7A group was significantly higher than that in the control group (**Figure 4N**).

In order to further verify that miR-6807-5p affected the odontogenic differentiation of DPSCs by silencing METTL7A, we

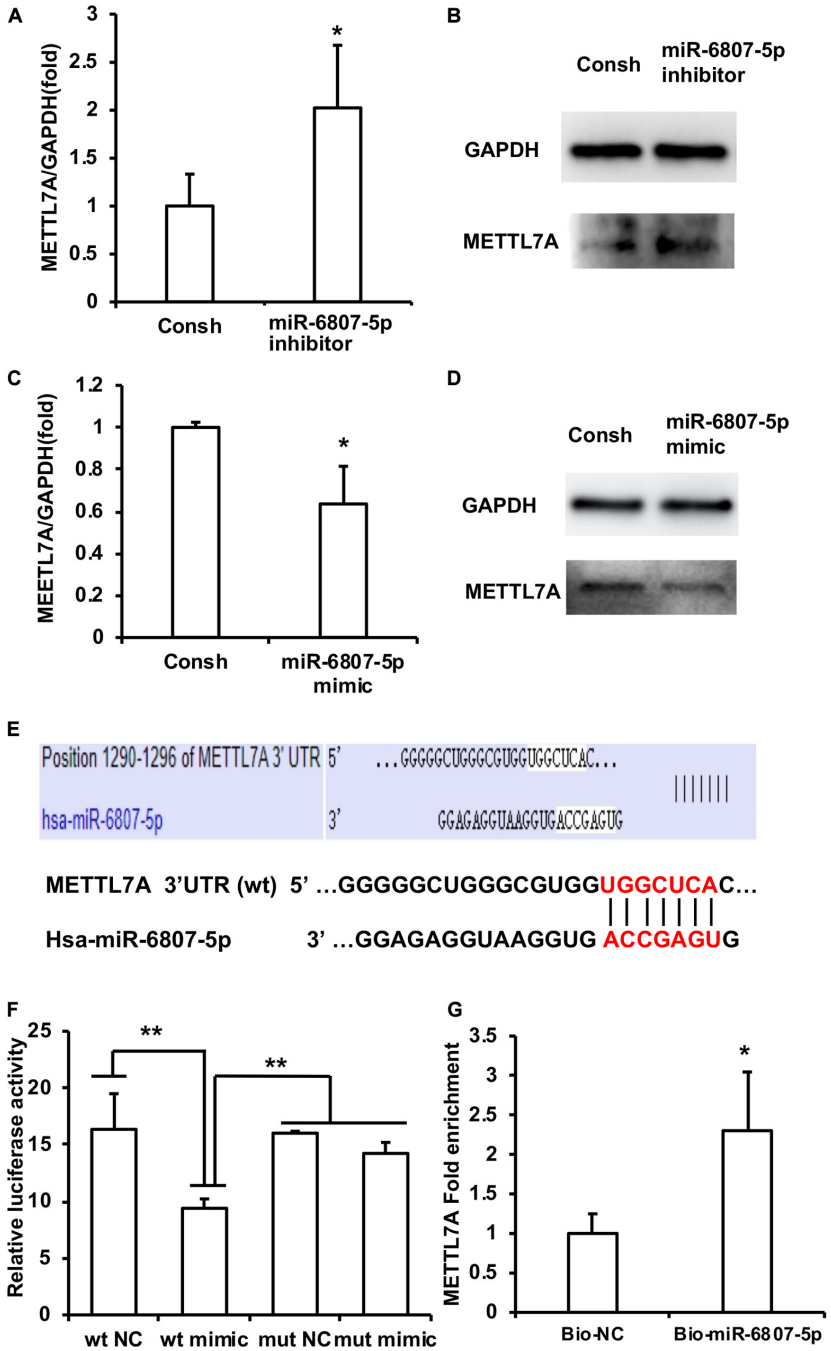

FIGURE 3 | METTL7A was identified as the downstream target gene of miR-6807-5p. **(A,B)** METTL7A was promoted in the miR-6807-5p inhibitor group detected by real-time RT-PCR and western blot. **(C,D)** METTL7A was repressed in the miR-6807-5p mimic group detected by real-time RT-PCR and western blot. **(E)** The binding site of miR-6807-5p in 3′UTR of METTL7A. **(F)** The miR-6807-5p binding to the 3′UTR of METTL7A was verified by the dual-luciferase reporter assay. **(G)** Results of the pull-down assay with biotinylated miRNA. GAPDH was used as internal control. Student's *t*-test or Tukey's *post hoc* tests used after one-way ANOVA were performed to determine statistical significance. All error bars represent the standard deviation ($n = 3$). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

designed a rescue experiment. The DPSCs were transfected with the lentivirus and retrovirus of Consh + Vector, Vector + miR-6807-5p mimic, and miR-6807-5p mimic + HA-METTL7A. Then, the transfected DPSCs were cultured in mineralized-inducing mediums. After 5 days of induction, the ALP activity in the DPSCs-Vector + miR-6807-5p mimic group was lower than that in the DPSCs-Consh + Vector and DPSCs-miR-6807-5p mimic + HA-METTL7A groups. There is no significant difference between the DPSCs-Consh + Vector group and the DPSCs-miR-6807-5p mimic + HA-METTL7A group

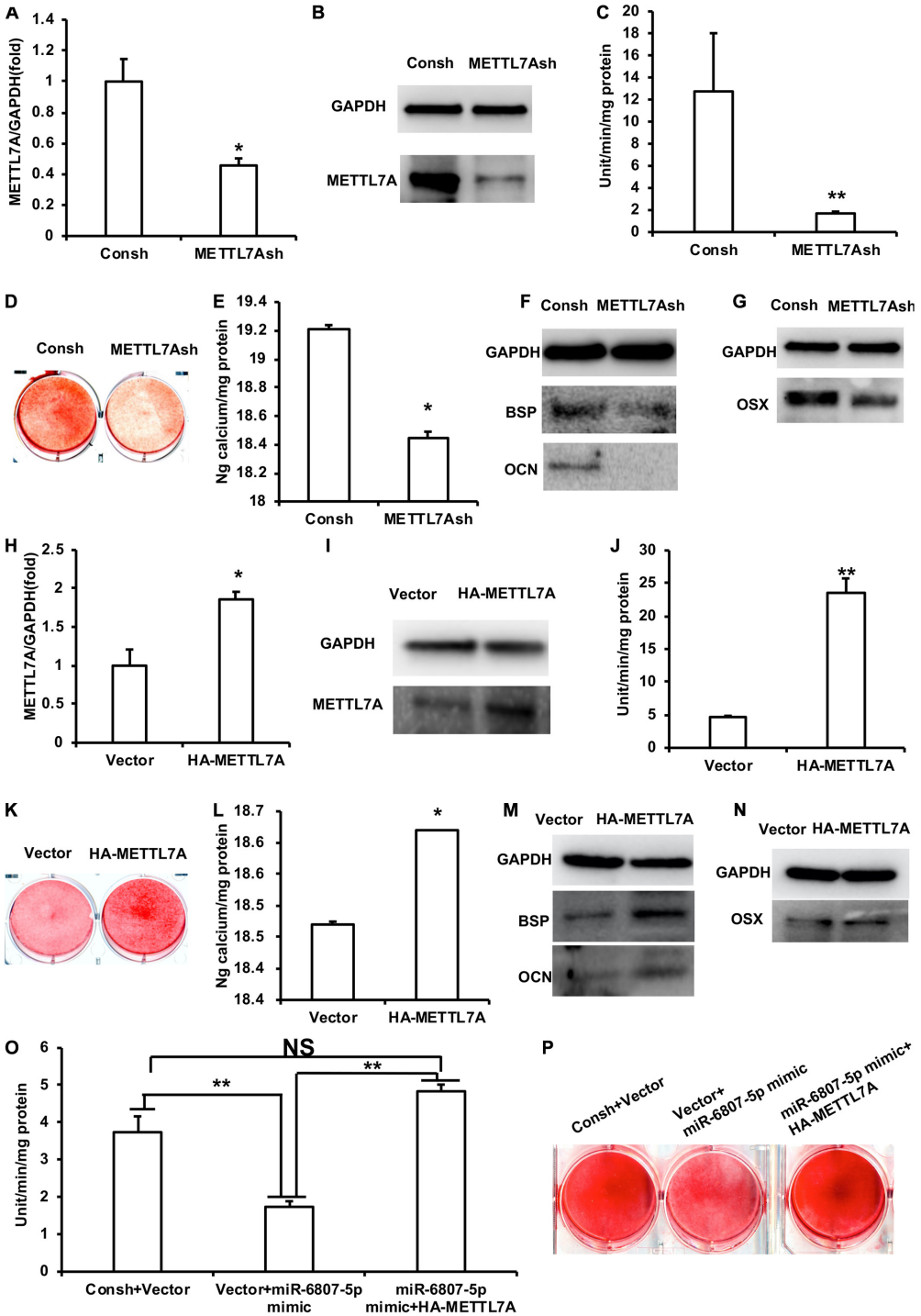

FIGURE 4 | METTL7A promoted the odontogenic differentiation of DPSCs. **(A,B)** The expression of METTL7A was detected by real-time RT-PCR and western blot. **(C)** ALP activity after 5 days of mineralized induction. **(D,E)** ARS and calcium ion quantification after 2 weeks of induction. **(F)** The expressions of BSP and OCN were detected by western blot after 1 week of induction. **(G)** The expression of OSX was weakened in the METTL7Ash group compared with that in the control group detected by western blot. **(H,I)** The expression of METTL7A was detected by real-time RT-PCR and western blot. **(J)** ALP activity after 5 days of mineralized induction. **(K,L)** ARS and calcium ion quantification after 2 weeks of mineralized induction. **(M)** The expressions of BSP and OCN were detected by western blot after 1 week of induction. **(N)** The expression of OSX was detected by western blot. **(O)** ALP activity of the Consh + Vector, Vector + miR-6807-5p mimic, and miR-6807-5p mimic + HA-METTL7A groups after 5 days of mineralized induction. **(P)** ARS of the Consh + Vector, Vector + miR-6807-5p mimic, and miR-6807-5p mimic + HA-METTL7A groups after 2 weeks of mineralized induction. GAPDH was used as internal control. Student's *t*-test or Tukey's *post hoc* tests used after one-way ANOVA was performed to determine statistical significance. All error bars represent the standard deviation ($n = 3$). $^{*}p \leq 0.05$, $^{**}p \leq 0.01$.

(**Figure 4O**). After 2 weeks of induction, ARS results revealed that the mineralization in the DPSCs-Vector + miR-6807-5p mimic group was lower than that in the DPSCs-Consh + Vector and DPSCs-miR-6807-5p mimic + HA-METTL7A groups. And there is no significant difference between the DPSCs-Consh + Vector group and the DPSCs-miR-6807-5p mimic + HA-METTL7A group (**Figure 4P**).

SNRNP200 Was Identified as a Co-binding Protein of METTL7A and Promoted the Odontogenic Differentiation of Dental Pulp Stem Cells

In order to further study the mechanism of METTL7A in the odontogenic differentiation of DPSCs, protein mass spectrometry was performed to identify the binding partner of METTL7A. The protein mass spectrometry results showed that there are 11 possible co-binding proteins of METTL7A including SNRNP200, AGO1, WDR1, and so on (**Supplementary Table 3**). Then we selected the candidate co-binding protein of METTL7A, SNRNP200, by analyzing the molecule weight of protein and the amount of peptide. Further, the Co-IP results showed that the association of SNRNP200 and METTL7A was decreased in the DPSCs-METTL7Ash group compared with that in the DPSCs-Consh group (**Figure 5A**). And the Co-IP assay also showed that the association of SNRNP200 and METTL7A was increased in the DPSCs-HA-METTL7A group compared with that in the DPSCs-Vector group (**Figure 5B**).

The DPSCs were transfected with the lentivirus of Consh and SNRNP200sh. After the transfected DPSCs were purified by puromycin for 3 days, real-time RT-PCR and western blot results showed that the expression of SNRNP200 was weakened in the DPSCs-SNRNP200sh group compared with that in the control group (**Figures 5C,D**). Then, the transfected DPSCs were cultured in mineralized-inducing mediums. After 5 days of induction, the ALP activity in the DPSCs-SNRNP200sh was significantly repressed compared with that in the control group (**Figure 5E**). After 2 weeks of induction, the ARS results revealed that the mineralization in the DPSCs-SNRNP200sh group was significantly repressed compared with that in the control group (**Figure 5F**).

DISCUSSION

With the development of tissue engineering, tooth tissue regeneration mediated by MSCs has become the most promising and ideal repair method. Therefore, it is very important to clarify the regulation method for odontogenic differentiation in seed cells.

In this study, ALP, ARS, and Calcium ion quantification showed that miR-6807-5p inhibited the mineralization of DPSCs. Western blot results showed that BSP and OCN, key indicators of osteogenesis/odontogenesis, were inhibited by miR-6807-5p. BSP localizes to the matrix of mineralization and involved in the formation of hydroxyapatite crystal in the bone and tooth (Zhang et al., 2009). OCN appears as an indicator at the end stage of osteoblast differentiation. It can regulate calcium homeostasis and osteogenic mineralization (An et al., 2016). The process of odontogenesis is highly similar to osteogenesis (Bosshardt, 2005). And the process of cementum formation by cementoblasts is similar to that of bone formation. In addition, to form mineralized nodules *in vitro*, cementoblasts express genes such as BSP and OCN (D'Errico et al., 2000; Wang et al., 2016). These results confirmed that miR-6807-5p inhibited the odontogenic differentiation of DPSCs. In order to investigate the expression of the key transcription factor of odontogenic differentiation, we detected the expression of OSX in DPSCs. OSX, also known as Sp7, is a key transcription factor in the osteogenic/odontogenic differentiation of MSCs. Studies revealed that OSX-inactivated mice had no bone formation (Nakashima et al., 2002; Baek and Kim, 2011). In this study, we found that miR-6807-5p inhibited the expression of OSX. In order to further verify, the function of miR-6807-5p, we performed the experiment of transplantation in nude mice. The *in vivo* result showed that miR-6807-5p inhibited the mineralization tissue formation and the expressions of DSPP and DMP1. DSPP and DMP1, two major phosphoproteins of non-collagenous proteins, can regulate dentin regulation (Liu Z. et al., 2021). DSPP and DMP1 are highly seen in odontoblast and dentine during tooth development and formation (Liu M.M. et al., 2021). Therefore, these results confirmed that miR-6807-5p inhibited the odontogenic differentiation of DPSCs.

In order to further study the mechanism of miR-6807-5p in the odontogenic differentiation of DPSCs, we performed protein mass spectrometry to find the co-binding protein of miR-6807-5p. The results identified that there were 10 possible co-binding proteins of miR-6807-5p including MYH9 and MYO1C. MYH9 and MYO1C are associated with bone formation and absorption (Desh et al., 2014; Kanzaki et al., 2017). Existing studies have not reported these proteins related to tooth formation. Thus, the role of the candidate co-binding protein of miR-6807-5p in odontogenic differentiation needs to be further studied.

Then we predicted the target genes of miR-6807-5p to further explore the mechanism of miR-6807-5p in odontogenic differentiation of DPSCs and identified four possible downstream targets including GREM1, TRMT10C, METTL1, and METTL7A. GREM1, an antagonist of bone morphogenetic protein-2 (BMP-2), can specifically bind to BMP-2 to block its function. It is necessary for bone development and maintenance of bone homeostasis (Canalis et al., 2012). TRMT10C, which encodes the precursor of the mitochondrial ribonuclease P subunit, is involved in the transcription of tRNA, RNA processing, and mitochondrial respiration (Karasik et al., 2021). METTL1 plays an important role in the regulation of mRNA output, splicing, and translation by affecting the modification of m7G RNA (Deng et al., 2020). METTL7A, methyltransferase-like 7A, is an integrated membrane protein anchored to the endoplasmic reticulum. Recent studies have found that METTL7A affected the survival ability and osteogenic differentiation of BMSCs by affecting the methylation status of some related genes (Lee et al., 2021). METTL7A is also a tumor suppressor gene with decreased expression in osteosarcoma (Jia et al., 2021). Moreover, these gene expressions were detected by real-time RT-PCR and western blot. It was found that only METTL7A was upregulated

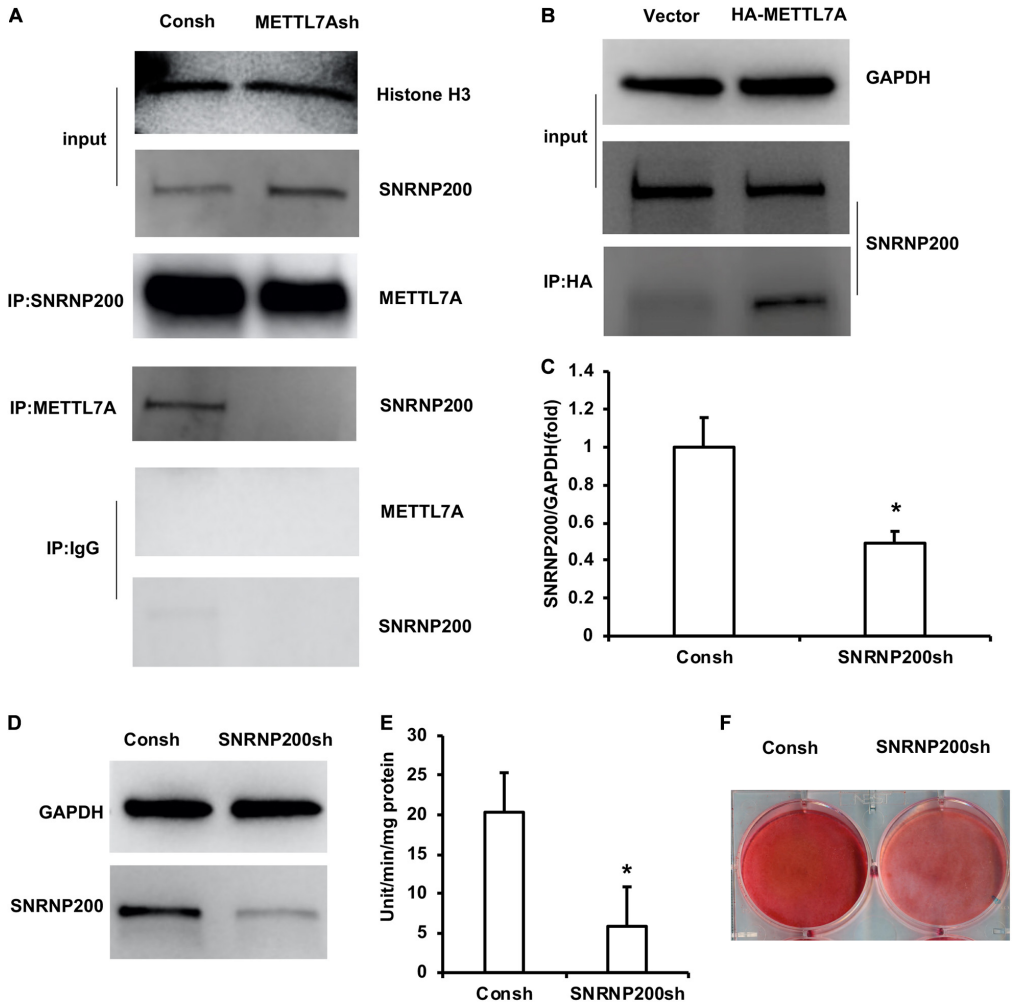

FIGURE 5 | SNRNP200, identified as a co-binding protein of METTL7A, promoted odontogenic differentiation of DPSCs. **(A)** The association of SNRNP200 and METTL7A in METTL7A-knockdown DPSCs detected by the Co-IP assay. **(B)** The association of SNRNP200 and METTL7A in METTL7A-overexpressed DPSCs detected by the Co-IP assay. **(C,D)** The expression of SNRNP200 was detected by real-time RT-PCR and western blot. **(E)** ALP activity after 5 days of mineralized induction. **(F)** ARS after 2 weeks of induction. GAPDH and histone H3 were used as internal control. Student's *t*-test was performed to determine statistical significance. All error bars represent the standard deviation ($n = 3$). $^*p \leq 0.05$.

in the miR-6807-5p inhibitor group and downregulated in the miR-6807-5p mimic group. We also confirmed that miR-6807-5p bound to the 3′UTR region of the downstream target gene METTL7A by the dual-luciferase reporter assay and pull-down assay with biotinylated miRNA. Furthermore, the results of ALP, ARS, and calcium ion quantification and the expressions of BSP, OCN, and OSX showed that METTL7A promoted the odontogenic differentiation of DPSCs. And METTL7A could rescue the ALP activity and mineralization ability in DPSCs, which are impaired by miR-6807-5p, indicating that miR-6807-5p inhibited the odontogenic differentiation of DPSCs via negatively regulating METTL7A.

In order to study the mechanism of METTL7A in the odontogenic differentiation of DPSCs, we analyzed the co-factors of METTL7A by protein mass spectrometry. We found 11 possible co-binding proteins of METTL7A including SNRNP200, AGO1, and WDR1. By comparing the molecular weight of protein and the amount of peptide, we found that SNRNP200 may be the candidate co-factor of METTL7A in DPSCs. SNRNP200 belongs to the pre-nuclear splicing gene. It is essential for the splicing of pre-miRNA. The defect of SNRNP200 may interfere in the recognition of transcripts, resulting in an abnormal splicing product (Absmeier et al., 2016). It is found that the mutation of SNRNP200 leads to retinopathy retinitis pigmentosa (Benaglio et al., 2011; Liu et al., 2012; Zhang et al., 2013; Cvačková et al., 2014). A study also revealed that SNRNP200 is also involved in the antiviral response (Tremblay et al., 2016). In acute myeloid leukemia, U-SNRNP200 complex was found to be exposed to the leukemia cell membrane, which provides a target for tumor therapy (Gillissen et al., 2018). In addition, SNRNP200 is also involved in the process of mammalian cell cycle (Ehsani et al., 2013).

In terms of osteogenesis, it has been previously reported that SNRNP200 knockdown can inhibit the osteogenic/odontogenic differentiation of SCAPs (Su et al., 2020). In this study, we found that knockdown of SNRNP200 inhibited the ALP activity and mineralization ability in DPSCs, indicating that SNRNP200 might promote the odontogenic differentiation of DPSCs. Therefore, we speculated that the SNRNP200 and METTL7A protein complex may affect the splicing of the key gene involving the odontogenic differentiation by influencing the methylation process of m6A. And thus these two proteins synergistically promoted the odontogenic differentiation of DPSCs. This speculation and regulation mechanism need to be further studied.

CONCLUSION

In conclusion, this study verified that miR-6807-5p inhibited the odontogenic differentiation of DPSCs via directly targeting METTL7A and repressing its expression. METTL7A might promote the odontogenic differentiation of DPSCs by associating with SNRNP200. This investigation provided the candidate targets for enhancing the odontogenic differentiation of dental MSCs and dental tissue regeneration.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Beijing Stomatological Hospital (Review No. 2011–02). The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Animal experiments were approved by the Beijing Stomatological Hospital, Capital Medical University (Ethics Committee Agreement, Beijing Stomatological Hospital Ethics Review No. KQYY-201804-003).

AUTHOR CONTRIBUTIONS

NW: responsible for data collection and collation, interpretation, and manuscript writing. XH and HY: responsible for data collection and collation. ZF and DX: responsible for conception, design, manuscript revision, confirmation, and so on. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.759192/full#supplementary-material

Supplementary Figure 1 | The gene expressions were detected by real-time RT-PCR. **(A–C)** There was no significant difference of the expression of GREM1 **(A)**, TRMT10C **(B)**, and METTL1 **(C)** in the miR-6807-5p inhibitor group compared with the control group. GAPDH was used as internal control. Student's *t*-test was performed to determine statistical significance. All error bars represent the standard deviation ($n = 3$).

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Chromatin Accessibility Predetermines Odontoblast Terminal Differentiation

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Embryonic development and stem cell differentiation are orchestrated by changes in sequential binding of regulatory transcriptional factors to their motifs. These processes are invariably accompanied by the alternations in chromatin accessibility, conformation, and histone modification. Odontoblast lineage originates from cranial neural crest cells and is crucial in dentinogenesis. Our previous work revealed several transcription factors (TFs) that promote odontoblast differentiation. However, it remains elusive as to whether chromatin accessibility affects odontoblast terminal differentiation. Herein, integration of single-cell RNA-seq and bulk RNA-seq revealed that *in vitro* odontoblast differentiation using dental papilla cells at E18.5 was comparable to the crown odontoblast differentiation trajectory of OC (osteocalcin)-positive odontogenic lineage. Before *in vitro* odontoblast differentiation, ATAC-seq and H3K27Ac CUT and Tag experiments demonstrated high accessibility of chromatin regions adjacent to genes associated with odontogenic potential. However, following odontoblastic induction, regions near mineralization-related genes became accessible. Integration of RNA-seq and ATAC-seq results further revealed that the expression levels of these genes were correlated with the accessibility of nearby chromatin. Time-course ATAC-seq experiments further demonstrated that odontoblast terminal differentiation was correlated with the occupation of the basic region/leucine zipper motif (bZIP) TF family, whereby we validated the positive role of ATF5 *in vitro*. Collectively, this study reports a global mapping of open chromatin regulatory elements during dentinogenesis and illustrates how these regions are regulated via dynamic binding of different TF families, resulting in odontoblast terminal differentiation. The findings also shed light on understanding the genetic regulation of dentin regeneration using dental mesenchymal stem cells.

Keywords: tooth development, odontogenesis, dental mesenchymal stem cells, transcription factors, epigenetics, H3K27ac, chromatin immunoprecipitation sequencing, RNA-seq

INTRODUCTION

Cell fate specification is achieved through spatiotemporal gene expression during embryonic development, tissue regeneration, or cell reprogramming (Spitz and Furlong, 2012). A set of tissue-specific transcription factors (TFs) regulate these genes at the transcriptional level. They potentially recognize and interact with their specific DNA-binding motifs in the genome to drive lineage-specific gene expression at different developmental stages (Lee and Young, 2013). Moreover, TFs are master regulators in gene regulatory networks (GRNs) to establish competency for different cell fates (Davidson, 2010). However, a majority of potential DNA-binding sites are inaccessible because the genomic DNA in eukaryotic cells is occluded by higher-order chromatin structures (Luger et al., 1997). Within this context, gene regulation occurs at gene regulatory regions in the opened chromatin, which allows for the binding of TFs and functioning of RNA polymerase (Calo and Wysocka, 2013). These types of opened chromatin regions, such as active promoters or enhancers, are characterized by histone modifications that flank nucleosome-free regions, including H3K4 methylation and H3K27 acetylation (Creyghton et al., 2010). The mechanism by which TFs recognize their binding motifs or influence the chromatin accessibility to initiate different biological processes remains largely unknown.

Dental papilla cells are cranial neural crest-derived mesenchymal populations, which form odontoblasts and dentin. Numerous TFs play crucial roles in odontoblastic differentiation via the regulation of gene expression programs, such as RUNX2, DLX3, SOX9, SOX2, and KLF6 (Li et al., 2011; Wang et al., 2014; Yang G. et al., 2017; Yang Y. et al., 2017; Chen et al., 2021). In our recent studies, three zinc-finger TFs, KLF4, SALL1, and ZEB1, have been revealed to regulate odontoblastic differentiation via different mechanisms. KLF4 regulates *Dmp1* and *Sp7* transcription through modulation of histone acetylation. Interaction of SALL1 with RUNX2 directly activates TGF-β2 to regulate the commitment of odontoblast lineages. Besides, ZEB1 alters the chromatin accessibility of *cis*-elements adjacent to genes including *Runx2* in the early stage and directly promotes *Dspp* transcription in the late stage (Tao et al., 2019; Xiao et al., 2021b; Lin et al., 2021). The previous findings affirm that chromatin accessibility is associated with odontoblast terminal differentiation. However, a global view of the interaction of lineage-determining TFs with dynamic changes in chromatin accessibility during odontoblast cell fate specification is elusive.

To fill this knowledge gap, we validated that postnatal day 0 (PN0) OC (osteocalcin)-positive odontogenic lineage in the first lower molar tooth germ mainly contributes to the crown odontoblast layer, which served as a potential *in vivo* odontoblast differentiation model. We employed this *in vivo* model and the frequently adopted *in vitro* odontoblast differentiation model to comprehensively analyze the mechanism of chromatin accessibility in odontoblast terminal differentiation.

MATERIALS AND METHODS

Animal maintenance

All C57BL/6 mouse experiments used for mDPC culture and subsequent ATAC-seq and RNA-seq were performed under the guideline and approval of the Institutional Animal Care and Use Committees at the School and Hospital of Stomatology attached to Wuhan University (protocol no.S07920070I). The OC-Cre (Zhang et al., 2013) and Rosa26-mTmG (Muzumdar et al., 2007) alleles used in this study were described. All experiments involving these two lines were performed with the approval of the Institutional Animal Care and Use Committees at the School of Life Science in Fujian Normal University (protocol no. 20210007). All animal experiments were performed in accordance to the ARRIVE guidelines 2.0.

Cell culture

We isolated primary dental papilla mesenchymal cells (mDPCs) from embryonic day 18.5 (E18.5) first molar tooth germ. A dissection needle was employed to remove the dental epithelium after digestion using 0.75 mg/ml of dispase (Becton, Dickinson and Co., Franklin Lakes, NJ, USA). The tooth germ was isolated from the mandible using forceps, dispersed, and digested with 0.25% trypsin-EDTA (Life Technologies, Carlsbad, CA, USA) at 37°C for 15 min. Cells were cultured in Dulbecco's modified Eagle medium (DMEM; Hyclone, Pittsburgh, PA, USA). To induce mineralization, mDPCs between passage 2 and 4 were cultured in an induction medium supplemented with 50 μg/ml of ascorbic acid (Sigma, St. Louis, MO, USA), 10 mmol/L sodium-glycerophosphate, and 10 nmol/L dexamethasone (Sigma). All the cells used in this study were maintained in 5% CO_2 at 37°C, and the medium was replenished every 2 days.

Plasmid construction dual-luciferase assay, and transfection

For enhancer activity assay, DNA fragments synthesized by Sangon Biotech (Shanghai, China) were inserted into the pGL3-promoter vector. Phusion® polymerase (NEB, USA) was used to clone the full-length of open-reading frame of ATF5, and subcloned into pcDNA3.1 (+). For dual-luciferase assay, we cotransfected mDPCs with pGL3 vector along with the pRL-TK plasmid and ATF5-OE plasmid or pcDNA3.1 (+) using Lipofectamine 2000 (Life Technologies). Four days posttransfection, the dual-luciferase assay was performed using the luciferase Assay System (Promega) following the protocol of the manufacturer. Triplicate wells were analyzed. Firefly luciferase activity from the whole-cell lysates was normalized using Renila activity internal control.

Lentivirus-expressing short-hairpin RNA (shRNA) against Atf5 (shAtf5) (top strand: GATCCGCGGGAGATCCAGTAC GTGAATTCAAGAGATTCACGTACTGGATCTCCCGCTTTT TTG; bottom strand: AATTCAAAAAAGCGGGAGATCCAG TACGTGAATCTCTTGAATTCACGTACTGGATCTCCCGCG) and empty control (control) were generated from HanBio

(HanBio, China). mDPC at passage was infected with lentivirus, 4 days after which knockdown efficiency was validated using qRT-PCR.

Single-cell RNA-Seq and downstream analysis

Single-cell RNA-seq was performed on the 10x Chromium platform using Chromium Single Cell 3′ Library and Gel Bead Kit v3 (10x Genomics; Annoroad Genomics, China). Following the quality check, the DNA library was sequenced on the Illumina Novaseq 6000 (Illumina, Annoroad Genomics, China). Filtered reads were mapped to mm10 transcriptome using Cell Ranger v3.0 (10x Genomics) (Zheng et al., 2017). The Seurat package (v3.0) (Stuart et al., 2019) was employed in R for downstream analysis. Briefly, raw count matrices were filtered to remove barcodes with less than 500 genes expressed; more than 8,000 genes expressed, and a there was a high percentage of UMIs from the mitochondria (>10%), leaving 6,720 cells for the first round of clustering. Counts were normalized and scaled using the SCTransform function (Hafemeister and Satija, 2019). The first round of dimension reduction and clustering was performed using "dim = 1:30." Dmp1+/GFP+ (GFP >1) served as a terminal odontoblast cluster for the OC-positive population. Considering the distance between other clusters and this cluster, we subset all clusters with "nearby" as odontogenic lineage for further analysis. Finally, 975 cells were picked as OC-positive odontogenic lineage. Dimension reduction and clustering were performed for the second round, and clusters were visualized using tSNE plots. We reconstructed cell differentiation trajectories using Monocle (Trapnell et al., 2014) (v3.0) from the Seurat object above. All cluster information was inherited from Seurat. Based on counts of genes in each cell, cell trajectories were imputed using the "order_cells" function. Distribution of regulons was generated in SCENIC packages (v 1.1.2) (Aibar et al., 2017) in R.

Transposase-accessible chromatin assay with high-throughput sequencing

We cultured mDPCs in a mineralization medium and normal culture medium for 0, 3, 5, 7, and 9 days. OC-positive and OC-negative tooth germ cells were harvested as follows: The mandible of PN0 OC-Cre and Rosa26-mTmG was treated with dispase to allow for the removal of dental epithelium using dissection needles. Fine forceps were then applied to isolate the tooth germ. To obtain a single-cell suspension, tooth germ was disassociated with trypsin and subjected to FACS sorting to acquire 10^5 OC-positive and 10^5 OC-negative cells. ATAC-seq libraries were prepared as described by Buenrostro et al. (2013) and indexed using a TruePrep DNA Library Prep Kit (TD501, Vazyme, Nanjing, China). Approximately 50,000 cells in each biological replicate were harvested and dissociated using a cell strainer via centrifugation ($750 \times g$ for 5 min) at room temperature. The cell pellets were resuspended in lysis buffer (10 mM Tris-HCl, pH 7.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.1% NP-40), then centrifuged at $500 \times g$ for 15 min at 4°C. The supernatant was discarded. The pelleted nuclei were immediately submitted to tagmentation reaction using Tn5 transposase (TTE Mix V50) for 30 min at 37°C. DNA purified using the Qiagen PCR purification MinElute Kit (Qiagen, Valencia, CA, USA) was eluted in 10 μl of elusion buffer, indexed, and amplified. All libraries were cleaned using VAHTS DNA Clean Beads and sequenced on the Illumina Novaseq 6000 (Illumina, provided by Annoroad Genomics, China). Three independent biological replicates were performed for each mDPC-D0 and D9, whereas two independent biological replicates were performed for each time point in the time-course ATAC-seq. However, one replicate was performed for OC-positive and OC-negative cells ATAC-seq.

Cleavage under targets and tagmentation library preparation

We cultured mDPCs in a mineralization medium and normal culture medium for 0 and 9 days. Cleavage under targets and tagmentation (CUT and Tag) libraries were prepared as previously described by Kaya-Okur et al. (2019) and indexed using an *In-Situ* ChIP Library Prep Kit (TD902, Vazyme, Nanjing, China). Approximately 10,000 mDPCs in each biological replicate were harvested and centrifuged ($600 \times g$) at room temperature for 3 min. The supernatant was washed and resuspended in Wash Buffer supplemented with protease inhibitors (Roche Complete Protease Inhibitor EDTA-Free Tablet, Sigma-Aldrich, St. Louis, MO, USA). Concanavalin A-coated magnetic beads in each sample were washed and resuspended in binding buffer. Then beads were added to the cells, gently vortexed, and incubated in a shaker for 10 min at room temperature. The unbound supernatant was removed. The bead-bound cells were resuspended in precooling antibody buffer [2 mM EDTA, 0.1% BSA in DIG Wash Buffer (0.05% digitonin in wash buffer)] and incubated with (1:50 dilution) primary antibody against H3K27ac (ab4729, Abcam, Cambridge, MA, USA) in a shaker overnight at 4°C. The primary antibody on the magnet stand was removed, and then a secondary antibody (Guinea Pig Anti-Rabbit IgG antibody, 611-201-122, Rockland Immunochemicals, PA, USA) was diluted (1:100) in DIG Wash buffer and incubated with cells at room temperature for 1 h. Cells were washed in DIG Wash buffer using the magnet stand to remove unbound antibodies. A dilution of hyperactive pA-Tn5 transposon complex (0.04 μM) was prepared in DIG-300 buffer supplemented with 0.01% digitonin and protease inhibitors. Then cells were incubated with pA-Tn5 transposon complex in a shaker at room temperature for 1 h. Subsequently, cells were resuspended in tagmentation buffer (10 mM $MgCl_2$ in DIG-300 buffer) and incubated at 37°C for 1 h. To terminate tagmentation, we added 10 μl of 0.5 M EDTA, 3 μl of 10% SDS, and 2.5 μl of 20 mg/ml proteinase K to each sample, followed by overnight incubation at 37°C. Purified DNA was amplified and indexed. The libraries were cleaned using VAHTS DNA Clean Beads (N411, Vazyme, Nanjing, China) and sequenced on the

Illumina Novaseq 6000 (Illumina, provided by Annoroad Genomics, China). We applied 150-bp pair-end sequencing with a sequencing depth of 6G base pair raw data (generated approximately 20 million mapped paired reads). Three independent biological replicates were performed for each mDPC-D0 and D9.

Cleavage under targets and tagmentation and Transposase-accessible chromatin assay with high-throughput sequencing library analyses

Raw reads of CUT and Tag and ATAC-seq were first subjected to trimmomatic (v.0.38) (Bolger et al., 2014) for adaptor trimming. We did a quality check using FastQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) before alignment to ensure proportionate quality libraries. Then the paired-end sequencing reads were aligned to the mouse genome (mm10) using Bowtie 2 (Langmead and Salzberg, 2012). SAMtools (Li et al., 2009) was applied to eliminate the PCR duplicates. DeepTools2 (Ramírez et al., 2016) was used to generate bigwig files. MACS2 (v.2.1.1) (Zhang et al., 2008) was applied for peak calling. Comparison of differentially accessible NFRs between different time points was achieved using DiffBind (DESeq2 v.1.26.0) (Love et al., 2014). We further applied the Homer package (Heinz et al., 2010) to identify the *de novo* motifs enriched in the NFRs of different mineralization time points. Genomic Regions Enrichment of Annotations Tool (GREAT) (McLean et al., 2010) was adopted to annotate differentially accessible NFRs and perform GO enrichment assay. Eventually, coverage plots for CUT and Tag and ATAC-seq results were generated using DeepTools2 and uploaded to the UCSC genome browser. All correlative graphs were plotted using R scripts in RStudio (v. February 1, 5001). For footprint, mapped reads at the groups were concatenated and subjected to CENTIPEDE (Pique-Regi et al., 2011), and cutting frequency near Dmp1 3′UTR was then visualized. Example scripts were uploaded to Github (https://github.com/Badgerliu/mDPC_epi_paper). All scripts are available upon request.

Integration of transposase-accessible chromatin assay with high-throughput sequencing and H3K27Ac cleavage under targets and tagmentation data

To identify odontoblast stage-specific active enhancers, we integrated H3K27Ac CUT and Tag peaks to ATAC-seq at the same time point following our previously published strategy (Liu et al., 2020). The regions flanked by two adjacent H3K27Ac peaks less than 1,500 bp were defined as "H3K27Ac-flanked" regions. D0- or D9-enriched NFRs were "intersected" with D0- or D9-enriched H3K27Ac peaks or "H3K27Ac flanked" regions using bedtools. The overlapped NFRs were termed as D0- or D9-enriched active enhancers.

Immunohistochemistry

The isolated mandibles of wild-type C57BL/6 mouse at PN2 (for anti-ATF5) and OC-Cre, and Rosa26-mTmG (for OC-positive lineage tracing) were fixed in 4% paraformaldehyde at 4°C for 24 h. The samples were then treated in 10% ethylenediaminetetraacetic acid (EDTA) for 1–2 days, dehydrated, and embedded in paraffin. Sagittal sections (5-μm thick) were dewaxed and rehydrated. After being boiled in 1 mM citrate buffer (pH = 6.0) for 15 min, slides were cooled down to room temperature. Subsequently, histological sections were blocked in bovine serum albumin (BSA) (Biosharp, China) and incubated with antibodies against GFP (1:100, Cat. No. ab13970, Lot No. GR89472–15; Abcam, MA, USA) or ATF5 (1:100; Cat. No.ab184923, Lot No. GR282324-13; Abcam, MA, USA) at 4°C overnight. For regular immune chemical color reaction, samples were reacted with polymer Helper and poly-HRP-anti-rabbit IgG or poly-HRP-anti-goat IgG at room temperature for 15 min after they were washed with phosphate-buffered saline (PBS). The samples were detected using a diaminobenzidine (DAB) reagent kit (Maixin) and counterstained with hematoxylin. For immunofluorescent analysis, we performed anti-GFP staining in Alexa 633 (anti-Chicken lgY, 1:500, Cat. No. A21103, Lot No. 2079359; Thermo Fisher Scientific, MA, USA) and counterstained with DAPI (Life Technologies, USA). Pseudocolor was performed using Fiji (Schindelin et al., 2012) to convert color of Alexa633 to GFP.

Quantitative reverse transcriptase PCR analysis

Total RNA from cells was extracted using HP Total RNA Kit (Omega biotech, Norcross, GA, USA), then reverse transcribed into cDNA using Reverse Transcription System (Life Technologies). qPCR was performed in CFX Connect Real-Time PCR system (Bio-RAD) using ChamQ SYBR qPCR Master Mix (Vazyme). Gapdh, Atf5, Alp, and Dmp1 were quantified with Gapdh as the internal normalization control. The RNA expression ratio was denoted as "mean ± standard deviation" from three independent biological replicates.

Western blot analysis

After different treatments, mDPCs were lysed in lysis buffer (Feiyi Technology, China) and centrifuged at 13,000 rpm for 10 min at 4°C. Total protein was quantified. After that, Western blot was performed with the following antibodies.

DMP1 (1:1,000; Cat. No. ab103203, Lot No. GR3212251-2; Abcam, MA, USA), DSP (1:1,000; Cat. No. NBP1-91612, Lot No. QC6694; NOVUSBIO, CO, USA), ATF5 (1:2,000; Cat. No. ab184923, Lot No. GR282324-13; Abcam, MA, USA), and β-ACTIN (1:8,000; BioPM, Beijing, China).

Statistical analysis

All results were presented as "mean ± standard deviation (SD)." One-way ANOVA was performed for multiple group comparisons, whereas a two-tailed *t*-test was performed for two groups. Values of $p < 0.05$ were considered statistically significant.

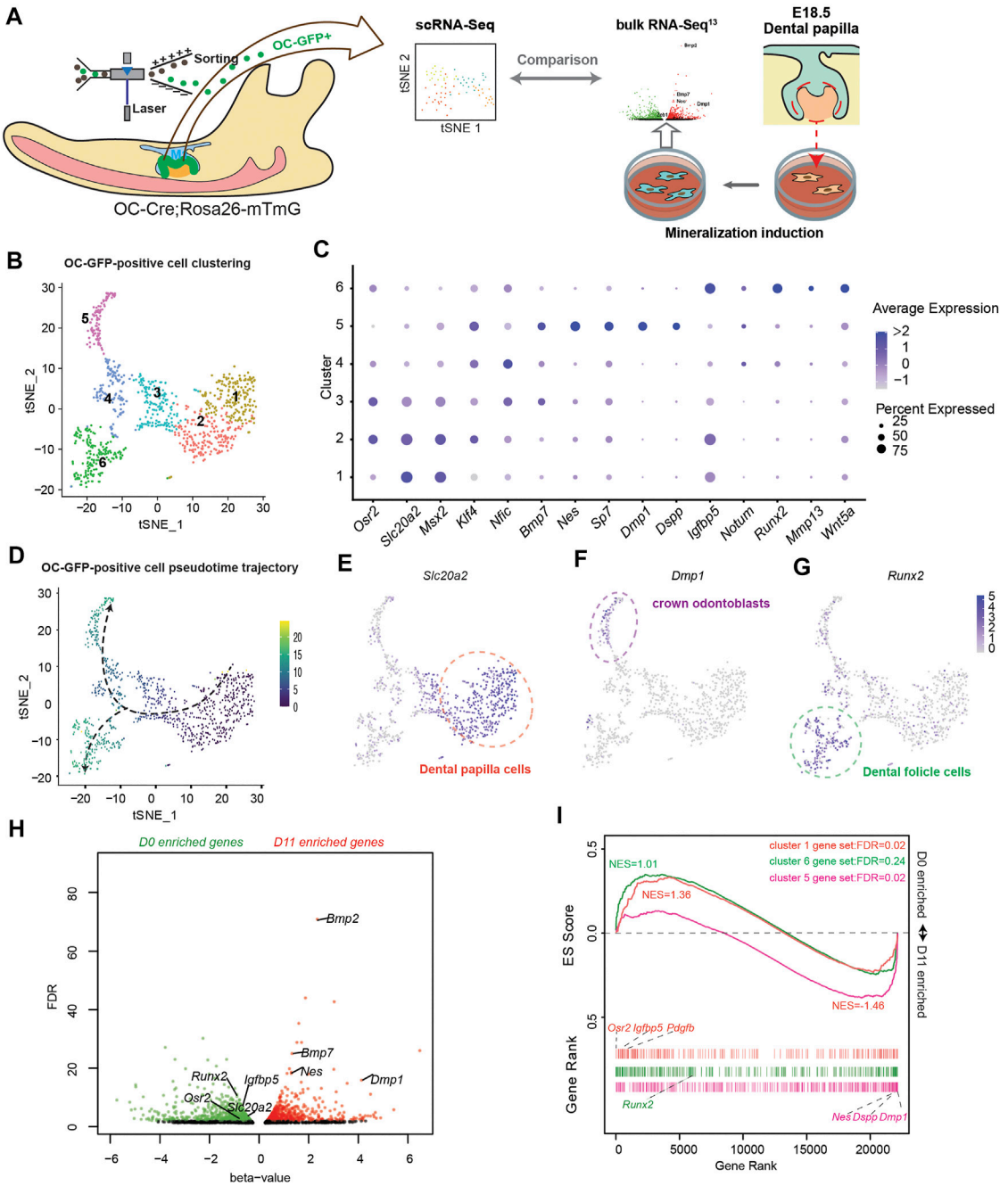

FIGURE 1 | Comparison between *in vitro* odontoblastic differentiation and *in vivo* osteocalcin (OC)-positive odontogenic lineage differentiation. **(A)** Study design comparing single-cell RNA-seq (scRNA-seq) using OC + odontogenic lineage from postnatal day 0 (PN0) first lower molar tooth germ and bulk RNA-seq of dental papilla cells before and after mineralization induction. **(B)** *t*-distributed stochastic neighbor embedding (tSNE) depicting the clustering of 975 single-cell transcriptional profiles obtained from FACS-sorted GFP-positive cells from OC-Cre and Rosa26-mTmG PN0 first lower molar tooth germ. **(C)** Dot-plot showing expression and enrichment of selected top genes identified in each cluster. The size of dot indicate the percentage of cells per cluster. **(D)** Pseudotime-trajectory of OC-GFP-positive scRNA-seq. **(E)** *Slc20a2* marks pre-odontoblast population in OC + odontogenic lineage. **(F)** *Dmp1* marks crown odontoblast population in OC + odontogenic lineage. **(G)** *Runx2* marks dental follicle cell population in OC + odontogenic lineage. **(H)** Volcano scatter plot showing the differentially expressed genes revealed in bulk RNA-seq from dental papilla cells before (D0) and after (D11) mineralization induction. Red dots indicate genes significantly enriched in D11, and green dots indicate genes significantly enriched in D0. **(I)** Gene set enrichment analysis (GSEA) using marker genes from cluster 1 (orange), cluster 6 (green), and cluster 5 (violet) as gene sets comparing expression profiles from D0 and D11 bulk-RNA-seq. NES, normalized enrichment score; FDR, false discovery rate, as generated in GSEA.

RESULTS

In vitro odontoblastic differentiation resembles crown odontoblast differentiation trajectory of OC-positive odontogenic cells.

Previous studies on odontoblast differentiation mainly employed an *in vitro* model using mouse dental papilla cells (mDPCs) induced by a mineralization medium. They reported significant upregulation of *Dmp1*, *Dspp* (Chen et al., 2009), and *Nestin* (*Nes*) (Kaukua et al., 2014), which are marker genes mainly expressed in the odontoblast layer [*Nes* is also expressed in the pericytes in dental pulp (Yianni and Sharpe, 2018)]. However, the same medium could also potentially induce osteogenic differentiation when bone marrow-derived mesenchymal cells (BMSCs) are used (Yu et al., 2021). Thus, whether this model can mimic *in vivo* odontoblast differentiation remains unclear. To clarify this, we first attempted to compare the transcriptomes of *in vitro* with *in vivo* odontoblast differentiation models (**Figure 1A**). Inspired by the specific Cre activity in the odontoblast layer of OC-Cre mice (Yun et al., 2016), we generated OC-Cre and Rosa26-mTmG mice to assess odontoblast differentiation *in vivo*. GFP activity was mainly localized at the odontoblast layer (**Supplementary Figures 2A, D**) and dental follicle cells in developing tooth root at postnatal day 0 (PN0) in the first lower molar. Also, some blood vessels were GFP positive (**Supplementary Figure 2C**). We isolated GFP-positive cells from PN0 first lower molar tooth germ of OC-Cre and Rosa26-mTmG mice, and performed single-cell RNA-seq. Considering the initial cluster and expression of *Dmp1* and Cd34, we isolated 975 cells associated with *Dmp1*, which was termed as "OC-positive odontogenic lineage." Six clusters were revealed (**Figure 1B**). Following the assessment of the significant differential marker genes (minimal percentage as 25% and $p < 0.01$; **Supplementary Table 1**), the cell distribution was revealed to resemble a subset of population single-cell RNA-seq reported from the apical halves of molar at PN7.5 (Wen et al., 2020). Clusters 1 and 2 were identified as dental papilla cells, expressing high levels of *Slc20a2*, *Osr2*, and *Dlx3* (**Figures 1C, E**; **Supplementary Figures 3, 4**) (Yamashiro et al., 2003). Cluster 5 was termed as odontoblast with explicitly high expression of *Dmp1*, *Nes*, and *Dspp* (**Figures 1C, F**; **Supplementary Figures 3 and 4**). *Runx2*, *Mmp13*, and *Bmp3* were highly enriched in cluster 6 (**Figures 1C, G**; **Supplementary Figures 3 and 4**); they were previously identified to be highly associated with dental follicle cells (Wen et al., 2020). Clusters 3 and 4 were regarded as transient status from OC-positive dental papilla cells to odontoblasts or dental follicle cells; they exhibited gradually low expression of *Slc20a2* and *Msx2*. Differentiation trajectory of these OC-positive odontogenic lineages based on gene expression changes was inferred (**Figure 1D**). Dental papilla cells (clusters 1 and 2) were the start point, whereas two destinations were crown odontoblasts (cluster 5) and dental follicle cells (cluster 6). These results demonstrated that OC-positive odontogenic lineage contributed to crown odontoblasts and dental follicle cells.

To make a transcriptome-wide comparison between *in vivo* and *in vitro* odontoblast differentiation, we adopted our previously published bulk RNA-seq profile using mDPCs treated with mineralization medium for 0 days (D0) and 11 days (D11) (Lin et al., 2021) (**Figure 1H**). *Zeb1* (Xiao et al., 2021a) and *Sall1* (Lin et al., 2021) were upregulated on day 11 and promoted odontoblastic differentiation. Also, the enrichment of *Osr2*, *Slc20a2*, and *Runx2* on D0 was not found in the crown odontoblast population (cluster 5) in the scRNA-seq profile. We applied gene set enrichment analysis (GSEA) with marker genes in clusters 1, 5, or 6 as three independent gene sets and found that genes enriched on D11 were positively correlated with cluster 5 gene set (NES = −1.46, FDR = 0.02, no. of permutations = 10,000), whereas D0 was positively correlated with cluster 1 gene set (NES = 1.36, FDR = 0.02). However, genes in the cluster 6 set exhibited an even distribution in both D0 and D11 (NES = 1.01) (**Figure 1I**) indicating it was not like the *in vitro* differentiation. These findings suggested that *in vitro* odontoblast differentiation using mDPCs from E18.5 concurs with *in vivo* odontoblast differentiation of OC-positive odontogenic lineage.

Identification of odontoblast-specific active enhancers

Activation and deactivation of *cis*-regulatory elements have been directly associated with transcriptional regulation (Heinz et al., 2010). We explored whether there are such elements that regulate transcription during odontoblast differentiation. The above *in vitro* and *in vivo* models were employed for ATAC-seq to identify open chromatin regions before and after odontoblast differentiation. The open chromatin regions in the *in vitro* models were annotated using H3K27Ac CUT and Tag to reveal active enhancers (**Figure 2A**). We found 27,484 and 26,215 nucleosome-free regions (NFRs) enriched in OC-positive and OC-negative cells (**Figure 2B**). We compared the ATAC-seq reads from *in vitro* odontoblast differentiation and found no significant difference between D0 and D11 ATAC-seq in the OC-positive enriched NFRs. However, the overall D0 and D11 ATAC-seq signals in the OC-positive enriched NFRs were significantly higher than those in OC-negative ones ($p < 0.001$, by Kolmogorov Smirnov test, *ks*-test) (**Supplementary Figure 6A**). Considering the transcriptome comparison between *in vitro* and *in vivo* differentiation, this is reasonable given that OC-positive odontogenic lineage covers the transition from D0 to D11. Besides, the shift in chromatin accessibility from D0 to D11 reflected detailed changes of *cis*-regulatory elements during odontoblast terminal differentiation. We, therefore, chose this *in vitro* model for further analysis.

As for the ATAC-seq from *in vitro* odontoblast differentiation model, there were 61,133 NFRs enriched in D0 (before mineralization induction) and 12,822 enriched in D9 [$\log_2$ (fold change) >0.5 or <0.5, FDR <0.01, by DESEQ2; **Supplementary Tables 2 and 3**]. The enrichment of ATAC-seq reads correlated with H3K27Ac CUT and Tag (**Figure 2B** and **Supplementary Figures 7A, B**). The NFRs overlapped or flanked by H3K27Ac signals at the same stage were defined as active enhancers (AEs); also, we found 12,221 AEs on D0 and 2,461 on D9 (**Supplementary Figure 7C** and **Tables 4 and 5**). In several

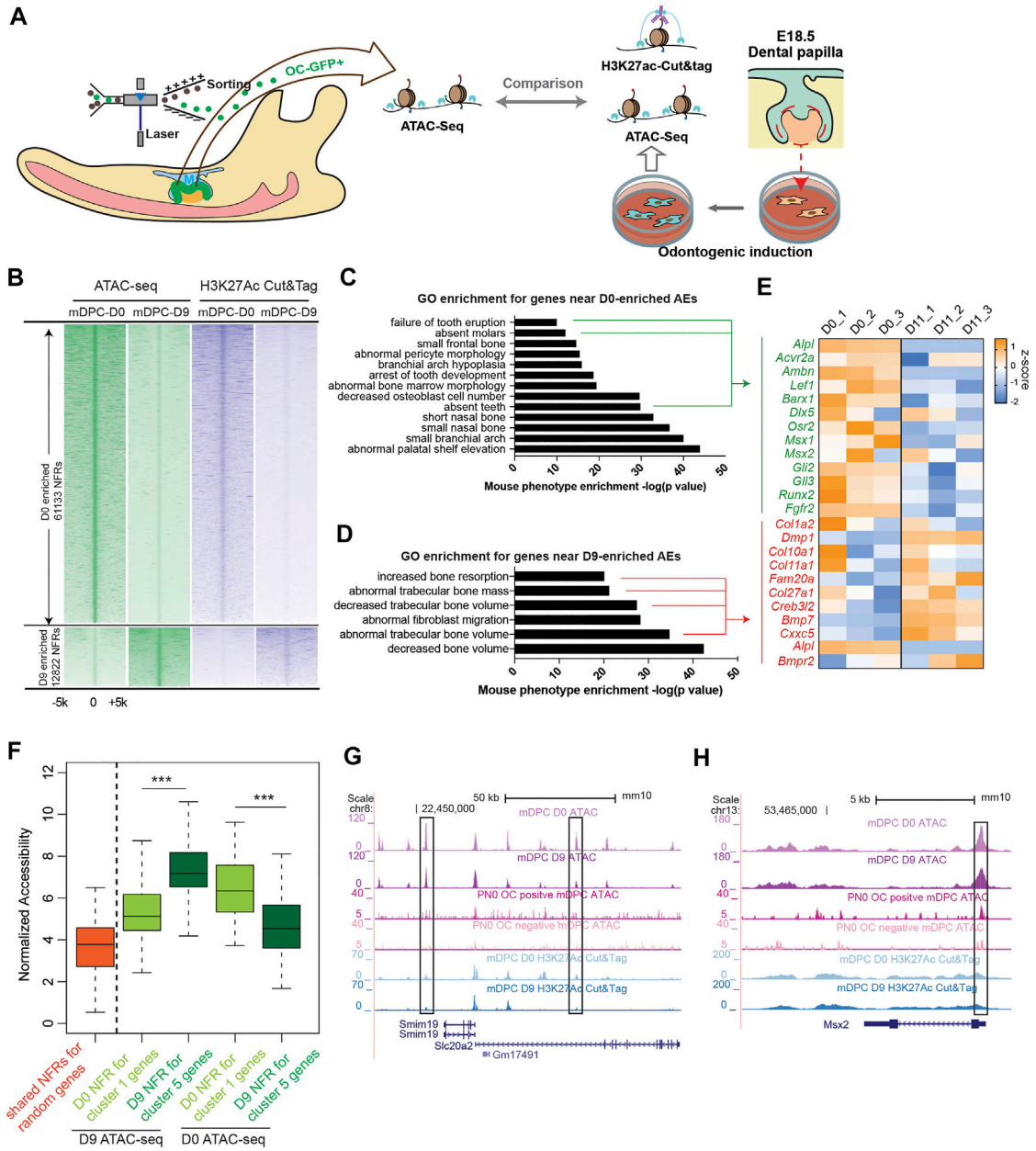

FIGURE 2 | Transposase-accessible chromatin assay with high-throughput sequencingATAC-seq) and H3K27Ac cleavage under targets and tagmentation (CUT and Tag) from both *in vitro* and *in vivo* odontoblast differentiation revealed chromatin early shaping predetermined dentinogenesis potential of dental papilla cells. **(A)** Study design comparing ATAC-seq from *in vitro* and *in vivo* odontoblast differentiation and annotation for active enhancers using H3K27Ac CUT and Tag. **(B)** Density plot of aligned ATAC-seq and H3K27Ac CUT and Tag peaks differentially enriched in D0 and D9 during mineralization of E18.5 dental papilla cells. Each line is centered on the nucleosome-free region (NFR) with significantly more reads in D0 or D9 ATAC-seq. Reads in H3K27Ac CUT and Tag are aligned to the same NFR. Gene ontology enrichment using "Mouse Phenotype Single KO" for D0- **(C)** and D9- **(D)** enriched active enhancers (AEs). **(E)** Expression changes of the overlapped genes from bulk RNA-seq profile in indicated GO terms visualized in heatmap. **(F)** Plot of accessibility scores (generated in DiffBind) of elements with differential accessibility associated with genes differentially expressed in cluster 1 and cluster 5, showing elements with increased accessibility in D9-*in-vitro*-differentiated odontoblast tend to be associated with genes enriched in cluster 5, and vice versa. ***$p < 0.001$, by Kolmogorov–Smirnov test. **(G, H)** UCSC genome browser view showing ATAC-seq and CUT and Tag peaks near the *Slc20a2* and *Msx2* loci.

regions near Dmp1 locus (**Supplementary Figure 7D**), the accessibility remained unchanged or decreased post odontoblast differentiation. However, the H3K27Ac CUT and Tag signals were significantly elevated. This is common during the establishment of enhancer activity: H3K27Ac modification follows the "opening" of chromatin regions (Calo and Wysocka, 2013). We analyzed the GO enrichment for the associated genes of D0- and D9-enriched NFRs in "Mouse phenotype single KO."

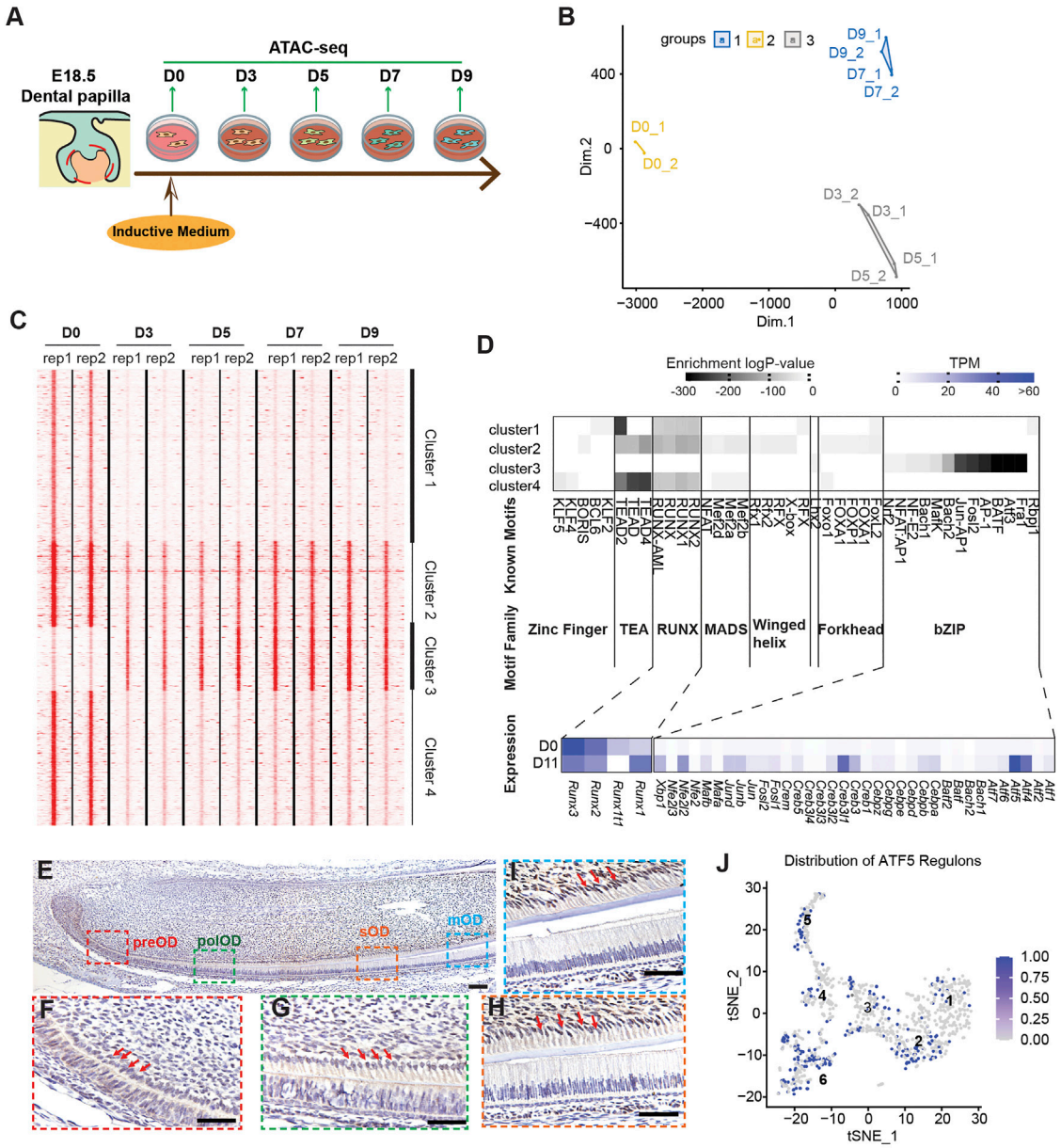

FIGURE 3 | Time–course ATAC-seq integrated with RNA-seq identified transcription factors (TFs) enriched in open chromatin regions associated with odontoblast terminal differentiation. **(A)** Study design for time-course ATAC-seq using *in vitro* odontoblast differentiation. **(B)** A multidimensional scaling (MDS) plot showing the difference between differential-enriched NFRs among D0, D3, D5, D7, and D9 ATAC-seq. **(C)** k-mean clustering of differential-enriched NFRs across replicates and stages, highlighting peak density profile. Each line is centered in NFRs enriched in different stages, with expansion from −5,000 to +5,000 bp. **(D)** Top enriched known motifs in each cluster as predicted via Homer analysis. The color gradient indicates the $\log_{10}$ (*p*-value) of enrichment analysis over the total background peaks using binomial testing. Hierarchy clustering of TF distribution based on $\log_{10}$ (*p*-value) was depicted in the heatmap. Coincidently, most of the clustered TFs belong to a specific TF family with a similar structure, such as zinc-finger, RUNX, forkhead, or bZIP. Of note, the bZIP TFs family was specifically enriched in cluster 3-enriched NFRs. The average expression of each member in the bZIP family on D0 and D11 bulk RNA-seq profiles was exhibited in the heatmap. **(E–I)** Immunohistochemistry of the expression pattern of ATF5 in PN2 murine lower incisor. ATF5 expression in the pre-odontoblasts (preOD) **(F)**, polarized odontoblasts (polOD) **(G)**, secretory odontoblast (sOD) **(H)**, and mature odontoblast (mOD) **(I)** were gradually increasing. Scale bar: 100 μm. Red arrows point to the odontoblast layer. **(J)** tSNE plot showing the distribution of cells with abundant ATF5-targeting genes (regulons) in OC-positive scRNA-seq profile.

Genes associated with D0-enriched NFRs were more enriched in odontogenic-related GO terms such as "failure of tooth eruption" and "absent teeth" (**Figure 2C**), including *Acvr2a*, *Lef1*, and *Msx1*. The expressions of these genes were low in D11 according to the RNA-seq profile. Transient transfection-based dual-luciferase assay in mDPCs confirmed elements near *Msx1* (*Msx1*-0.6; mm10 chr5: 37,824,275–37,824,941) and *Fgfr2* (Fgfr2+96; mm10 chr7: 130,167,270–130,167,658; and

Fgfr2-34; chr7: 130,298,850–130,299,791) exhibited higher enhancer activity on D0 compared with D11, despite the possible loss of plasmids during odontoblast differentiation (**Supplementary Figure 8**). Moreover, genes associated with D9-enriched NFRs were highly associated with mineralization GO terms, such as "increased bone resorption" and "decreased trabecular bone volume/mass" (**Figure 2D**). RNA-seq profile demonstrated that the genes, *Dmp1* and *Creb3l2*, were also significantly upregulated on D11 (**Figure 2E**). We performed *in vitro* validation and found that two elements targeting these genes (Col1a2-8.3; chr6: 4,497,230–4,497,895; and Bmp7+67; chr2: 172,872,129–172,872,743) exhibited high enhancer activity (**Supplementary Figure 9**).

We also found that differentially accessible NFRs associated with cluster 5 genes (in scRNA-seq) had significantly higher average accessibility from mDPCs D9 than those from mDPCs D0, and vice versa (**Figure 2F**). Particularly, Msx2 and Slc20a2 were highly expressed in cluster 1, and there were more intense signals of ATAC-seq along with H3K27Ac in the mDPC D0 group (**Figures 2G,H**). Collectively, this analysis suggested that chromatin accessibility was associated with the terminal differentiation of odontoblast.

Time-course ATAC-seq reveals that dynamic changes in open chromatin regions predetermine terminal differentiation of odontoblast.

Because NFRs enriched in mDPC-D9 groups are associated with odontoblast terminal differentiation, we explored whether these regions were regulated by any specific TFs. We performed a more detailed time-course ATAC-seq during *in vitro* odontoblast differentiation at 2-day intervals (**Figure 3A**). Following the analysis of differentially enriched NFRS in each group, we found that the accessibility of NFRs changed dramatically from D0 to D3 (**Figure 3B**), much earlier than the upregulation of odontoblast-specific genes, *Dmp1* and *Dspp*, which are always, in most cases, increased around day 5 or day 7 post-induction (Tao et al., 2019). There were 5,526 NFRs differentially enriched between the D0 and D3 group, 48 between D3 and D5, 131 between D5 and D7, and no different NFRs between D7 and D9 (**Supplementary Figure 10**). High-resolution analysis across all the merged consensus peaks from D0 to D9 using k-means clustering revealed four clusters of elements with two major trends of chromatin accessibility, with the NFRs in clusters 1, 2, and 4 gradually "closed", and cluster 3 gradually "open" (**Figure 3C**). The promoter of *Gli1* (**Supplementary Figure 11A**) and an element near *Klf4* (**Supplementary Figure 11B**) gradually lost accessibility during differentiation. GO enrichment assay for NFRs revealed that genes near cluster 1 (including *Gli1*) were associated with "incisor/tooth morphology" indicating their odontogenic function, whereas genes near cluster 3 were associated with "bone mass" (**Supplementary Figure 12**). These underpinned cluster 3 are the very NFRs associated with odontoblast terminal differentiation, given by mineralization as the major biological process.

We applied Homer to identify the known TFs motifs enriched in the four clusters, which allowed us to find the specific TFs that regulate NFR activity. We found a well-separated pattern of the TF family in the four clusters of NFRs. Zinc Finger, TEA, RUNX, MADS, and Forkhead TF families were exclusively enriched in clusters 1, 2, and 4, whereas the basic region/leucine zipper (bZIP) family members were enriched in cluster 3. To identify the possible missing TF members in the bZIP family attributed to the incomplete database, we examined the changes in expression level and abundances of all the recorded bZIP TF (from UniProtKB database) (UniProt, 2019) referring to our bulk RNA-seq data. Most of the bZIP family members detected in bulk RNA-seq were upregulated post odontoblast differentiation. In contrast, RUNX family members, enriched only in clusters 1, 2, and 4 were downregulated (**Figure 3D**). Of the bZIP family members, we selected *Atf5* for further validation. IHC results revealed that ATF5 was highly expressed in the secretary odontoblasts and mature odontoblasts, but weakly expressed in the pre-odontoblast in the odontoblast layer of the lower incisor from PN0 mouse (**Figures 3E–H** and **Supplementary Figure 14**). Additionally, SECNIC imputation demonstrated that the regulons of ATF5 in the scRNA-seq profile were mainly distributed in clusters 2, 3, 4, 5, and 6, which depicted its positive role in the differentiation of odontoblast and dental follicle cells (**Figure 3J**). Furthermore, we scanned all the target NFRs of ATF5 motifs in cluster 3 and found that the genes associated with these NFRs mediated "ossification" and "bone development" (**Supplementary Figure 13**). Taken together, our time-course ATAC-seq experiments with motif analyses revealed that the dynamic changes in chromatin accessibility occur earlier than the transcriptional changes and predetermined odontoblast-related gene transcription.

ATF5 promotes odontoblastic differentiation partially by binding to a Dmp1 enhancer

We further characterized the function of ATF5 in the *in vitro* odontoblast differentiation model. We applied lentivirus-mediated shAtf5 transduction with scrambled plasmid as a control. qPCR and Western blot demonstrated that *Atf5* knockdown significantly reduced RNA levels of *Dmp1* and *Alp* on day-9 post induction. A significantly lower protein level of DSPP was reported in the sh*Atf5* group on day 9 (**Figure 4A** and **Supplementary Figure 15**). Alizarin red results demonstrated that mineralization was significantly inhibited in the *Atf5*-knockdown group (**Figure 4B**). Based on our previous analysis of the target genes of the ATF5 motif in cluster 3 NFRs, Dmp1 was selected as a target of ATF5 for further exploration. We found an element in the downstream of *Dmp1* (Dmp1+13; chr5: 104,216,600–104,216,700) with ATF5 motif (**Figure 4C**), accessibility of which was significantly increased. CENTIPEDE-based Tn5 transposase footprinting assay (Pique-Regi et al., 2011) was performed using two concatenated replicates from mDPC D0 and D9 ATAC-seq. Despite the fact that there were more mapped reads in D0 than D9, footprint revealed a higher cut frequency of Tn5 flanking this motif on D9 mDPC ATAC-seq (**Figure 4D**), an implication that this site was protected by protein. Furthermore, ATF5 overexpression significantly increased the enhancer activity of Dmp1+13

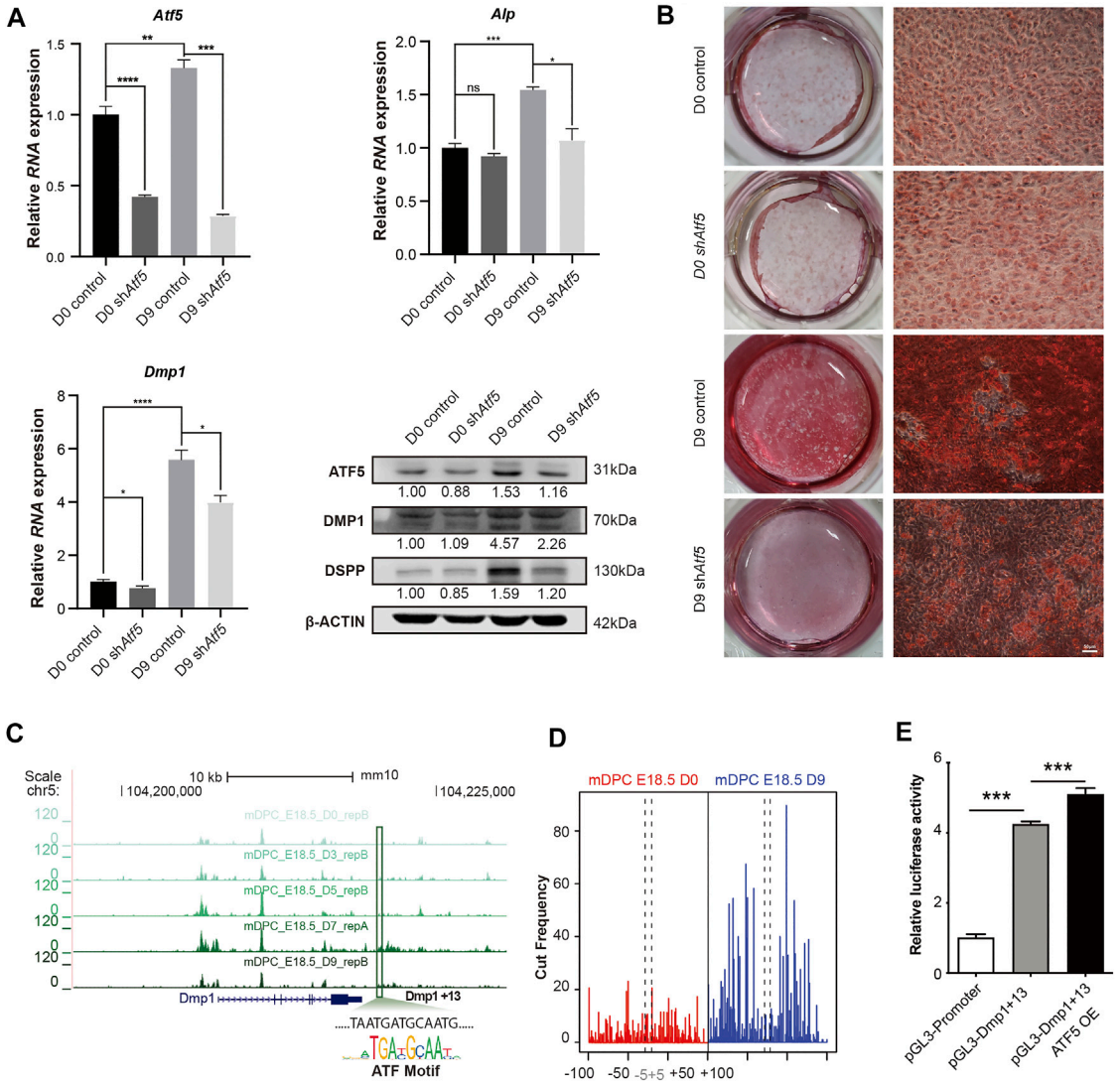

FIGURE 4 | ATF5 promotes odontoblast terminal differentiation by activating a *Dmp1*-related enhancer. Primary mDPCs of E18.5 mice cultured in mineralization induction medium for 0 days (D0) and 9 days (D9) after infection with lentivirus-expressing shRNA against Atf5 and empty control. **(A)** qRT-PCR and Western blot showing expression of related odontoblast markers in different conditions. **(B)** Alizarin red staining showing fewer mineralization nodules on D9 in sh*Atf5* group compared with control. **(C)** A chromatin region in the downstream of *Dmp1* (Dmp1+13) gradually gained accessibility (belonged to cluster 3 in **Figure 3**) in time–course ATAC-seq with a typical binding motif for the ATF family. **(D)** Tn5-transposase footprint based on CENTIPEDE revealed the ATF-binding motif in **(C)** "protected" on D9 ATAC-seq. **(E)** Dual-luciferase assay showing the enhancer activity of Dmp1+13 could be promoted by the overexpression of ATF5 in mDPC cells. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

(**Figure 4E**). Collectively, these findings affirmed that ATF5, a major TF enriched in odontoblast-related NFRs, potentially promoted odontoblast terminal differentiation partially via the induction of *Dmp1*-related enhancer activity.

DISCUSSION

The mechanism by which TFs interact with chromatin to regulate gene expression and influence cell fates is one of the leading questions in the genome and developmental biology. A previous study combining *in vivo* genetic lineage tracing and bulk RNA-seq along with ChIP-seq against histone modifications in postnatal dental pulp perivascular-derived mesenchymal stem cells illustrated that odontoblast-specific genes, such as *Dspp* and Dmp1, were in a transcriptionally permissive state inhibiting by RING1B (Yianni and Sharpe, 2018). However, during embryonic development, how the fate of odontoblast lineage is initiated remained unclear. The variation in regional accessibility is the first step for chromatin landscape alternation (Calo and Wysocka, 2013). In this study, we described the transcriptome changes during odontoblast terminal differentiation via single-cell RNA-seq from OC-positive odontogenic lineage combined with bulk RNA-seq from mDPC induced by mineralization medium.

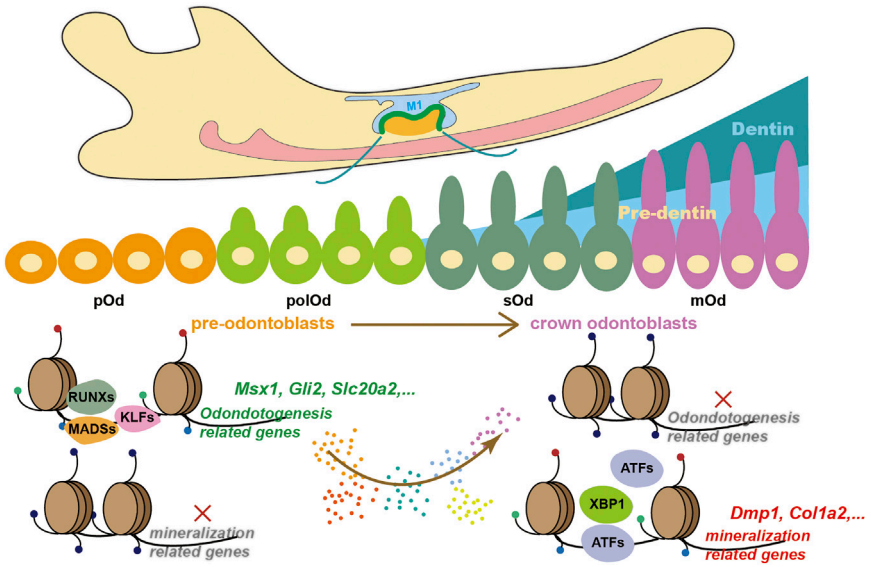

FIGURE 5 | A proposed working model describing how chromatin accessibility predetermines odontoblast terminal differentiation.

Following the identification of marker genes, we found that the increase in chromatin accessibility of these markers, such as *Dmp1* (Balic and Mina, 2011) and *Dspp* (Chen et al., 2004), occurred much earlier than the initiation of transcriptional upregulation. This integrated analysis underpinned the predecisive roles of chromatin accessibility during odontoblast terminal differentiation (**Figure 5**).

At the end of differentiation, marker genes upregulated along with the enriched NFRs were associated with the major biological process of odontoblast, including GO terms such as "bone mass" and "bone volume." Because several odontoblast markers and the same induction medium are shared with osteoblast, it is unclear whether the *in vitro* induction is via odontoblast or osteoblast differentiation. *Dmp1* and *Dspp* are two marker genes for odontoblast differentiation; however, loss of either of them causes bone defect (Verdelis et al., 2008; Sun et al., 2010). Our integrated analysis between single-cell RNA-seq from odontogenic lineage and bulk RNA-seq from *in vitro* odontoblast induction revealed that mineralization using mDPC isolated from E18.5 lower molar is comparable with crown odontoblast differentiation at the transcriptional level. *Runx2* is a positive regulator of osteoblast differentiation but is nearly undetectable after the bell stage of a tooth (Chen et al., 2009) and was significantly down-regulated during *in vitro* differentiation. Also, NFRs with *Runx2* gradually lost accessibility during this biological process. These results imply that "downregulation of *Runx2*" may be a crucial aspect to differentiate between odontoblast and osteoblast differentiation. We also noticed that there were more open chromatin regions in the odontoblast *in vitro* at D0 than OC-positive and -negative cells. This may be due to the batch effects in sequencing and culture condition. Nevertheless, the *in vivo* model is the best validation method for whether a gene potentially promotes odontoblast differentiation.

Other than terminal differentiation, we revealed that NFRs near odontogenic genes, including *Gli2*, *Msx1*, and *Runx2*, were gradually "closed." These genes have been extensively explored for their roles in early odontogenesis (Alappat et al., 2003), and any defect would lead to hereditary tooth abnormality (Hardcastle et al., 1999) or tooth agenesis (Mundlos et al., 1997). The loss of these odontogenic potential may be attributed by the long-term *in vitro* culture or the differentiation, whereby the differentiation of dental papilla cells becomes irreversible. Compelling evidence shows that dental mesenchymal cells (Yoshikawa and Kollar, 1981) or dental pulp stem cells (Hu et al., 2014) after PN0 cannot form tooth when recombined with dental epithelium before E11.5. In our recent work, we analyzed the role of Zeb1 and Sall1 in the regulation of odontoblast lineage and found that these 2 TFs can only alter odontogenic-related NFRs in mDPCs from E16.5 but not PN0 lower molars. These results suggested that despite the effect of culture, mDPCs at late embryonic stages (i.e., PN0) lose their odontogenic potential as depicted by the loss of accessibility of chromatin regions.

Assessment of transcription factor motifs enriched in ATAC-seq peaks can yield insights into the transcriptional regulatory network (Miraldi et al., 2019). Previously, we made a direct imputation on the relationship between the most possible transcription factors based on the highest expression (Liu et al., 2020). Herein, we surprisingly found that the enrichment and expression of TFs in the same family (with similar domains) exhibited a distinct stage-dependent pattern. For instance, KLF4 and KLF5, and other zinc finger TFs were enriched in clusters 1, 2, and 4 NFRs associated with the

expression of odontogenic genes. We had previously revealed that other zinc-finger TFs not enriched also contribute to early chromatin accessibility maintenance, such as ZEB1 (Xiao et al., 2021a) and SALL1 (Lin et al., 2021). Given the similar binding motifs of all the transcription factors belonging to the same family, it is reasonable to hypothesize that the functional redundancy among zinc-finger TF robustly maintains the odontogenic potential. Such redundancy is common in other tissues, for instance, the function of *tfap2a* and *tfap2c* in melanocyte differentiation (Li and Cornell, 2007).

In summary, the present study outlined the landscape of chromatin accessibility during odontoblast terminal differentiation, broadening our understanding of how chromatin associates and predetermine the fate of odontoblast lineage. Also, our findings could serve as a consensus for understanding dentinogenesis using dental mesenchymal stem cells. Moreover, several TF families have been revealed and are associated with chromatin accessibility in a stage-dependent manner. ATF5, for instance, promotes this process. However, a detailed functional analysis of the interaction between a specific TF or TF family and chromatin regions (such as enhancers) needs more *in vivo* validation.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: National Genomic Data Center, ngdc.cncb.ac.cn, CRA004359.

ETHICS STATEMENT

The animal study was reviewed and approved by the Institutional Animal Care and Use Committees at the School and Hospital of Stomatology attached to Wuhan University (protocol no. S07920070I) and the Institutional Animal Care and Use Committees at the School of Life Science in Fujian Normal University (protocol no. 20210007).

AUTHOR CONTRIBUTIONS

QZ contributed to the conception of the study, data acquisition, and critically revised the manuscript. ZH contributed to the conception and design of the study, data acquisition, analysis and interpretation of the data, and critically revised the manuscript. HZ contributed to the conception, data acquisition, drafting the manuscript and critical revision of the manuscript. CL, YL, YX, YC, FP, and ZC contributed to the study design and critically revised the manuscript. HL contributed to the study design, conception, data acquisition, analysis and interpretation, and drafted and critically revised the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2021.769193/full#supplementary-material

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