Stem Cells International

Epigenetic Regulation of Dental Tissue-Derived Stem Cells

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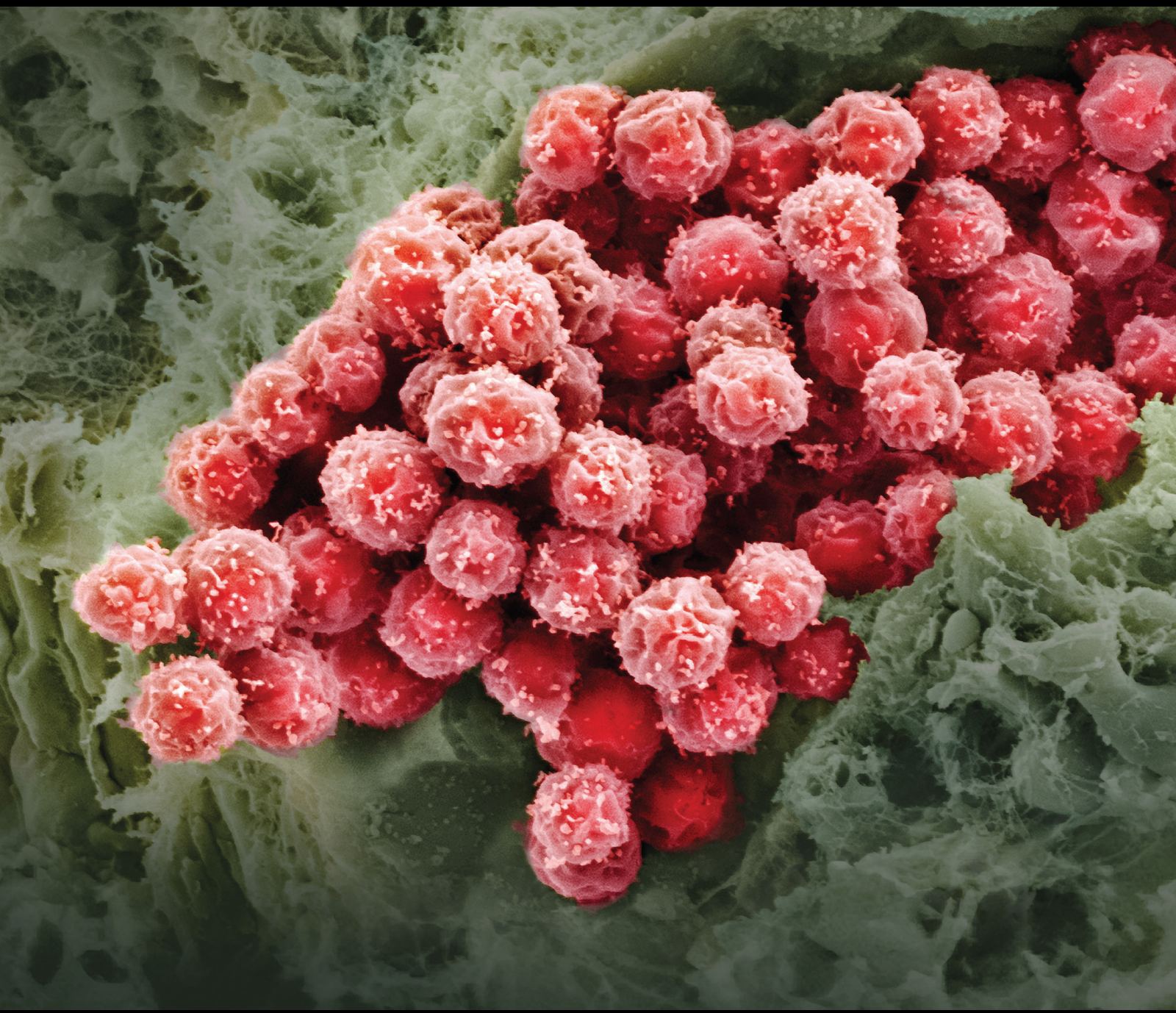

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Research Article

Single-Cell Transcriptome Analysis Defines Expression of Kabuki Syndrome-Associated KMT2D Targets and Interacting Partners

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Objectives. Kabuki syndrome (KS) is a rare genetic disorder characterized by developmental delay, retarded growth, and cardiac, gastrointestinal, neurocognitive, renal, craniofacial, dental, and skeletal defects. KS is caused by mutations in the genes encoding histone H3 lysine 4 methyltransferase (KMT2D) and histone H3 lysine 27 demethylase (KDM6A), which are core components of the complex of proteins associated with histone H3 lysine 4 methyltransferase SET1 (SET1/COMPASS). Using single-cell RNA data, we examined the expression profiles of *Kmt2d* and *Kdm6a* in the mouse dental pulp. In the incisor pulp, *Kmt2d* and *Kdm6a* colocalize with other genes of the SET1/COMPASS complex comprised of the WD-repeat protein 5 gene (*Wdr5*), the retinoblastoma-binding protein 5 gene (*Rbbp5*), absent, small, and homeotic 2-like protein-encoding gene (*Ash2l*), nuclear receptor cofactor 6 gene (*Ncoa6*), and Pax-interacting protein 1 gene (*Ptip1*). In addition, we found that *Kmt2d* and *Kdm6a* coexpress with the downstream target genes of the Wingless and Integrated (WNT) and sonic hedgehog signaling pathways in mesenchymal stem/stromal cells (MSCs) at different stages of osteogenic differentiation. Taken together, our results suggest an essential role of KMT2D and KDK6A in directing lineage-specific gene expression during differentiation of MSCs.

1. Introduction

Kabuki syndrome (KS) is a rare genetic disorder caused by mutations in the histone modifier genes encoding histone H3 lysine 4 methyltransferase (*KMT2D*) and histone H3 lysine 27 demethylase (*KDM6A*) (OMIM: #147920 and #300867) [1, 2]. The main clinical manifestations of KS include dysmorphic facial features, skeletal abnormalities, intellectual disability, hearing loss, and retarded postnatal growth. In addition, KS frequently associates with various dental abnormalities such as abnormal tooth number, hypodontia, microdontia, widely spaced teeth, and enamel hypoplasia [3–5].

Mutations in *KMT2D* are the most common cause of KS and account for 75% of cases, whereas mutations in *KDM6A* cause up to 5% of cases [6–8]. In mice, *Kmt2d* and *Kdm6a* are essential during early embryonic development and exhibit a broad and distinct expression pattern in most adult tissues [6, 7]. As part of the complex of proteins associated with histone H3 lysine 4 methyltransferase SET1 (SET1/COMPASS), KMT2D and KDM6A physically associate with a protein module comprised of the WD-repeat protein 5

(WDR5), retinoblastoma-binding protein 5 (RBBP5), absent, small, and homeotic 2-like protein-encoding protein (ASH2L) and Dpy-30 histone methyltransferase complex regulatory subunit (DPY30) and nuclear receptor cofactor 6 (NCOA6), Pax-interacting protein 1 (PTIP), and PTIP-associated protein 1 (PA1) [6]. Recent research has shown that association of KMT2D with the histone acetyltransferases p300 and CBP encoded by *EP300* and *CREBBP* is capable of establishing active enhancer states enriched in histone H3 lysine 4 monomethylation and histone H3 lysine 27 acetylation (H3K4me1/H3K27ac) to facilitate long-distance gene activation [9]. In addition to p300/CBP, the interplay between KMT2D and the SWI/SNF related, matrix associated, and actin-dependent ATP-dependent chromatin remodeling factors (SMARCA4 and SMARCB1) promotes cell type-specific enhancer activation [10]. Research has demonstrated that haploinsufficiency of KMT2D is sufficient to lead to the classical KS phenotype [11]. Mechanistically, haploinsufficiency of KMT2D causes structural changes in chromatin, which affects the mechanical properties of the nucleus [12]. By contrast, haploinsufficiency of *KDM6A* leads to postnatal growth restriction, microcephaly, cerebral atrophy, seizures, facial dysmorphism, and cleft palate [13]. KDM6A plays an important role in definitive endoderm differentiation through modulating the Wingless and Integrated (WNT) signaling pathway [14]. A recent study also established that KDM6A controls human neural differentiation and dendritic morphology [15]. KDM6A is capable of changing the composition of bivalent promoters by removing histone H3 lysine 27 trimethylation (H3K27me3) marks, which in turn leads to selective upregulation of neural genes. These findings are also supported by work by Dhar et al. showing that KDM6A is required for the activation of bivalent genes during mouse embryonic stem cell differentiation [16].

Previously, using single-cell RNA sequencing (scRNA-seq), we characterized the cellular composition of the mouse incisor dental pulp [17]. Our study revealed distinct patterns of cell state heterogeneity in mesenchymal stem/stromal cells (MSCs) undergoing different stages of differentiation, including differentiated cells representing osteoblasts and odontoblasts. In this study, we examined the expression profile of *Kmt2d* and *Kdm6a* in different subpopulations of pulp cells. Our investigation revealed that genes encoding members of the WRAD protein complex display partially overlapping expression patterns in MSCs. We also noted that members of the WNT and sonic hedgehog signaling (SHH) pathways, which are known downstream targets of KMT2D [18], exhibit similar expression patterns across distinct subclasses of pulp cells. Collectively, our analysis sheds new light on the role of KMT2D and KDK6A in the lineage commitment of MSCs.

2. Materials and Methods

2.1. scRNA-seq. Incisor dental pulps from 6-day wild-type mice were obtained as described previously [19]. The cell suspension was then assessed with a Countess II FL Automated Cell Counter (Thermo Fisher Scientific Inc., Waltham, MA). A total of 8,000 cells were loaded into for capture into single channels of the 10× Genomics Chromium Controller (10× Genomics, Pleasanton, CA). After cell lysis, complementary DNA was synthesized and amplified for library preparation and sequencing with the HiSeq 4000 (Illumina, San Diego, CA).

2.2. Genome-Wide ATAC-seq, hMeDIP-seq, and Bulk RNA-seq. We used the Active Motif (Carlsbad, CA) service to perform Assay for Transposase-Accessible Chromatin using sequencing (ATAC-seq), hydroxymethylated DNA immunoprecipitation sequencing (hMeDIP-seq), and bulk RNA-seq. The extraction of RNA was performed with the RNAeasy Mini/Midi kit (Qiagen, Germantown, MD). Whole-transcriptome analysis was performed with the Illumina NextSeq 500. The Burrows-Wheeler Aligner (BWA) algorithm with default settings was used to map the paired-end sequencing reads to the mouse genome. For the hMeDIP-seq experiment, we applied the Monarch Genomic DNA Isolation kit (New England Biolabs, Ipswich, MA) to isolate genomic DNA. The sonicated DNA was then ligated to the Illumina adaptors. The antibody AM39791 to 5hmC was used to produce DNA demethylation tags. Next, the libraries were generated from immunoprecipitated DNA and sequenced with the NextSeq 500. Input DNA without the immunoprecipitation step was used as a control.

2.3. Computational Analysis. The sequencing reads with more than one mismatch were excluded. The STAR aligner was used, and only reads with MAPQ scores greater than 255 were included. The 10× Genomics barcodes and a unique molecular identifier threshold were used for filtering and generation of a pulp digital counts matrix. The expression pattern of the pulp cells was measured by dispersion and dimensionality reduction with uniform manifold approximation and projection (UMAP) [20–22]. The neighborhood clustering graph was performed with the Leiden algorithm [23]. The neighborhood graph was corrected using the batch remover BBKNN [24]. The Illumina base call files were converted to FASTQ format and aligned to the mm10 genome. The MACS2 peak-calling program was used for determining chromatin accessibility across the genome [25]. The BWA algorithm with default settings was used to map hMeDIP-seq reads. The MACS peak finding algorithm was used to map methylated segments (18). The 5hmC enrichment was presented as average of values for all target regions. The STAR aligner and the Subread package were used for RNA-seq fragments, feature counts (FPKM assignment to genes), and DESeq2 differential analysis [26–28].

3. Results

3.1. Expression of Members of the SET1/COMPASS and PRC2 Complexes Associated with KMT2D and KDM6A. KMT2D and KDM6A function as components of the transcriptional activation complex SET1/COMPASS to establish open chromatin domains associated with active enhancers (H3K4me1-rich) and promoters (H3K4me2/3-rich) [6]. Protein-protein interaction networks are critical for a system-level understanding of gene regulatory processes. By

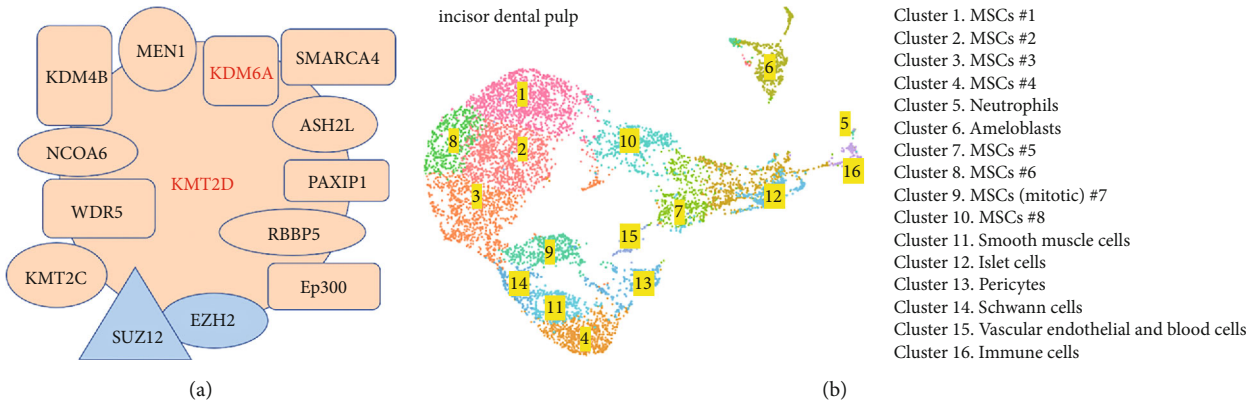

Figure 1: The interaction network of KMT2D and KDM6A and scRNA-seq clustering analysis of the mouse incisor dental pulp. (a) STRING interaction network analysis revealed that both KMT2D and KDM6A associate with other components of the SET1/COMPASS complex, including KMT2C, ASH2L, WDR5, RBBP5, KDM4B, p300, NCOA6, SMARCA4, PAXIP1, and MEN1. In addition, this analysis revealed that KMT2D and KDM6A physically interact with EZH2 and SUZ12, key components of the PRC2. (b) The mouse incisor dental pulp is composed of 16 distinct cell types that represent MSCs at different stages of osteogenic and odontogenic differentiation as well as pericytes, ameloblasts, smooth muscle cells, islet cells, Schwann cells, vascular endothelial, and blood cells.

analyzing the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) interaction network (https://string-db.org), we discovered that both KMT2D and KDM6A have a specific set of interacting partners (Figure 1(a)). Among the members of the SET1/COMPASS complex, we identified KMT2C, ASH2L, WDR5, RBBP5, KDM4B, p300, NCOA6, SMARCA4, PAXIP1, and MEN1. The STRING analysis revealed that enhancer of zeste homolog 2 (EZH2) and suppressor of zeste 12 protein homolog (SUZ12), key components of the polycomb repressive complex 2 (PRC2), physically interact with KMT2D and KDM6A. Previously, we reported the results of an scRNA-seq analysis to define the expression pattern of developmental genes in the incisor dental pulp [17]. Based on the expression of key genes, we grouped the pulp cells into 16 clusters (Figure 1(b)). In the current study, we performed a more in-depth analysis of the *Kmt2d* and *Kdm6a* expression. *Kmt2d* is mainly expressed in clusters 1, 2, 3 8, 9, 13, 14, and 15, whereas *Kdm6a* displayed a broader expression domain with enrichment in clusters 1, 2, 3, 4, 6, 8, 9, 13, 14, 15, and 16 (Figure 2). Additionally, we analyzed the expression of genes encoding critical partners of KMT2D and KDM6A within the SET1/COMPASS complex. Similar to the expression pattern of *Kdm6a*, *Ash2l* is enriched in clusters 1, 2, 3, 4, 6, 8, 9, 13, 14, 15, and 16. *Wdr5* is expressed in clusters 2, 3, 8, and 9, while *Rbbp2* and lysine demethylase 4b gene (*Kdm4b)* are relatively weakly expressed in dental pulp. *Ep300* is vigorously expressed in clusters 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 13, 14, 15, and 16. *Ncoa6* has limited expression and is mainly enriched in clusters 2 and 9. *Smarca4* exhibits a wide expression range in clusters 1, 2, 3, 4, 6, 8, 9, 10, 13, 14, 15, and 16. The expression of *Paxip1* is limited to cluster 9, whereas *Men1* is enriched in clusters 1, 2, 3, 8, 9, and 14. In addition to the members of the SET1/COMPASS complex, we analyzed the expression profiles of genes encoding EZH2 and SUZ12. *Ezh2* is enriched in clusters 1, 2, 3, 4, 6, 8, 9, 13, 14, and 15. *Suz12* displays a very similar expression profile with enrichment in clusters 1, 2, 3, 4, 6, 8, 9, 13, 14, 15, and 16 (Figure 2).

3.2. Expression of Downstream Targets of KMT2D Associated with the WNT and SHH Signaling Pathways. We analyzed the expression profiles of members of the WNT and SHH signaling that are known to be downstream targets of KMT2D. We detected expression of *Wnt4* in clusters 1, 2, 6, 8, 9, and 10 (Figure 3). By contrast, *Wnt5a* is more vigorously expressed in clusters 1, 2, 3, 4, 8, 9, 10, 11, 13, and 14, and *Wnt5b* expression is limited to cluster 9. *Wnt6* is enriched in clusters 3, 6, and 9. The expression of *Wnt10a* is restricted to clusters 6 and 9. *Axin2* encoding axis inhibition protein 2 is only enriched in cluster 9. The *Ctnnb1* gene encoding b-catenin displayed very broad and vigorous expression in the incisor dental pulp, with enrichment in clusters 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 13, 14, 15, and 16. *Tle2* encodes transducing-like protein 2, which acts as a transcriptional repressor [29]. We identified high expression of *Tle2* in clusters 1, 2, 3, 4, 7, 8, 9, 10, 11, 13, and 14. *Lef1* and *Tcf7*, which encode transcription factors of the WNT pathway, showed a relatively restricted range of expression; *Lef1* is detected in clusters 4, 9, and 11, whereas *Tcf7* is only enriched in clusters 3, 4, 9, 11, and 14. We also analyzed the expression patterns of members of SHH signaling such as *Gli1*, *Gli3*, and *Ptch1*. *Gli1* exhibits predominant expression in clusters 3, 4, 6, 9, 11, and 14. *Gli3* has a broad expression pattern with a relatively high expression in clusters 1, 2, 3, 4, 6, 8, 9, 11, 13, and 14. *Ptch1* is also broadly expressed, enriched in clusters 1, 2, 3, 4, 6, 7, 8, 9, 10, 11, 13, 14, and 15 (Figure 3).

3.3. Chromatin Accessibility of the KMT2D- and KDM6-Associated Factors and Downstream Target Genes. According to the scRNA-seq data, *Rbbp3*, *Kdm4b*, and *Axin2* exhibit relatively weak expression in the dental pulp (Figures 2 and 3). We next investigated the genomic structure of these genes for specific epigenetic marks and chromatin accessibility. Open chromatin regions and the DNA demethylation mark 5hmC are reliable indicators of active

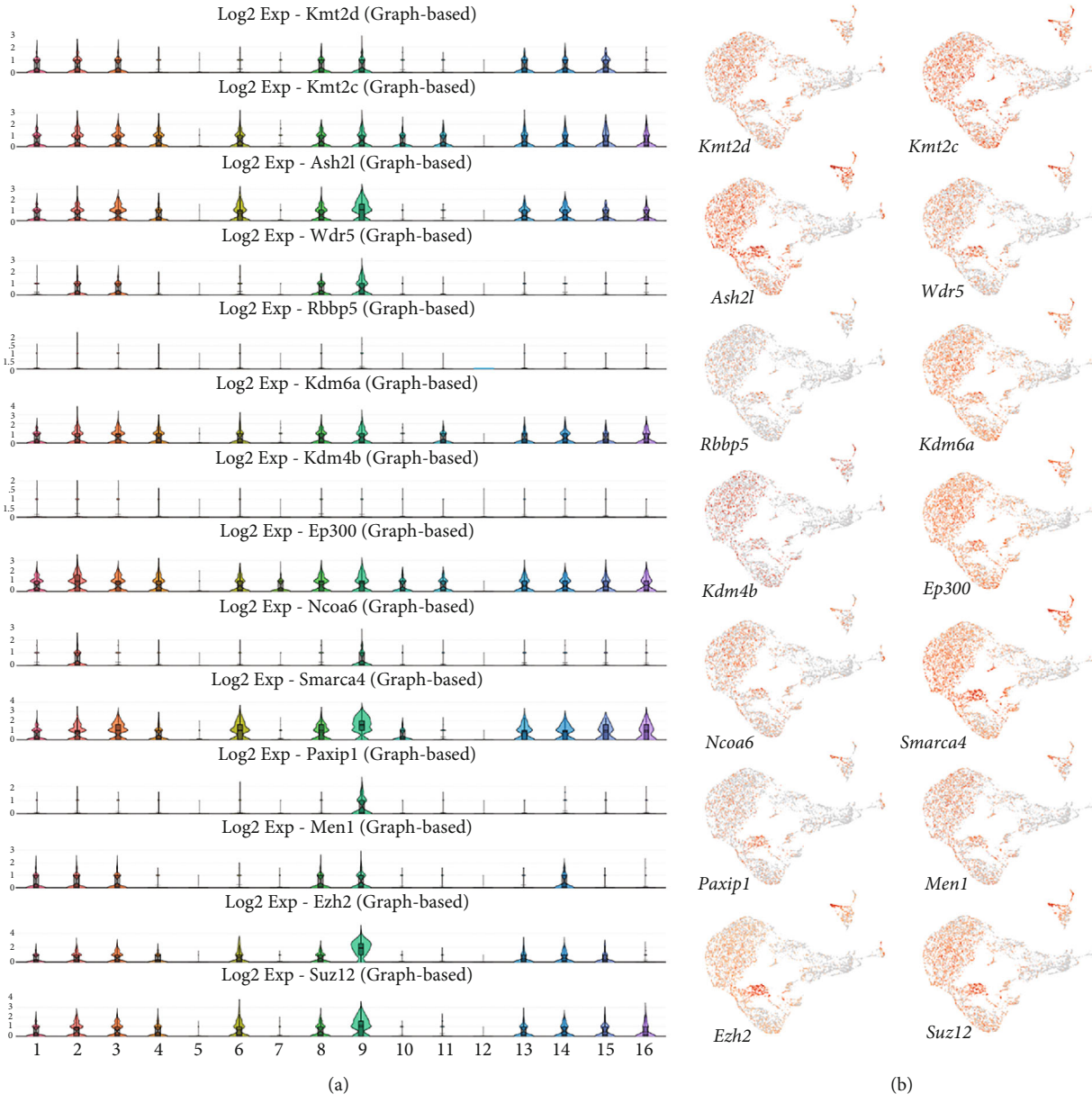

Figure 2: Expression of genes encoding proteins associated with KMT2D and KDM6A. (a) Violin plots of genes encoding KMT2C, ASH2L, WDR5, RBBP5, KDM4B, p300, NCOA6, SMARCA4, PAXIP1, MEN1, EZH2, and SUZ12. (b) UMAP visualization of genes encoding components of the SET1/COMPASS and PRC2 complexes.

genomic states. Previously, using ATAC-seq and hmeDIP-seq assays, we identified accessible chromatin regions and genome-wide enrichment of 5hmC in the mouse dental pulp [30, 31]. By analyzing these datasets, we found that the vast majority of genes encoding protein partners and downstream targets of KMT2D and KDM6A exhibit open chromatin enriched in 5hmC (data not shown). Our analysis also revealed that, despite the weak expression of *Rbbp3*, *Kdm4b*, and *Axin2*, the genomic regions across these genes retain open chromatin configurations (Figure 4). Additionally, we detected specific enrichment of 5hmC in *Rbbp3*, *Kdm4b*, and *Axin2*. Collectively, these data suggest that even relatively weakly expressed genes acquire active chromatin states in the mouse dental pulp.

4. Discussion

Our study revealed that genes encoding KMT2D and KDM6A and other components of the SET1/COMPASS activation complex display overlapping expression patterns within the mouse dental pulp. Sixteen clusters of cells represent MSCs at different stages of osteogenic and odontogenic differentiation as well as pericytes, ameloblasts, smooth muscle cells, islet cells, Schwann cells, vascular endothelial cells, and blood cells [17]. We identified a core set of genes that are common in clusters 1, 2, 3, 8, and 9, which represent the five subpopulations of MSCs (Figure 5). These genes encode KMT2D, KDM6A, KMT2C, ASH2L, p300, and SMARCA4, which are key components of the SET1/

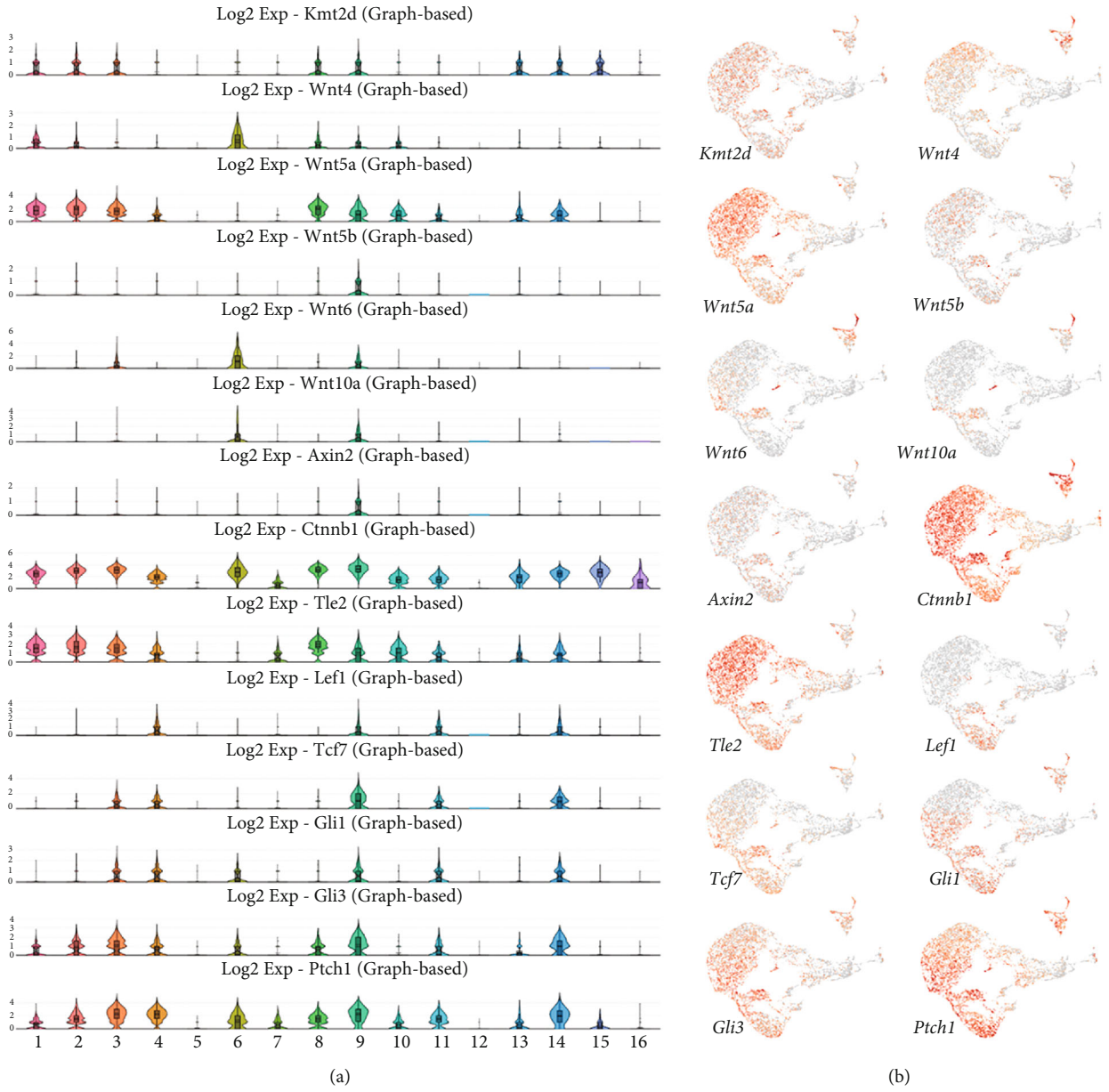

Figure 3: Expression of genes encoding downstream targets of KMT2D and KDM6A. (a) Violin plots of genes encoding *Wnt5a*, *Ctnnb1*, *Tle2*, *Gli3*, and *Ptch1*. (b) UMAP visualization of genes encoding components of the WNT and SHH signaling pathways.

COMPASS complex (Figure 6). The mouse STRING database revealed that KMT2D and KDM6A also interact with EZH2 and SUZ12, which are subunits of the PRC2. We analyzed the expression patterns of *Ezh2* and *Suz12* in the incisor dental pulp and found that both genes exhibit vigorous expression in all pulp clusters including MSCs.

A recent investigation reported the involvement of KMT2D in regulating WNT/β-catenin and SHH signaling in dental epithelium [18]. Both *KMT2D* and *KDM6A* are coexpressed in the dental epithelium of human tooth germs [32]. Hence, we examined the expression profiles of the canonical targets of KMT2D in the dental pulp. We deduced that *Wnt5a*, *Ctnnb1*, *Tle2*, *Gli3*, and *Ptch1* represent a common set of genes for all five clusters of MSCs (Figures 6 and 7).

KMT2D is a major mammalian histone H3K4 methyltransferase that interacts with transcription factors and chromatin remodeling proteins to mediate transcriptional activation [33]. Specific nonsense and frameshift mutations in the *KMT2D* sequence have been reported to lead to KS, which is characterized by a dysmorphic face, postnatal growth retardation, skeletal abnormalities, midfacial hypoplasia, cleft lip/palate, and mental problems [34, 35]. Common dental abnormalities in KS patients are ectopic upper molars, screwdriver-shaped upper incisors, delayed tooth eruption, widely spaced teeth, enamel hypoplasia, missing

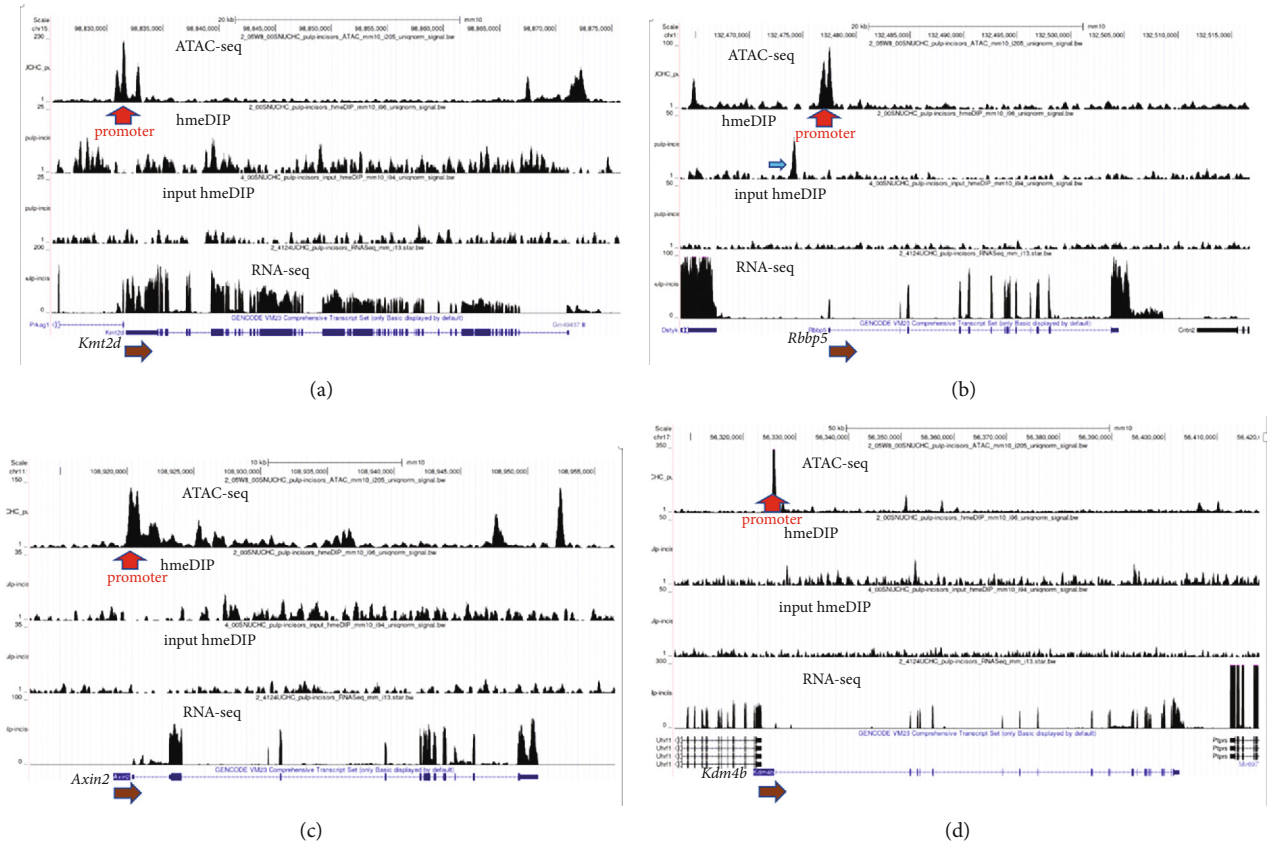

Figure 4: Chromatin structures of *Kmt2d*, *Rbbp5*, *Axin2*, and *Kdm4b* in the mouse incisor dental pulp. (a) ATAC-seq analysis indicated that the chromatin is accessible in *Kmt2d*. Strong peaks were detected in the transcription start site (TSS) marked with a red arrow. hmeDIP-seq results showed 5hmC enrichment in gene bodies (compare to the input), which correlates well with RNA expression. The brown arrow indicates the direction of transcription. (b) The hmeDIP-seq and ATAC-seq peaks are enriched in the promoter (blue arrow) and TSS (red arrow) of *Rbbp5*. The brown arrow indicates the direction of *Rbbp5* expression. (c) The ATAC-seq and hmeDIP-seq peaks are enriched in the gene body of *Axin2*. A strong ATAC-seq signal was also detected in the TSS (red arrow). The direction of *Axin2* expression is marked with a brown arrow. (d) A strong ATAC-seq peak is present in the TSS (red arrow) of *Kdm4b*. 5hmC is enriched within the gene body of *Kdm4b*. The brown arrow indicates the direction of *Kdm4b* expression.

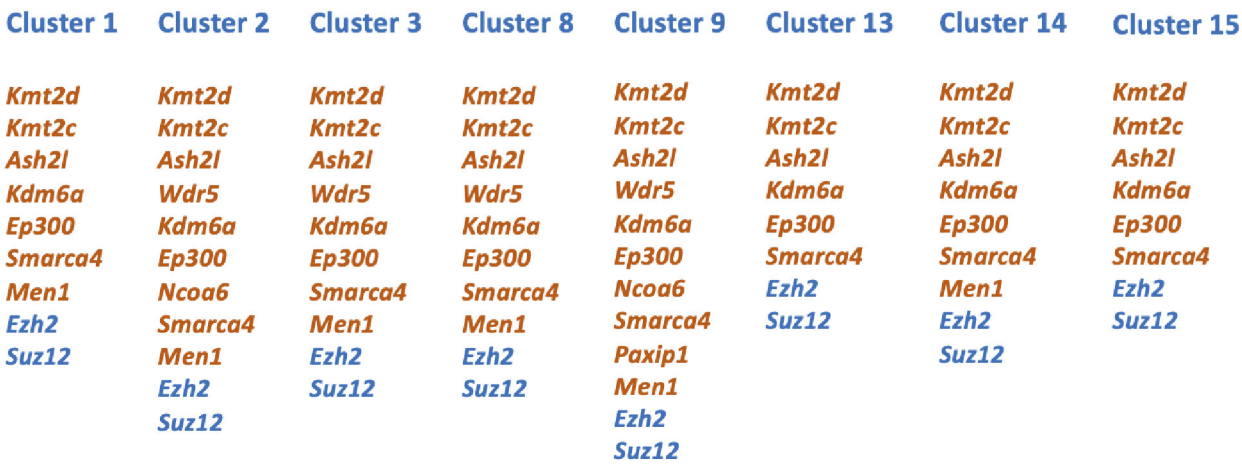

Figure 5: Core set of epigenetic factors that are common in five subpopulations of MSCs, pericytes, Schwann cells, and vascular endothelial cells. These factors are KMT2D, KDM6A, KMT2C, ASH2L, p300, and SMARCA4, which are the key components of the SET1/COMPASS complex (brown), as well as EZH2 and SUZ12 (blue), which are subunits of the PRC2.

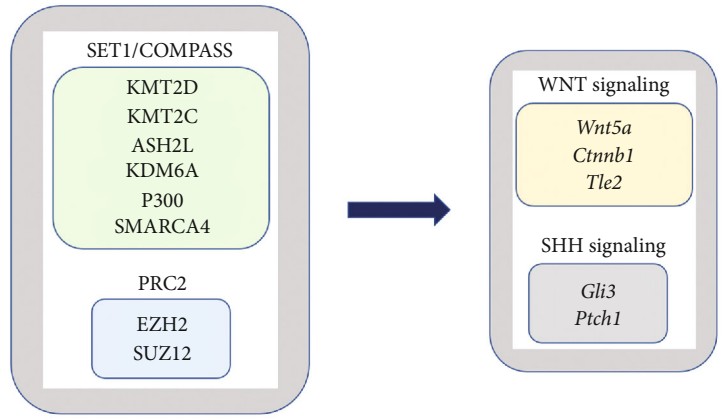

Figure 6: KMT2D and KDM6A form functional interactions with other components of the SET1/COMPASS complex and the PRC2 to control the expression of downstream targets of the WNT and SHH signaling pathways.

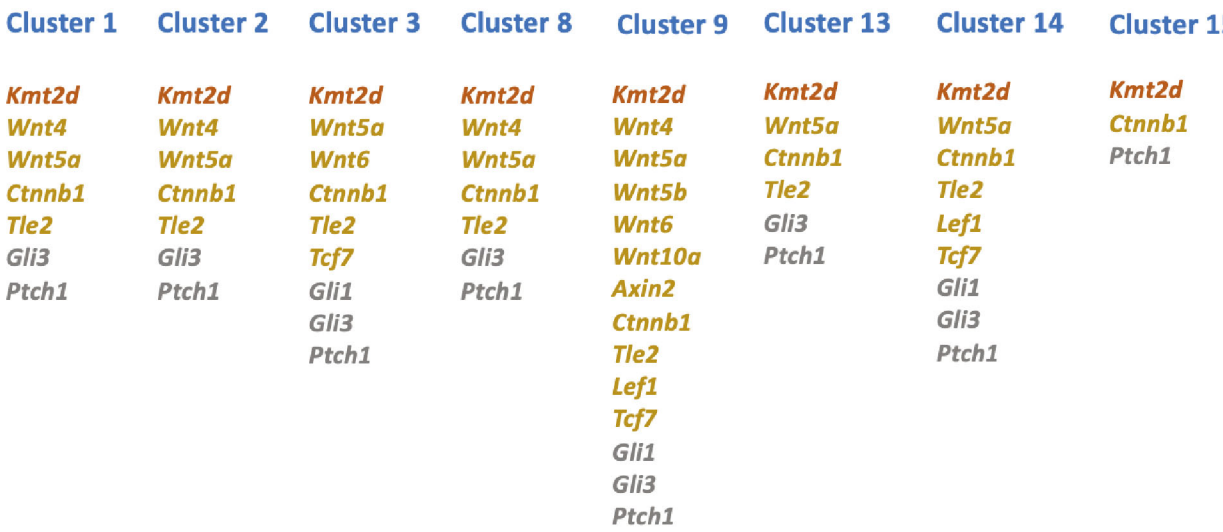

Figure 7: Core set of genes that are common in five subpopulations of MSCs. These genes are *Wnt5a*, *Ctnnb1*, *Tle2*, *Gli3*, and *Ptch1*. *Kmt2d* is marked in brown. Members of the WNT signaling pathway are shown in light brown and members of SHH signaling are in grey.

teeth, high-arched palate, micrognathia, small dental arches, hypodontia, microdontia, severe maxillary recession, congenital absence of teeth, and malocclusion [4, 5, 35–38].

With the assistance of histone H3K27 acetyltransferases CBP and p300, KMT2D is involved in enhancer activation and cell-type-specific gene expression during differentiation [33]. In chondrocytes, KMT2D regulates the expression of *Shox2* [39]. Research has established that a decrease in *Shox2* expression in *Kmt2d*-depleted mouse chondrocytes can release *Sox9* inhibition, thereby causing chondrocyte differentiation. *Kmt2d* is expressed in the developing mouse calvarial osteoblasts, epithelia, and neural tissues [40]. Moreover, the heterozygous loss of *Kmt2d* impairs the neuromuscular junction, muscle cell differentiation, and myofiber regeneration [2]. In addition, a growing body of research indicates that defects in neural crest development are a major cause of KS [41, 42]. Mouse knockout studies have revealed that *Kmt2d* and *Kdm6a* are required for proper differentiation of cranial neural crest cells [42, 43]. *Kmt2d* depletion in *Xenopus* impairs neural crest formation, which is accompanied by reduced levels of H3K4me1 and H3K27ac, a hallmark feature of active chromatin states [41]. Interestingly, in *Danio*, inactivation of *Kmt2d* affected all examined tissues, whereas ablation of *Kdm6a* had a more selective impact on craniofacial and heart development [44]. These findings provide mechanistic insights into the pathogenesis of KS and indicate KMT2D as a key regulator of development and differentiation.

The dental pulp-derived MSCs exhibit multipotent differentiation capacity and are considered a promising stem cell source for tissue engineering and regenerative medicine [45]. Over the past decades, a growing body of scientific evidence shows that epigenetic mechanisms play a major role in lineage specification and gene regulatory networks underlying MSC differentiation [46–48]. Therefore, further research toward identifying the molecular pathways through which KMT2D controls gene expression in dental pulp stem

cells will provide a more refined understanding of the mechanisms underlying the pathogenesis of KS.

Data Availability

All datasets from this study will be made available upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors' Contributions

This project was conceptualized by D.B. Experiments were carried out by P.R., A.V., and B.E. The analysis was performed by B.E., D.B., and P.J. The manuscript was drafted by B.E. and D.B. and reviewed by D.G. and M.M. Project supervision was carried out by D.B. All authors have read and agreed to the published version of the manuscript.

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Review Article

The Role of Epigenetic in Dental and Oral Regenerative Medicine by Different Types of Dental Stem Cells: A Comprehensive Overview

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Postnatal teeth, wisdom teeth, and exfoliated deciduous teeth can be harvested for dental stem cell (DSC) researches. These mesenchymal stem cells (MSCs) can differentiate and also consider as promising candidates for dental and oral regeneration. Thus, the development of DSC therapies can be considered a suitable but challenging target for tissue regeneration. Epigenetics describes changes in gene expression rather than changes in DNA and broadly happens in bone homeostasis, embryogenesis, stem cell fate, and disease development. The epigenetic regulation of gene expression and the regulation of cell fate is mainly governed by deoxyribonucleic acid (DNA) methylation, histone modification, and noncoding RNAs (ncRNAs). Tissue engineering utilizes DSCs as a target. Tissue engineering therapies are based on the multipotent regenerative potential of DSCs. It is believed that epigenetic factors are essential for maintaining the multipotency of DSCs. A wide range of host and environmental factors influence stem cell differentiation and differentiation commitment, of which epigenetic regulation is critical. Several lines of evidence have shown that epigenetic modification of DNA and DNA-correlated histones are necessary for determining cells' phenotypes and regulating stem cells' pluripotency and renewal capacity. It is increasingly recognized that nuclear enzyme activities, such as histone deacetylases, can be used pharmacologically to induce stem cell differentiation and dedifferentiation. In this review, the role of epigenetic in dental and oral regenerative medicine by different types of dental stem cells is discussed in two new and promising areas of medical and biological researches in recent studies (2010-2022).

1. Introduction

The epigenetic chromatin state will change without deoxyribonucleic acid (DNA) sequence alterations, leading to gene regulation. This occurs when a signal is received from an initiator, such as noncoding RNAs (ncRNAs), depending on the environmental changes around the cell. An initiator determines which regions of a chromosome need to be affected, thus changing gene expression. Histone modifications and DNA methylation, epigenetic maintainers, allow chromatin to remain markedly epigenetically altered [1]. It is known that stem cells are crucial to the regeneration of damaged tissues, and they come from the embryonic stem cells or the postnatal origin (adult stem cells). The epigenetic state of embryonic stem cells permits self-renewal or differentiation into any pluripotent cell type, unlike adult stem cells, which have more restricted lineage potential (multipotent) [2]. The dental pulp or periodontium, primarily a source of dental stem cells (DSCs), functions as a source of mesenchymal stem cells (MSCs). DSCs positively expressed markers related to MSCs in vitro containing cluster of differentiation (CD)146, 105, 90, 73, 59, 44, 29, 13, and STRO-1. Contrarily, some hematopoietic markers (CD45, 34, 31, and 14) were negatively expressed [3]. In many tissues,

including the dental pulp, the renewal of stem cells depends on epigenetic mechanisms to histone proteins. Epigenetics, or the modification of gene expression by environmental factors, is unaffected by any DNA sequence changes [2]. DNA methylation, histone modifications, ribonucleic acid (RNA) modifications, and ncRNAs are generally responsible for epigenetic modifications. The enzyme DNA methyltransferases (DNMTs) are responsible for DNA methylation, one of the well-explored epigenetic modifications. As a result of DNMTs, the 5-methylcytosine existing in CpG islands can be methylated into DNMTs. It is also possible to reverse DNA methylation using enzymes from the ten-eleven translocation (TET) family [3]. DNA methylation regulates the expression of phosphatase and tensin homolog (PTEN), which contributes to tumor progression and resistance to chemotherapy. It was demonstrated that overmethylation of the PTEN promoter inhibits PTEN expression in tamoxifen-resistant breast carcinoma cells and activates Protein Kinase B (AKT) and 5-Azacytidine (5-Aza) methylation of the PTEN promoter and making cells more susceptible to tamoxifen's cytotoxicity. The study demonstrated that although PTEN is essential for modulating auditory progenitors in mice and hematopoietic stem cells in zebrafish, the regulation of PTEN expression by DNA methylation could serve different purposes, as a functional part to manage lineage tumorigenesis and commitment in human adult stem cells. In comparison with adipose MSCs, placental MSCs, hematopoietic stem cells, skin fibroblasts, and osteoblasts, PTEN is not a molecule or functional marker for alveolar bone marrow-derived mesenchymal stem cells (BMSCs). Studying dental MSC characteristics, including the meager tumorigenic potential and individual cell fate of dental MSCs, is essential for developing new functional signatures between dental pulp stem cells (DPSCs) and BMSCs [4]. Furthermore, the Xi and Chen findings exhibited that PTEN is decreased in cultures of human osteosarcoma cells in comparison with osteoblasts [5]. Gong et al. showed that PTEN is less expressed in osteosarcomas than in adjacent tissues in human cell lines [6]. Furthermore, the Freeman et al. experiment briefly stated that the PTEN loss was expected in osteosarcoma [7].

Chromatin is reconstituted or relaxed by histone methyltransferases. Histone H3 is the most widespread histone modification. Repressive Histone H3 lysine 9 (H3K9) and Histone H3 lysine 27 (H3K27) precisely and dynamically regulate the transcriptional regulation of target genes. Also, Histone H3 lysine 79 (H3K79), Histone H3 lysine 4 (H3K4), and Histone H3 lysine 36 (H3K36) are correlated with activation of transcription. A variety of internal modifications of messenger RNA (mRNA), such as N^6-methyladenosine (m6A), N^1-methyladenosine (m1A), 5-methylcytosine, and 5-hydroxymethylcytosine, are crucial to mRNA stability. Methyltransferases catalyze the transformation of m6A from one state to another, while demethylases reverse it [3]. DNA methylation and posttranslational modifications are the most studied epigenetic modifications, particularly acetylation, methylation, and phosphorylation of histone proteins. Gene expression changes caused by epigenetic modifications can be targeted by drugs that inhibit enzymes, which can either inherit or accumulate during life [2]. Throughout development, the capacity of cells to specialize and differentiate increases. Stem cells in skeletal muscle, bone marrow, and fat use this process to decide whether they should undergo self-renewal or transition into new cells. Hence, adult stem cells can preserve the homeostasis of tissues by repairing and self-renewal or replacing damaged tissues through differentiation. In recent years, scientists have become increasingly interested in the possibility of replacing damaged cells within an organism. These cell populations are currently of particular interest to researchers trying to understand their regenerative ability and the potential use of these cells for immunotherapy or to treat various diseases. Although it has not been determined whether all organs have stem cells to maintain tissue turnover, many viable stem cells are available. A wide range of adult stem cells has been found in the recent past, including the cartilage, skin, intestine, blood, mammary epithelial cells, and dental pulp [8].

2. The Role of Epigenetic in Regenerative Medicine

Genetic and epigenetic mechanisms contribute to changes in gene expression programs without affecting DNA sequence. As a mammal develops, the zygote undergoes numerous differentiation events to produce various types of cells. Epigenetic mechanisms are required to acquire cell-type-specific gene expression programs during differentiation [9]. Qualitatively, epigenetic mechanisms may induce plastic, short-term changes in gene activity and quantitatively, resulting in more stable, long-term changes in gene activity. Multiple cell divisions can be transmitted epigenetic memories in cells that have been stably reprogrammed. Gene regulatory regions can be altered by the presence and activity of ectopically expressed transcription factors. Certain chromatin features have been shown to hinder the process of reprogramming the cell, and therefore, overcoming this barrier is an essential feature of the reprogramming step [10]. Over the past few years, advances in genome-wide association studies have greatly benefited regenerative medicine. Researchers were able to identify multiple somatic mutations affecting epigenome organization and the role of epigenome modifications in the development of cancers. In silico genomic feature annotations and association analysis of genetic association, linkage disequilibrium, and enriched genomic features, referred to as a Bayesian approach, identified more than 200 breast cancer-related signals [11]. Transient, genome-wide epigenomic remodeling has recently been shown to have regenerative capabilities in the formation of organoids and the regeneration of the liver following damage. The study of epigenetic genome modifications involves more than simply the regeneration of tissue and stem cells but also the prognosis and metastasis of multiple types of cancer, specifically relating to tumor microenvironments, immune control, tissue-level mechanical forces, and other cell-intrinsic mechanisms, including transcriptomics and metabolomics [12]. Moreover, epigenetic reprogramming processes essential for developing an embryo from a

fertilized egg and establishing cellular totipotency provide clues about epigenetic mechanisms with potential for regenerative therapies [13]. Nevertheless, detailed epigenomic profiling of the human body enabled researchers to determine epigenetic effects on disease development, such as regions of DNA free of nucleosomes specifically targeted by regulatory factors [14]. Also, epigenetic changes have demonstrated a prominent role in the metastasis of various types of cancer, and epigenetic modifications have contributed to the prognosis and diagnosis of various diseases such as neurodegenerative disorders. Therefore, mitochondrial RNAs have other changes apart from the epigenetic changes associated with mitosis [15].

3. The Current Used Dental Stem Cells (DSCs) in Epigenetic Modifications

In the connective tissues after birth, there are several multipotent MSCs. These bone marrow multipotent stromal cells were initially shown to proliferate *in vitro* as colony-forming unit-fibroblasts. Some stem cells share similar characteristics among tissues (adipose tissue, liver, and existing blood in the cord). In addition, they have been discovered in the follicle of the newly formed dental embryo and the root apex of growing permanent teeth, in addition to the periodontal ligament and the pulp of permanent and mature deciduous teeth [16]. DSCs have been isolated from various alternative sources of human dental tissues, such as stem cells from apical papilla (SCAPs), human exfoliated deciduous teeth (SHEDs), periodontal ligament stem cells (PDLSCs), and dental follicle precursor cells (DFPCs). Adult stem cells used for regenerative endodontic procedures, such as DFPCs and PDLSCs, can be restricted due to their lack of odontogenic mineralizing properties or, as in the case of SHEDs and SCAPs, their scarcity. A source of SHEDs is the exfoliated pulp tissue of deciduous teeth. Rather than stem cells, pulp tissue is typically used to store the cells obtained from exfoliated or extracted deciduous teeth. SHEDs may be derived incubatively via fluorescence-activated cell sorting and pulp tissue enzymatic digestion and expanded to follow the adequate number. It is relatively easy to obtain these cells, but several investigators have proved harvesting difficult. Some have reported that low volumes of SHEDs could not be obtained because of infection or lack of viable tissue [2]. MSCs are known to have regenerative potential abound in dental tissue. They include porcine DPSCs, SHEDs, PDLSCs, SCAPs, and dental follicle stem cells (DFSCs), which possess easy accessibility. There have been broad properties for DSCs in regenerative medicine due to their potential for osteogenic, adipogenic, and chondrogenic differentiation and their ability to form mineralized tissues. A possible approach will be to use bioscaffolds or biomaterials infused with growth factors. A second tack is to study the effects of natural compounds on dental-derived stem cells, such as polydatin and beer polyphenols. Stem cells in the dental pulp have been established by demonstrating pulp healing postdefect and maintaining homeostasis of related tissues. Porcine DPSC cultures promote endothelial, fibroblastic, and osteogenic (type I collagen, Alkaline phosphatase, osteocalcin, osteopontin, and osteonectin) markers. A variety of bone matrix proteins and odontoblast-specific markers such as dentin sialophosphoprotein are absent from porcine DPSCs tissue, confirming its undifferentiated status. As well as their multilineage differentiation potential, DPSCs can also be identified by the expression of surface antigens. Their surface antigens include STRO-1, CD90, 44, 73, 105, and 271. Although these are not specific markers of DPSCs, they provide further evidence of DPSCs *in vivo*. Characteristics of CD34 and 117 as hematopoietic lineage markers and glia-2, a marker of neurovascular origin, are promoted in DPSCs for the DPSCs' regenerative potential [17]. The current studies of epigenetic regulation of DSCs are summarized in Table 1 in regenerative medicine.

Cell differentiation and development rely on epigenetic modifications that regulate gene expression without altering DNA sequences. Epigenetic influences on embryonic stem cells have gained attention recently [18]. DNA methylation and histone modification as epigenetic modulatory mechanisms have been defined, the most extensively investigated. It has been shown that controlling DPSCs' self-renewal and differentiation can be therapeutic. In the last few years, intensive research has investigated how ncRNAs seem to function as epigenetic regulators of gene expression. Because ncRNAs are implicated in regulating gene expression during health and disease, concluding inflammatory, and reparative processes, they are of particular importance, as a diagnostic biomarker or as part of a dental therapy program; epigenetic modifications may be an effective, promising option [19]. An organism is formed through two remarkably organized procedures: expanding the cell number and changing the phenotypic characteristics with significant spatial-temporal accuracy while growing its organs and tissues. The genetic information for entire cells in an organism is similar. To specify a cell lineage or stimulate cell fate alterations, specific transcription factors must cross-react with each other and apply cross-antagonistic effects. Similarly, stem cell renewal is modulated via epigenetic mechanisms that alter the accessibility of chromatin and determine the direction of cell identity through transcriptional events [8]. Ai et al. showed that DNA methylation patterns are associated with bone formation. They found that DPSCs, DFPCs, and PDLSCs had analogous patterns for DNA methylation. The PDLSCs were more osteogenic-related factors transcriptionally active than DPSCs and DFPCs, exerting a suitable osteogenic capacity both *in vitro* and *in vivo*. Also, DNA methylation modulates the DSCs' odontogenic differentiation by regulating osteogenic differentiation. TET1 inhibited DPSC proliferation and odontogenic differentiation, indicating that demethylation of DNA affects dental tissue regeneration [3]. Epigenetic mechanisms such as methylation of cytosine remain in DNA, changing the posttranslational histone cores, and intercepting transcriptional information and translation can activate epigenetic mechanisms at different levels (Figure 1).

DNA methylation is an epigenetic tag widely studied for its role in transcriptional repression of promoters, chromosome compaction, and cellular memory, among other effects. In particular, DNMTs such as DNMT3a, DNA

Table 1: The current studies of epigenetic regulation of dental stem cells in regenerative medicine.

Cell type	Method	Outcomes	Ref/ year
Induced pluripotent stem cell (iPS cell) generation from dental pulp stem cells (DPSCs)	A reprogramming scheme was investigated for iPS generation from DPSCs	Sufficient iPS cell generation from DPSCs, improving clinical and industrial utilization of iPS knowledge to use in therapies	[53]/ 2018
DPSCs	Examination of regulating Notch/Wnt signaling and stimulation of adipo-/osteocytes differentiation in DPSC cell lines	Wnt signaling could participate in the safer progression and lower destructive reprogramming platforms in DPSCs to utilize in cell therapy	[35]/ 2020
DPSCs and bone marrow-derived mesenchymal stem cells (BMSCs)	Examination of similar genetic and epigenetic mechanisms between the osteogenic differentiation of DPSCs and BMSCs	Common epigenetic and genetic mechanisms are concluded in the osteogenic differentiation of BMSCs and DPSCs	[54]/ 2021
Human MSCs	Identification of miR-34a aims protein systems as an osteoblastic regulator of [51] human MSC differentiation	miRNA-34a showed particular dual modulatory impacts on both proliferation and differentiation of human MSC. Additionally, miR-34a suppression could be a novel therapeutic approach for increasing the formation of bone tissue	[55]/ 2014
BMSCs were isolated from C57/BL mice	Investigation of miRNA function in the regulation of osteogenesis procedure in the inflammatory condition	miR-34a reverses proinflammatory cytokine effects and stimulates osteogenic differentiation, exhibiting that therapy based on miR-34a might be a suitable method for stimulating the regeneration of bone tissues	[39]/ 2019
Human DPSCs	Evaluation of different differentiation circumstances. K^+ channels initiation is assumed to modulate the Ca^{2+} content, which is intracellular, tolerating to change cell cycle to human DPSC differentiation induction	Epigenetic reprogramming and cell cycle regulation via a promotion with remarkable K^+ facilitated differentiation of human DPSCs into neuron-like cells. Hence, human DPSCs have the practical function as neuron-like cells via alteration of cells cycle	[56]/ 2020
Human DSCs	Investigation regulating the specification of signals tissue and lineage of cells epigenetically through evaluation of miRNA activity behind dental stem cells (DSCs)	miRNA-modulated pathway for the human DSC differentiation and a chosen network of miRNAs that control DSC osteogenic differentiation	[57]/ 2014
DPSCs	Investigation of immunomodulatory abilities of DPSCs by cocultured from elderly and young donors	Decrement of IL-6 and HGF expressions are necessary for the bone and dental tissue regeneration and downregulate highly in elder DPSCs	[58]/ 2021
DPSCs	Assessment of multipotential differentiation abilities of DPSCs	Ferutinin activated and promoted osteogenic differentiation of DPSCs, as a promising effective stem cell therapy for osteoporosis	[59]/ 2020
DPSCs	Explore the osteogenic, adipogenic, and resistance to oncogenic transformation of DPSCs in comparison with BMSCs	Several epigenetic factors widely implied tumorigenesis lineage and commitment, which might be considered when progressing stem cell therapeutic uses	[4]/ 2019
Periodontal ligament stem cells (PDLSCs)	To characterize DPSCs and their mechanism of differentiation cells from human DPSCs and PDLSCs, explore a miRNA array based on LNA	miR-720 reduced DPSC proliferation as distinguished via immunocytochemical assessment against ki-67 and stimulated odontogenic differentiation, like Alkaline phosphatase and mRNA levels of osteopontin. Also, outcomes demonstrated that miR-720 is a modulator miRNA for the DPSC differentiation	[60]/ 2013
Human DPSCs	Odontoblast-related genes changes were explored epigenetically via the alteration of the mitogen-activated protein kinase (MAPK) signaling pathway in cell lines	Cell proliferation downregulated response to MS-275 using, while it did not affect cytotoxicity in 5 and 10 nM and induced odontoblast-like cells differentiation	[61]/ 2020
DSCs	Evaluate KDM6B knockdown in DSCs and its effect on Alkaline phosphatase function and mineralized nodules formation	Outcomes exhibited participation of HDMs in the epigenetic modulation of odontogenic differentiation of DSCs. Lysine demethylase 6B (KDM6B) may indicate a promising beneficial aim in the tooth repairment and regeneration of craniofacial tissues	[62]/ 2013

Table 1: Continued.

Cell type	Method	Outcomes	Ref/ year
DPSCs	N^6-methyladenosine (m^6A) methylation biological actions were assessed in DPSCs	m^6A methylated hallmarks in DPSCs and modulatory participation in the cells cycle. It can use as a therapeutic approach in vital pulp therapy	[63]/ 2021
Mesenchymal stem cells (MSCs) derived from bone marrow and dental tissues	Address the function of long noncoding RNAs (lncRNAs) in osteogenesis modulation of MSCs derived from bone marrow and dental tissues. Also, lncRNAs as therapeutic aims for MSC-related diseases were investigated	lncRNAs involved in the bone marrow and dental tissue-derived MSCs' osteogenic differentiation could aim as prognosis and therapeutic parameters. Nevertheless, the lncRNA precise actions remain elusive	[64]/ 2018
MSCs	Investigate trichostatin A effects on osteogenic differentiation and resolve inflammation on MSCs derived from inflamed and normal gingival tissues	Anti-inflammatory properties and stimulation repairment of periodontal tissue were shown via trichostatin A as a candidate for therapeutic approaches in repairing periodontal tissues	[65]/ 2020
DPSCs	Explore genome-wide gene expression microarray and DNA methylome investigation to clarify molecular changes by DNA methylation alterations correlated with DPSC exposure to ethanol	Findings proved that significant alcohol usage might affect cellular processes that cause decreased mineral deposition, resulting in osteoporosis/-penia, dental abnormalities, and hallmark conditions for different fetal conditions induced via alcohol	[23]/ 2016
DPSCs	Examine the interference with N6-adenosine-methyltransferase 70 kDa subunit (METTL3) in DPSCs inhibits cell proliferation and osteogenic differentiation	Findings provide new ideas for using stem cells in clinical applications and for treating metabolic bone diseases by altering epigenetic modifications	[66]/ 2021
Human DPSCs	H19 mechanisms and impacts were assessed in human DPSC odontogenic differentiation	Provide novel visions of how the S-adenosylhomocysteine hydrolase (SAHH)/H19 axis functions in the odontogenic differentiation of human DPSCs. It would help develop therapies for the regeneration of dentin following stem cells	[67]/ 2018
Human dental follicle stem cells (DFSCs)	Examine Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2) and histone H3 lysine 27 (H3K27) trimethylation expression during osteogenesis of human DFSCs	EZH2 promoted the Wnt/β-catenin pathway by modulating the level of H3K27 trimethylation on stimulators of genes in these signaling pathways	[68]/ 2018
Human DPSCs	Assessment of trichostatin A on differentiation and proliferation of odontoblast along with its capacity in the forming of dentin and odontoblast differentiation in vivo during tooth progression	Trichostatin A conducted vital function in odontoblast differentiation and proliferation of human DPSCs in dental progression phases	[69]/ 2013
DSCs	Investigate the DSC niche cell types and the miR-200 class effects on the fates of DSCs	miR-200 modulates signaling pathways necessities for cell differentiation, progression of the cell cycle, and DSC niche maintenance	[70]/ 2021
DPSCs	Evaluate the regulatory role of Krüppel-like factor 2 (KLF2) during osteoblast DPSCs differentiation via assessing the KLF2 levels and autophagy-related molecules in cells	Chromatin immunoprecipitation evaluation showed that the functional epigenetic biomarkers and KLF2 were elevated in the stimulator region of autophagy-related 7 (ATG7)	[71]/ 2020
Human DFSCs	Investigate genes' functions and regulatory mechanisms (HOXA transcript antisense RNA, myeloid-specific 1 (HOTAIRM1), and homeobox A (HOXA)) in human DFSCs	HOTAIRM1 stimulated the human DFSC osteogenesis via modulating homeobox A2 (HOXA2) through DNA methyltransferase 1 (DNMT1). HOXA2 exhibited necessary actions in human DFSCs, same as HOTARIM1. Nonetheless, the HOTARIM1 modulatory pattern within the HOXA group remains unknown	[72]/ 2020
Mice DPCs	Identify Spalt-like transcription factor 1 (SALL1), polarizing and secretory odontoblasts, in vivo	SALL1 effectively modulates the odontoblast lineages commitment via connection with runt-related transcription factor 2 (RUNX2) and straight activation of transforming growth factor beta-2 (TGFβ-2) at an initial phase	[73]/ 2021

Table 1: Continued.

Cell type	Method	Outcomes	Ref/ year
Human DPSCs and BMSCs	Investigate corepressor CBFA2/RUNX1 partner transcriptional corepressor 2 (CBFA2T2) expression was remarkably increased in response to BMP2 treatment during osteogenic differentiation of human DPSCs and BMSCs	CBFA2T2 is required for BMP2-induced osteogenic differentiation of MSCs by inhibiting euchromatic histone lysine methyltransferase 1-(EHMT1-) mediated histone methylation at RUNX2 stimulator	[74]/ 2018
Human DPSCs	Histone H3 lysine 4 (H3K4) trimethylation and H3K27 trimethylation spatiotemporal patterns were examined in the mice model. Human DPSCs induced during odontogenic differentiation, H3K27 trimethylation demethylases (UTX and JMJD3), and H3K4 trimethylation methylases	During dental MSCs differentiation, Wnt family member 5A (WNT5A) transcription activities were modulated via stability among H3K27 trimethylation/H3K4 trimethylation, Jumonji domain-containing protein-3, and H3K4 trimethylation methylase stimulator	[75]/ 2018
Human DFSCs	Explore the osteogenic differentiation of human DFSCs and chromodomain helicase DNA binding protein 7 (CHD7) expression	CHD7 regulates the osteogenic differentiation of human hepatocytes by modulating the transcription of parathyroid hormone 1 receptor (PTH1R). Also, overexpression of PTH1R partially restores osteogenic differentiation in CHD7-knockdown human DFSCs	[76]/ 2020
Human DPSCs	Investigate the photobiomodulation therapy (PBMT) role on viability, human DPSCs migration, and its correlation to epigenetic mechanisms such as acetylation of histones	There is a correlation between PBMT and high viability and human DPSCs migration, related to the histone acetylation upregulation. Also, PBMT is an appealing adjuvant therapy for regenerative endodontic treatment	[77]/ 2020
Human DPSCs	Evaluate epigenetic reprogramming via the histone deacetylase 3 (HDAC3) and histone deacetylase 2 (HDAC2) selective inhibitors, MI192, to stimulate the osteogenic capacity of human DPSCs for bone regeneration	By reprogramming epigenetic factors of hDPSCs with HDAC2- and HDAC3-specific inhibitors, MI192 improves osteogenic differentiation, implying the feasibility of this method for bone augmentation	[78]/ 2021
Dental pulp and dental follicles	Differentiation profiles and epigenetic states of dental follicle/dental pulp, two odontogenic neural crest-derived ancestor populations, were examined	The results showed to highlight the crucial function that epigenetic regulation conducts in the odontogenic terminal differentiation neural crest cells	[79]/ 2013
PDLSCs	Examine the periodontal regeneration effect on the heterogeneous nuclear ribonucleoprotein L (HNRNPL) mechanism in the osteogenesis of PDLSCs induced by strontium chloride ($SrCl_2$)	There may be some implications for the treatment of periodontitis patients who have osteoporosis simultaneously by understanding the different functions of HNRNPL and SET domain containing 2, histone lysine methyltransferase (SETD2)	[80]/ 2019
PDLSCs	Assess the lysine demethylase 6A (KDM6A) function in chondrogenic differentiation of PDLSCs and the underlying mechanisms related to epigenetic	In the destruction of inflammatory tissue such as osteoarthritis, it was expected an improvement in MSC-mediated regeneration of cartilage through upregulation of KDM6A or the use of EZH2-inhibitors	[81]/ 2018
Cranial neural crest cells	Kat2a and 2b genes function as histone acetyltransferases and were examined in the progression of craniofacial in zebrafish and the *Gcn5* in mice	As a result of regulating H3K9 acetylation, these outcomes proposed that Kat2a and 2b are essential to the growth and cartilage and bone differentiation in both mice and zebrafish	[82]/ 2018

methyltransferase 3b (DNMT3b), and DNMT1 are involved in these modifications, which modulate the chromatin conformation during embryonic stem cell differentiation and somatic cell reprogramming. Interestingly, stem cells' genomes are largely euchromatic, while the genomes of somatic cells are enriched in heterochromatic conformation [8]. In a study on PDLSCs, Yu et al. revealed that the subpopulation of Alkaline phosphatase $^{+}$ demonstrated greater CD146 and STRO-1 expression than Alkaline phosphatase $^{-}$ cells. In addition, some stemness-associated genes were expressed in Alkaline phosphatase $^{+}$ cells (OCT4, NANOG, and SOX2) compared to Alkaline phosphatase $^{-}$ cells [20]. To derive PDLSC populations that are homogenous, Alvarez et al. investigated the surface markers, including CD271/ 140α, 51, and STRO-1/CD146. As a result of CD271-positive cells having a more significant dental/osteogenic potential, they were associated with a tremendous increase in osteogenic gene expressions, such as distal-less homeobox 5 (DLX5), bone gamma-carboxyglutamate protein, and runt-related transcription factor 2 (RUNX2) [21]. Researchers found that activated yes-associated protein (YAP) inhibits apoptosis in human PDLSCs, stimulates

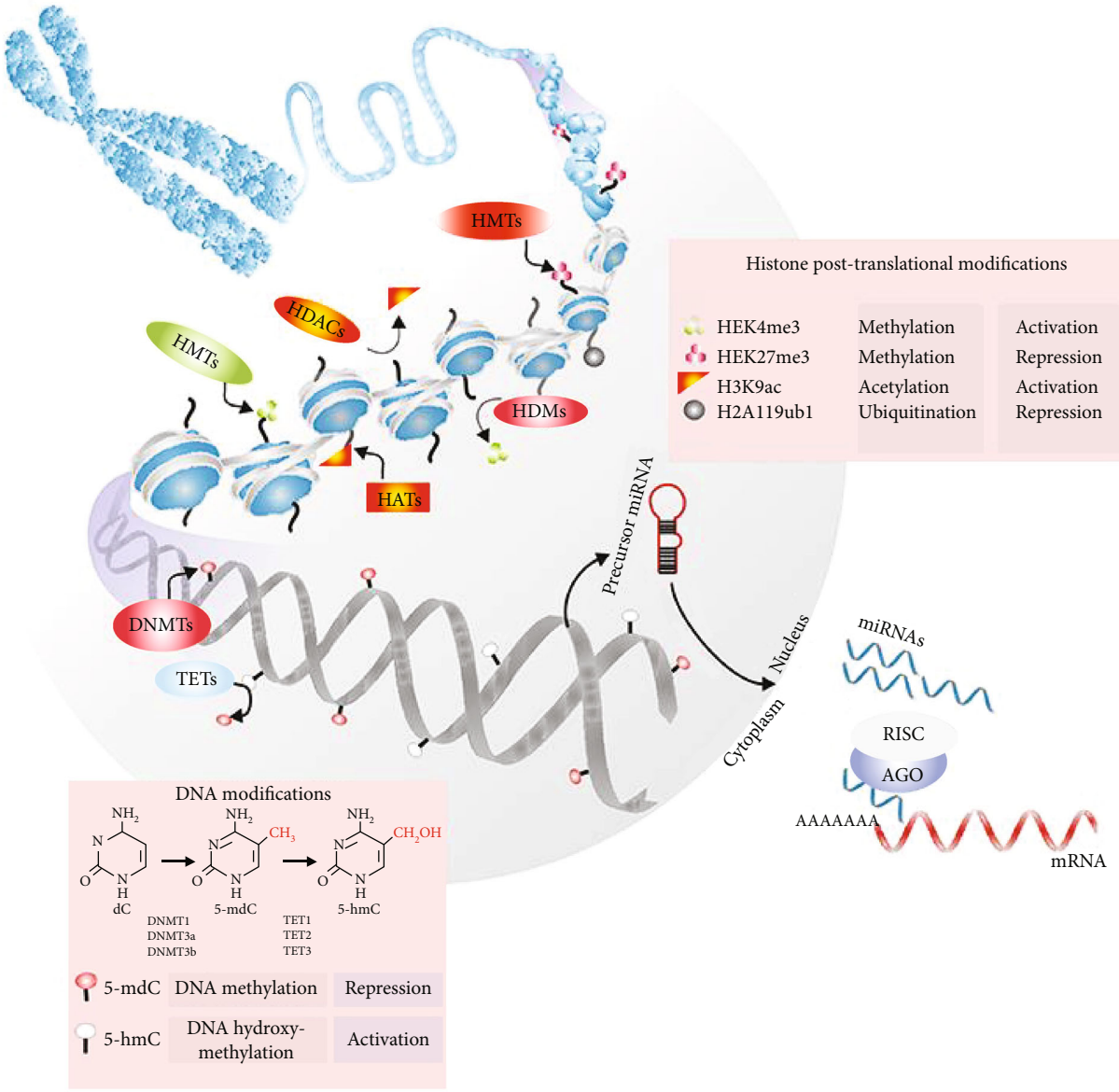

Figure 1: DNA methylation, histone posttranslational modifications (HPTMs), and ncRNAs are the most common epigenetic markers in chromatin remodeling and restructure. A methyl group (-CH3) attach to 5-methyl-deoxy-cytidine (5-mdC) by DNA methyltransferases (DNMTs). The family members of the ten-eleven translocations (TET) can remove DNA methylation marks by converting 5-mdC to 5-hydroxymethylcytosine. Histone methyltransferases and HDACs have been shown to regulate cell methylated, acetylated, and ubiquitinated histone patterns. mRNA can be repressed during transcriptional repression by ncRNAs, such as miRNAs. The Dicer breaks down miRNA precursors in the cytoplasm, and the miRNA is then filled and attached to mRNA targets. Inhibition, degradation, and/or destabilization of the miRNA-mRNA interactions depend on their base pairing [8].

proliferation, and expedites the cell cycle and retardation of senescence [22]. Another experiment revealed that high glucose levels led to increased DNA methylation levels in PDLSCs, blocking their ability to differentiate into osteoblasts. Nevertheless, 5-Aza-2-deoxycytidine could inhibit the canonical Wnt signaling pathway and increase Alkaline phosphatase, osteocalcin, and osteopontin genes, restoring osteogenic differentiation capacity in PDLSCs [23]. Also, Yu et al. reported that TET1 and 2 reductions cause the hypermethylation of the Dickkopf Wnt signaling pathway inhibitor 1 precursor, which actuated the pathway, upregulation of FasL expression, and betterment the PDLSCs immune regulation properties. TET1/TET2-reduced PDLSCs demonstrated remarkable upregulation of therapeutic ability in the colitis mice model [24].

3.1. Postnatal Human Dental Pulp Stem Cells (DPSCs). DPSCs are isolated from a postnatal human dental pulp to regenerate a reparative dentin-like complex and differentiate into different cell types. These cells are unique among stem cells because they can be separated into diverse cells, including neural progenitors, odontoblasts, melanocytes, chondrocytes, osteoblasts, smooth muscle cells, and adipocytes. DPSCs are an auspicious tissue type for dental tissue

engineering and craniomaxillofacial regeneration due to their high proportion of prevalence, low morbidity, well differentiation, and biomaterial toleration. In addition to osteogenic and adipogenic differentiation, DPSCs also show neurogenic differentiation, similar to SHEDs, making them very attractive for clinical use [25]. Since the DPSC high differentiation plasticity marks them an ultimate stem cell source for cellular treatment, regeneration, and engineering of tissues for multiple disorders, they are currently being explored. Stem cells originate from the dental pulp of permanent teeth, which contains wisdom teeth surgically removed that do not contribute to the occlusion of permanent teeth. Genetic and congenital disorders are often characterized by defects in the fetal or postnatal stages [16].

DPSCs usually differentiate into various cell types and have also proven to retain many of their characteristics after cryopreservation for two years. As a result, many studies are being conducted on the DPSC differentiation and their clinical potential. The multidirectional differentiation capacity and the easy accessibility of DPSCs make them an excellent candidate for use in tissue engineering and disease. Studies have demonstrated DPSC formation of an immunocompromised complex resembling dentin and pulp. DPSCs in scaffold-free and prevascularized microtissue spheroids may also effectively regenerate vascularization in dental pulp tissues. Also, they could provide a model for dentin regeneration and the treatment of endodontics. The potential for clinical application of DPSCs goes beyond treating dental problems to treating other medical disorders, such as craniofacial defects, nervous system injuries, muscle regeneration, osteoarthritis, myocardial infarction, Alzheimer's disease, diabetes, Parkinson's disease, liver diseases, and stress urinary incontinence [1]. Periodontal disorders, hypodontia, enamel development, and odontogenic differentiation have been associated with epigenetic changes. Duncan et al. presented research on the potential therapeutic potential of inhibiting histone deacetylases and DNA methyltransferases in dental pulp as a regenerative endodontic application. It has been shown that permissive chromatin associated with transcriptional upregulation is instrumental in developing DPSCs into mature odontoblasts. However, there is still limited knowledge about epigenetics and how it leads to specialized cell lineages in DPSCs, despite some progress [25]. Schwann cell markers are expressed after incubation through c-Kit$^+$/STRO-1$^+$/CD34$^+$ DPSC induction, which was demonstrated by Carravale et al. The incorporation of the DPSC collagen scaffold resulted in sensory neurons being regenerated and myelination occurring in the rats' sciatic nerve injury model [26]. Alraies et al. recognized alterations between high (A3)/low (A1 and 2) proliferative ability DPSC populations [27]. A paper by Young et al. elucidated that murine DPSC clones could differentiate into oligodendrocytes and neuron-like cells *in vitro*. Interestingly, only those DPSCs that express remarkable levels of nestin gene expression differentiate successfully into neurofilament-positive neuron-like cells and microtubule-associated protein 2 [28]. As a result of transplantation of CD146$^+$ cells into immune-compromised beige mice, Matsui et al. found that dentin/pulp-like structures formed. Moreover, CD146$^+$ cells also possessed higher mineralization properties compared to nonseparated cells, CD146$^-$ or 146$^{+/-}$ cells. DPSCs transplanted with human mitochondria are immunohistochemically detected to contain dentin matrix protein-1 (DMP1), dentin sialophosphoprotein, and dentin matrix protein-2 (DMP2) [29]. The induction of c-Kit$^+$/STRO-1$^+$/CD34$^+$ DPSCs exhibited superior levels of commitment than that of DPSCs c-Kit$^+$/STRO-1$^+$/CD34$^-$, which may be evidenced via β-III tubulin expression and the shift from neuron-like shapes and appearance to spheroid-like appearances [30].

In long-term culture, production of SA-β-gal and biomarkers concluding p16, 21, interleukin- (IL-) 1β, 6, 8, and growth-related oncogene alpha (Groα) was shown to increase for mobilized DPSCs with age as determined by their revulsive reaction to the colony-promoting factor of granulocytes from different donors. A model of ischemic hindlimb damage and ectopic teeth roots revealed that aged mobilized DPSCs had the similar regenerative potential to young mobilized DPSCs [31]. Salkin et al. demonstrated that transforming growth factor-beta 1 (TGF-β1) transfection promotes proliferation and prevents apoptosis and cellular senescence, suggesting a potential therapeutic intervention. They proposed that the overexpression of TGF-β1 along with gene transmission might lead to the enhanced DPSCs' biological abilities and replace the external delivery of recombinant proteins into the cells [32]. 5-Aza was used by Nakatsuka et al. to assess the Myod potential of DPSCs in mice. DNA demethylation caused via 5-Aza and forced Myod-1 expression stimulated expression of transcription factors related to muscle-specific [33]. The results of Paino et al.'s study showed that histone deacetylase 2 (HDAC2) silencing could increase the expression of osteocalcin and bone sialoprotein in DPSCs, similar to the effect of valproic acid [18]. The expression profile of circular RNAs was revealed in DPSCs during odontogenic differentiation by Chen et al. 43 circular RNAs were upregulated during dental differentiation, while 144 circular RNAs were downregulated. Signaling pathways regulating pluripotency in MSCs are abundant in these differentially expressed genes, such as the TGF and Wnt signaling pathways [34].

Somatic cells' epigenome is improved with heterochromatin, permanently silencing more genes. Stem cells have a particular genome in euchromatic conformation; mainly, differentiated cells' genome is a mixture of euchromatic and heterochromatic forms. In addition, DNA methylation levels in DPSCs are low both *in vitro* and *in vivo*, and histone acetylation levels are high in DPSCs, which may weaken the interaction between chromatin and DNA, allowing the expression of genes to proceed. Conversely, loss of acetylation mediated by HDAC results in a closed heterochromatin conformation, suppressing transcription. Recent findings have associated histone methylation with chromatin remodeling in DPSCs. Trimethylation marks of H3 are the most characterized, and these marks serve as gene activators for transcriptional activation and repressors for transcriptional silencing for transcriptional repression. Moreover, cell stemness and differentiation genes contain bivalent histone methylation marks, which can activate H3K4 trimethylation

and repress H3K27 trimethylation histone methylation in DPSCs. The bivalent domain-containing genes in the stem cells would allow them to respond more rapidly to environmental changes by repressing specific genes at the same time while activating others [35]. DNA methylation, histone modifications, and ncRNAs are crucial in controlling DPSCs' fate. Genetic control is mediated by signaling pathways and transcription factors, and epigenetic control is mediated by DNA methylation, histone modifications, and ncRNAs. The manipulation of DPSCs' fate toward pulp–dentin regeneration is possible with an epigenetic modulation understanding in DPSCs [17]. Among the most well-researched epigenetic modifications, DNA methylation is often associated with gene silencing and stem cell fate regulation. Several studies have also found particular regulatory effects of DNA methylation in DPSCs [1]. Recent studies have observed complex epigenetic networks for porcine DPSCs, including long noncoding RNAs (lncRNAs), microRNAs (miRNAs), and DNA methylation. The lncRNA G043225 stimulates odontogenic differentiation through direct interactions with fibrillin 1 and miR-588. lncRNA H19 generally suppresses DNMT3b activity, decreases the DLX3 methylation level, and therefore causes the advancement of porcine DPSC odontogenic differentiation. In the same way, miR-675 inhibits the DNMT3b-mediated methylation of DLX3 in DPSCs for promoting human DPSC odontogenic differentiation. In addition, colon cancer-associated transcript 1/lncRNA increases cell differentiation and proliferation by suppressing the miR-218 signaling pathway [17]. H3K4 trimethylation activates remodeling acetylases of histone and enzymes along with the increment of transcription. In contrast, histone H3K27 trimethylation functions in the opposite way. In this regard, H3K27 trimethylation and H3K4 trimethylation conduct on progressive genes to provide bivalent domains genome together. It is believed that the differentiation-relevant genes that up- and downregulate through opposing modifications of the associated histones are "locked away" but kept "poised," ready for activation once the appropriate signals come. Almost half of the bivalent domains in the mouse genome have been associated with connecting areas for the OCT4, NANOG, and/or SOX2 transcription factors. Conspicuously, most domains ultimately revert to H3K27 trimethylation or H3K4 trimethylation based on their lineage [12].

3.2. Dental Pulp-Derived Mesenchymal Stem Cells. Teeth are composed of several types of cells, including odontogenic and undifferentiated progenitor cells, within a remarkably vascularized connective tissue center and undifferentiated stem cells, which also include multipotent osteoblasts that have both significantly proliferative properties *in vitro* as well as *in vivo*. During early embryonic development, mesenchymal crest cells transfer to the branchial arches of the nervous system, indicating that the MSCs that make up dental pulp are descended from neural crest cells. The three prominent human teeth are deciduous, permanent, and supernumerary, contributing to developmental pathways and morphological properties; however, they vary molecularly. In a study of dental pulp cells isolated from these teeth, multipotent MSCs were observed. However, several differences were observed at the molecular and cellular levels. Rodent nerve injury models showed that these transplanted cells inhibited apoptosis and inflammation that interfered with repairment while differentiated into oligodendrocytes, which are mature, to stimulate neuroregeneration [16]. Reduction expression of STRO-1 and regulation of transcription factors, NANOG, OCT4, and nestin, was observed with an upregulation in gingival-derived mesenchymal stem cell (GMSC) passage by Ranga Rao and Subbarayan [36].

3.3. Human-Exfoliated Deciduous Teeth Stem Cells (SHEDs). Deciding teeth exfoliate spontaneously upon extrusion by their permanent successors or are surgically removed before breaking out permanent successors. These extracted or exfoliated deciduous teeth involve residual pulp tissues concluding dental MSCs, first discovered as SHEDs approximately 2 decades ago. They may be accessed with a slightly invasive process. The SHED multipotency is indicated by their shared adipose, osteogenic, and chondrogenic origins with BMSCs. SHEDs express MSCs and embryonic stem cell biomarkers, lack hematopoietic signaling biomarkers, and involve CD11b/c and 45. Patient-derived SHEDs are ideally constructed from a child in the process of a genetic condition that does not affect the child's natural teeth. When an individual has twenty deciduous teeth, the maximum number of teeth that could hypothetically be attained from a described child in the process of genetic disorder is twenty. As a result, there are twenty chances for SHEDs to be collected from a child. This is deciduous teeth benefit, which is more common in children, over wisdom teeth which differ in that adults have a maximum of four sets. Consequently, patients with genetic disorders may have fewer deciduous teeth to establish disease models derived from SHEDs. This represents a severe disadvantage. For these reasons, professional dental care for children with severe consequences is vital for their quality of life, oral health, and setting up patient-derived SHED models of genetic conditions [16]. According to Inada et al., two of the five primarily isolated SHEDs had higher OCT3/4 expression and had higher Alkaline phosphatase activities. These two lines proliferated faster and were simpler to program into induced pluripotent stem cells (iPS cells) [37].

4. The Role of Histone Modifications in DSC Differentiation

Heterochromatin modifications, including histone modifications, are also essential factors in the fate of DSCs. Lysine demethylase 3B (KDM3B) increases osteogenic differentiation of SCAPs. Lysine demethylase 4B (KDM4B) removes H3K9 trimethylation via attaching to DLX stimulators, contributing to aim gene expression [3]. It was reported by Yang et al. that DLX5 and KDM4B are modulated by SCAP-positive feedback loop. Moreover, DLX5 increases the osteogenic differentiation genes DMP1, dentin sialophosphoprotein, and osteopontin, encouraging their expression. The nude mice model investigation also implied that DLX5 promotes osteogenesis by upregulating KDM4B in SCAPs. At

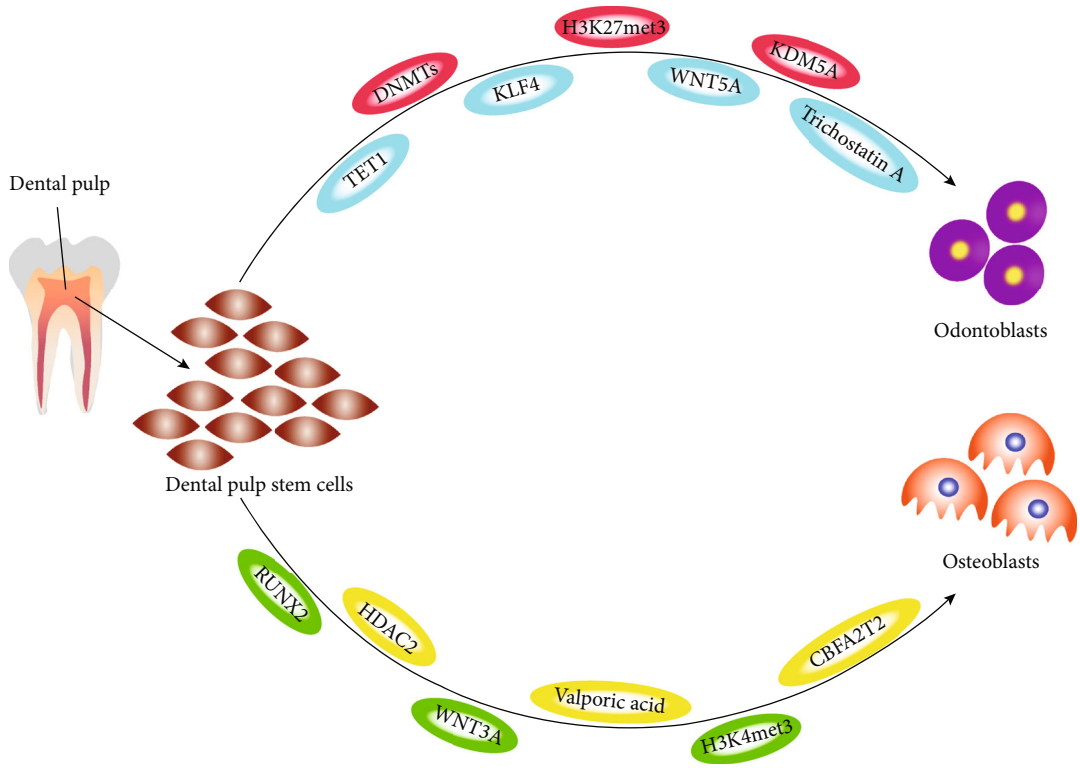

Figure 2: The pathways of dental pulp stem cell (DPSC) differentiation. Yellow and red ovals block differentiation pathways, and green and blue ovals stimulate the differentiation of DPSCs. Abbreviations: DNMTs: DNA methyltransferases; TET: ten-eleven translocations; H3K27met3: histone H3 lysine 27 trimethylations; H3K4met: histone H3 lysine 4 trimethylation; KDM5A: lysine demethylase 5A; HDAC2: histone deacetylase 2; KLF4: Krüppel-like factor 4; WNT5A: Wnt family member 5A; WNT3A Wnt family member 3A; RUNX2: runt-related transcription factor 2.

the same time, lysine demethylase 5A (KDM5A) inhibits the DPSC capacity to differentiate to the dentin morphotype via the elimination of H3K4 trimethylation from the dentin sialophosphoprotein, DMP1, and stimulators of osteocalcin [38]. Transcription factors can modulate access to target genes by altering at least 12 amino acid residues in histones. Also, acetylation and methylation, two histone modifications, are vital for various biological procedures, concluding determination of cell fate and transcriptional modulation [8]. It is also possible to control gene expression epigenetically through ncRNAs, including lncRNA, siRNA, and miRNA; however, ncRNA amount exceeds mRNA transcription coding. Small interfering RNA (siRNA)/miRNAs function by controlling them for aiming mRNA strands. The marked mRNA strands are then cleaved. It is not clear with certainty where miRNAs function; nonetheless, a minimum of one-third of genomes are expected to be modulated via the coding of miRNAs. Both miRNAs and siRNAs process several targets, but the former has just one aim. The extent to which miRNAs affect gene expression is still being determined [19]. In addition, several miRNAs may be involved in regulating differentiation in these dental tissues, including miR-99a, miR-210, and miR-218. Considering the relation between miR-218 lower levels and higher RUNX2 levels, it would appear that the expression of miR-218 is essential to managing RUNX2 expression, a transcription factor crucial for osteogenic differentiation. Findings suggest that miR-218 modulates RUNX2 expression to regulate osteogenic sequences in human dental tissue-derived MSCs. The regulation of osteogenic differentiation capacity by miRNA differential profiles remains to be determined. The expressions of miR-101 and -21 are implicated in osteogenic differentiation of periodontal ligament stem cells; both miRNAs enhance the mineralization ability of periodontal ligament stem cells by regulating the expression of periodontal ligament associated protein 1 (Figure 2). Meanwhile, several miRNAs were distinctively expressed among the odontoblast differentiation of DPCs, such as miR-32, -586, and -885-5 (Figure 2). Further, increased expression of noninductive stemness markers, such as osteopontin, dentin sialophosphoprotein, osteocalcin, and dentin sialophosphoprotein, is maintained by overexpression of p300. These results indicate that p300 interacts with stemness markers and conducts a vital role in noninductive conditions. It has been shown that acetylation of H3K9 increases in the genes' specific regions associated with odontogenic potential when p300 is overexpressed (Figure 2) [8]. Nakatsuka et al. revealed that miR-34a suppressed the process of osteogenesis via inhibiting the cell cycle and proliferation of cells [33] and that the miR-34a suppression could expedite this process BMSC osteogenesis. In contrast, Xin et al. implied that the upregulation of miR-34a might facilitate the process of BMSC osteogenesis and regression of proinflammatory cytokine by aiming tumor necrosis factor-alpha (TNF-α) [39].

5. The Role of Dental Stem Cell's Self-Renewal and Differentiation in Regenerative Medicine

DSCs possess the potential for self-renewal and multidifferentiation similar to MSCs. Research and clinical advances have been made in dental pulp regeneration during the past few years. In regenerative endodontics, several strategies have been proposed, with different scaffolds, growth factors, and stem cells, emphasizing important aspects of dentistry such as disinfection and dentin conditioning [40]. DPSCs were first reported in 2000. Since then, they have become the most commonly used DSCs for developing cell-based therapies for dental and systemic diseases. Dental pulp tissue, which can be harvested from extracted teeth, appears to contain potential stem cells that can be used in clinical applications in the future. A laboratory led by Yaegaki has recently induced a new type of cell, hepatocytes, from dental pulp cells isolated from full-grown wisdom teeth and exfoliated deciduous teeth [41]. The researchers from Nakashima et al. recently published a pilot study that demonstrated the potential benefit of mobilizing DPSCs for pulp regeneration in human teeth that have undergone pulpectomy. Clinical and laboratory evaluations revealed that the pulp responded positively with no toxicity despite the short sample size. An ongoing randomized, controlled study in a single center is currently being conducted to evaluate the efficacy of using autologous SHEDs to revitalize young necrotic permanent teeth [42]. The clinical applications of DPSCs have been demonstrated in several studies, showing that the cells can promote bone regeneration and neo-bone formation in cranial defects, suggesting their potential impact on regenerative medicine, including bone conditions [43]. Additionally, other studies have examined how DSCs differentiate into nerve tissues and pancreatic cells and their efficacy as a source of iPS cell proliferation [44, 45]. DSCs have recently become more popular due to the extension of dental applications to other fields of medicine. Researchers continue to explore the potential of DSCs to differentiate other cell types. This may provide better quality treatments for diseases such as diabetes mellitus, Alzheimer's, Parkinson's, myocarditis, and other devastating conditions [46].

6. The Role of Pharmacological Agents in Epigenetic Modifications

Molecular design of multitargeting epigenetic agents has become a popular method for developing epigenetic therapies by specifically targeting several unrelated cellular targets at once (at least one of which is the epigenetic enzyme). Multiaction drugs simplify treatment regimens by reducing adverse drug reactions, reducing the likelihood of drug resistance, and facilitating easy administration. Zinc-dependent HDAC inhibitors are commonly used to target more than one cancer-related target. Additionally, methyltransferase and demethylase enzymes are also commonly targeted, as are acetyllysine-binding bromodomains [47]. There are pharmaceutical agents which can generally change histone acetylation epigenetically. These molecules, known as histone deacetylase inhibitors (HDACis), have changed gene transcription, induced pleiotropic cell effects, and affected stem cells fate. Several types of HDACi effectively induce differentiation, proliferation, and anti-inflammatory properties, concluding with valproic acid, trichostatin A, and butyric acid. Recent studies have demonstrated that HDACis perform an epigenetically vital function in regulating DPSC differentiation and self-renewal by working in a balance with histone acetyltransferases. This HDACi suppresses N-terminal deacetylation located in the histone tail within the nucleosome, leading to modification of chromatin structure and increased transcription. There are eighteen human HDAC enzymes, each possessing a distinct, complex, but often overlapping role, which has yet to be fully explained. HDACs express themselves in the cytoplasm, nuclear (noncytoplasmic), or tissue-restricted (cytoplasmic) locations. Consequently, pan-HDAC inhibitors that target all 18 enzymes are being explored more than isoform-specific suppressors. Some pan-HDACis were performed to accelerate differentiation and dental pulp cell population mineralization and dedifferentiate immature stem cell populations in specific environments and concentrations [2]. The histone posttranslational modifications, which occur on particular amino acid histone proteins remains, are another critical epigenetic mechanism. The modification types in histone tails contain methylation, deimination, acetylation, sumoylation, parylation, ubiquitination, isomerization, citrullination, and phosphorylation. The modifications impact transcription, replication, DNA repair, and chromatin structure. These modifications may also serve as potential targets for anticancer drugs [47]. Several clinical trials combining HDAC inhibition with protein kinase inhibition have led to the concept of combining both actions into one compound. Interestingly, Zang et al. have shown that HDACi combined with pazopanib results in positive antitumor effects [48]. Based on the osimertinib structure, another epidermal growth factor receptor (EGFR) inhibitor that is approved, researchers designed and synthesized dual inhibitors of HDAC and EGFRs [49]. In comparison with vorinostat (approved HDAC inhibitor), some of the designed compounds inhibited HDAC at a greater level. These were regarded as moderate to low EGFR inhibitors. Another approach was to pair ruxolitinib with vorinostat to create dual Janus kinase (JAK)-HDAC inhibitors. As a result of a pyrazole substituted pyrrolopyrimidine (compound number 24) being highly potent and selective against a panel of 97 kinases, Yao et al. reported IC50 below 20 nM for inhibiting JAK1 and HDAC1 and 2, 3, 6, and 10 [50]. In an experiment by Kuang et al., hypoxia-primed porcine DSCs were implanted with a synthetic polymer to construct a three-dimensional rat model in an in situ model. Histologically pulp-like tissues were generated and vascularized. Another group of scientists conducted BMP2-treated DPSC culture onto the amputated dog canine pulp, which are autogenously transplanted [51]. It was supposed that preconditioned porcine DPSCs would guide differentiation precisely and ensure optimal functional regeneration of pulp.

Autologous SHEDs must undergo long-term follow-up despite the promising and cheerful results obtained by Xuan et al. Additionally, allogenic DPSCs are more effective and safer than self-derived ones. Further research is needed on allogenic DPSCs [52].

7. Conclusion

It is crucial to summarize the current epigenetic cues to advance clinical research into DSCs. Epigenetic modifications influence several critical signal pathways, ultimately responsible for DSC fate. In addition, by deciphering the epigenetic code of DSCs, regenerative therapies could be directed at DSCs from bench to bedside, thus making DSCs more accessible. Bioengineering relies heavily on stem cells, so it is essential to elucidate how cell fate regulation affects differentiation. Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit (EZH2) blocks the differentiation of bone, muscle, neural, and hematopoietic precursor cells in MSCs. As well as inhibiting NANOG activity in embryonic stem cells, EZH2 suppresses the activity of POU class 5 homeobox 1 - two pluripotency genes - proposing that modifiers of histone are phase- and cell-specific and act differently based on the cell type and differentiation state. To reach clinical application, several regulatory obstacles must be overcome. Firstly, it is vital that off-target effects are considered. Almost all HDACis, like TSA and valproic acid, are paninhibitors with no specific selectivity, so they upregulate target proteins. In immunomodulation, for instance, the expression of anti-inflammatory factors can be overexpressed while an individual miRNA upregulates proinflammatory cytokines. Secondly, epigenetic therapeutics must be thoroughly investigated and screened to minimize unwanted effects before being used in clinical settings to minimize the possibility of neoplastic transformation during regeneration. Lastly, technical and financial support must be endorsed to bank DPSCs and reserve the "biological insurance." Also, it is required to develop standardization and optimization of manufacturing protocols to guarantee the cell source quality at all stages of cryopreservation, isolation, collection, and expansion.

8. Future Direction

Stem cells are essential for dentin-pulp regeneration, signaling molecules that regulate cell fate, scaffolds that provide a favorable microenvironment, and stem cells with pluripotency capacity. DPSCs are regulated by complex epigenetic networks of histone modifications, ncRNAs, and DNA methylation that promote migration, self-renewal, and multidifferentiation. Epigenetic regulation in these processes will help improve DPSC migration, self-renewal, and multidifferentiation during the regeneration of pulp tissues. There have been positive results with HDACis on bone regeneration in animal models, including trichostatin A. Therefore, HDACis could regenerate pulp and dentine *in vivo*. Today, DNMT inhibitors and ncRNAs do not exhibit any regenerative potential *in vivo*. A suitable prospect is to understand the promising functions of epigenetic modulation in DSC fate and identify novel therapeutic targets for DSC-mediated regeneration. It is critical to assess how to ensure the DSC stemness in standard culture conditions. During long-term cell culture, DSCs may lose some of their potential. Diomede et al. reported that 5-Aza induced the GMSC differentiation into embryonic lineages in 48 hours. The GMSCs exhibited three germ layers and secreted markers related to embryonic development after being treated with 5-Aza. According to the above results, future translational medicine can benefit from epigenetic regulation. Additionally, it has been shown that 5-Aza treatment induces the differentiation of GMSC into different embryonic lineages other than neural precursor cells post extended expansion, indicating that 5-Aza may have a potential role in the preservation of DSCs stemness in the future. DNMT suppression increased Krüppel-like factor 4 (KLF4) levels and accordingly enhanced the rate of DSCs odontoblastic differentiation. Furthermore, stem cell differentiation can realize this great potential for regenerative medicine in specific tissues in the future. While there is a significant amount of research in domains such as delivery methods, off-targets, and neoplastic transformation, these issues must be addressed before epigenetic strategies are optimized for dentin-pulp regeneration. Small molecules can be used to manipulate epigenetic factors of DPSCs to promote differentiation and regeneration in the search for functional pulp regeneration approaches.

Abbreviations

RNA: Ribonucleic acid
ncRNAs: Noncoding RNAs
DSCs: Dental stem cells
DNA: Deoxyribonucleic acid
MSCs: Mesenchymal stem cells
DPSCs: Dental pulp stem cells
SHEDs: Stem cells from human exfoliated deciduous teeth
PDLSCs: Periodontal ligament stem cells
DFPCs: Dental follicle precursor cells
BMSCs: Bone marrow-derived mesenchymal stem cells
SCAPs: Stem cells from apical papilla
GMSCs: Gingival-derived mesenchymal stem cells
CD: Cluster of differentiation
DNMTs: DNA methyltransferases
TET: Ten-eleven translocations
PTEN: Phosphatase and tensin homolog
AKT: Protein kinase B
5-Aza: 5-Azacytidine
H3K9: Histone H3 lysine 9
H3K27: Histone H3 lysine 27
H3K79: Histone H3 lysine 79
H3K4: Histone H3 lysine 4
H3K36: Histone H3 lysine 36
mRNA: Messenger RNA
m6A: N6-methyladenosine
m1A: N1-methyladenosine
DFSCs: Dental follicle stem cells
DLX5: Distal-less homeobox 5
RUNX2: Runt-related transcription factor 2

YAP: Yes-associated protein
DMP1: Dentin matrix protein-1
DMP2: Dentin matrix protein-2
Groα: Growth related oncogene alpha
IL: Interleukin
TGF-β1: Transforming growth factor beta 1
HDAC2: Histone deacetylase 2
lncRNAs: Long noncoding RNAs
miRNAs: MicroRNAs
DNMT3b: DNA methyltransferase 3b
iPS cells: Induced pluripotent stem cells
KDM3B: Lysine demethylase 3B
KDM4B: Lysine demethylase 4B
KDM5A: Lysine demethylase 5A
siRNA: Small interfering RNA
TNF-α: Tumor necrosis factor-alpha
HDAC: Histone deacetylase 2
HDACi: Histone deacetylase inhibitor
EGFR: Epidermal growth factor receptor
JAK: Janus kinase
IC50: The half maximal inhibitory concentration
EZH2: Enhancer Of Zeste 2 Polycomb Repressive Complex 2 Subunit
KLF4: Krüppel-like factor 4.

Data Availability

All data are included in the article.

Conflicts of Interest

The authors declare that they have no competing interests.

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Research Article

Long Noncoding RNA IGFBP7-AS1 Promotes Odontogenesis of Stem Cells from Human Exfoliated Deciduous Teeth via the p38 MAPK Pathway

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Stem cells from human exfoliated deciduous teeth (SHED) are attractive seed cells for dental tissue engineering. Epigenetics refers to heritable changes in gene expression patterns that do not alter DNA sequences. Long noncoding RNAs (lncRNAs) are one of the main methods of epigenetic regulation and participate in cell differentiation; however, little is known regarding the role of lncRNAs during SHED odontogenic differentiation. In this study, RNA sequencing (RNA-seq) was used to obtain the expression profile of lncRNAs and mRNAs during the odontogenic differentiation of SHED. The effect of IGFBP7-AS1 on odontogenic differentiation of SHED was assessed by alkaline phosphatase (ALP) staining, alizarin red S (ARS) staining, quantitative reverse transcription PCR (qRT-PCR), Western blot, and *in vivo*. The level of p38 and p-p38 protein expression was examined by Western blot, and the result was verified by adding the p38 inhibitor, SB203580. The expression profiles of lncRNAs and mRNAs were identified by RNA-seq analysis, which help us to further understand the mechanism in odontogenesis epigenetically. IGFBP7-AS1 expression was increased during odontogenic differentiation on days 7 and 14. The ALP staining, ARS staining, and expression of odontogenic markers were upregulated by overexpressing IGFBP7-AS1 *in vitro*, whereas the expression of osteogenesis markers was not significantly changed on mRNA level. The effect of IGFBP7-AS1 was also verified *in vivo*. IGFBP7-AS1 could further positively regulate odontogenic differentiation through the p38 MAPK pathway. This may provide novel targets for dental tissue engineering.

1. Introduction

Hard tissue defects in the maxillofacial tissues, including defects of bone tissue caused by tumors and malformations from birth, as well as tooth defects caused by dental caries and dental trauma, usually require bone reconstruction or dental tissue regeneration. However, existing treatment methods are associated with major disadvantages [1–3]. Recently, biological tissue engineering has provided a feasible method for the regeneration of dental hard tissue [4, 5]. There are three basic elements involved in tissue engineering: (1) cells, (2) growth factors/signals, and (3) scaffolds [6]. The selection of seed cells is the basic issue in tissue engineering. Stem cells from human exfoliated deciduous teeth (SHED) are attractive seed cells for tissue engineering. The use of SHED in dental tissue engineering may be advantageous over traditional stem cells for the following reasons: (1) SHED have a higher rate of proliferation *in vitro* than human bone marrow mesenchymal stem cells (hBMSCs) [7] or dental pulp stem cells (DPSCs) [8]; (2) SHED have a greater capacity to form bone and dentine [9]; and (3) SHED are isolated from deciduous exfoliated teeth, the only disposable organ of the body, which is associated with fewer ethical concerns. In addition, SHED are particularly convenient and safe as autologous seed cells for dental tissue engineering in adolescents with mixed dentition who suffer from pulp necrosis or pulpitis. In our previous study, we aimed to use the steroid, betamethasone, to achieve SHED odontogenesis [10]; however, we found that the osteoclast process was also simultaneously activated. Thus, the purpose of our present

study is to elucidate the mechanism by which SHED-directed differentiation can be induced to promote odontogenesis.

Epigenetics refers to heritable changes in gene expression patterns that do not alter DNA sequences. What is more, DNA methylation, chromatin remodeling, and noncoding RNAs are the three main methods of epigenetic regulation. Long noncoding RNAs (lncRNAs), the length of which is over 200 bp, are noncoding RNAs and have been reported to exert their epigenetic functions through different ways such as chemically remodeling chromatin [11]. lncRNAs have been suggested to participate in various biological and pathological processes, such as cell differentiation and proliferation, and cancer development [12]. Previous research has reported the lncRNA expression profiles during mesenchymal stem cell osteo/odontogenesis [13, 14]. lncRNA DANCR has been reported to inhibit the odontogenic differential process of DPSCs through the Wnt/β-catenin pathway [15]. In contrast, lncRNA H19 has been shown to improve odontogenic differentiation epigenetically via DLX3 methylation in DPSCs [16] or through the MAPK pathway in stem cells from apical papilla (SCAP) [17]. However, the role of lncRNAs is little known during the odontogenic differentiation of SHED.

Insulin-like growth factor-binding protein 7-antisense 1 (IGFBP7-AS1) is an antisense lncRNA located on chromosome 4 in humans, and the antisense transcript of insulin-like growth factor-binding protein 7 (IGFBP7). IGFBP7-AS1 is reported to be significantly associated with overall survival in patients with glioblastoma, and IGFBP7-AS1 knockdown inhibited the function of glioma cells in viability, migration, and invasion [18]. No studies have considered the role of IGFBP7-AS1 in odontogenic differentiation so far.

In the present study, the expression profiles of lncRNA during odontogenesis of SHED were analyzed by high-throughput RNA sequencing (RNA-seq), which help us to further understand the epigenetic mechanism in odontogenesis. IGFBP7-AS1 was identified as the top differently expressed lncRNA during odontogenic differentiation and selected for further study. The role and mechanistic pathway of IGFBP7-AS1 in the odontogenic differentiation of SHED were investigated, and we aimed to identify a new target for improving tooth regeneration using SHED-based tissue engineering.

2. Methods

2.1. Cell Culture and Odontogenic Differentiation. SHED were kindly provided by Oral Stem Cell Bank operated by Beijing Tason Biotech Co. Ltd. (http://www.kqgxb.com) from children (age from five to seven) and cultured as previously described [10]. Our experiments were consented with the Ethics Committee of the Peking University School and Hospital of Stomatology, Beijing, China (Approval Number: PKUSSIRB-201732003). SHED at stages P3-P6 were used for subsequent experiments. To induce odontogenic differentiation, SHED were exposed to osteogenic media (OM) comprised of 0.01 mM dexamethasone disodium phosphate, 0.1 mM L-ascorbic acid phosphate, and 1.8 mM monobasic potassium phosphate (Sigma-Aldrich, MO, USA) after reaching 70%-80% confluence. The OM was changed every two days.

2.2. Alkaline Phosphatase (ALP) Staining. SHED were cultured in 12-well plates (3×10^4 cells/well) with or without OM, and ALP staining was performed on day 7 using an ALP staining kit according to the manufacturer's protocol (CWbiotech, Beijing, China). Briefly, phosphate-buffered saline (PBS) was used to rinse the cultured cells and the cell layer was fixed in 4% paraformaldehyde for 30 min. Then, it was washed with dH_2O and incubated in an alkaline solution for 10 min at room temperature.

2.3. Alizarin Red S (ARS) Staining. SHED were cultured in 12-well plates. When the cells reached 70%-80% confluency, they were exposed to OM and cultured for an additional 14 days. Briefly, the cells were fixed in 4% paraformaldehyde for 15 min and subsequently stained with 0.1% ARS (pH 4.0-4.6) for 20 min. The integrated density was also measured from histochemical slides.

2.4. RNA Isolation and Quantitative Reverse Transcription PCR (qRT-PCR). TRIzol (Invitrogen, Carlsbad, CA, USA) was used to extract the total RNA, and 1 μg of total RNA was converted to cDNA using a PrimeScript RT Reagent Kit (TaKaRa, Shiga, Japan). qPCR was performed as the previous study described [10]. Each genetic analysis was performed in triplicate, and the primers that were used are listed in Table 1.

2.5. RNA Sequencing. The RNA samples were prepared using a total of 3 μg of RNA per sample as the input material. The NEBNext Ultra RNA Library Prep Kit for Illumina (NEB, San Diego, CA, USA) was used to generate the sequencing libraries according to the manufacturer's recommendations. Briefly, mRNA was purified from the total RNA using poly-T oligo-attached magnetic beads, and fragmentation was performed using divalent cations under an elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5x). First-strand cDNA synthesis was performed using a random hexamer primer and M-MuLV reverse transcriptase (RNase H). Second-strand cDNA synthesis was subsequently performed using DNA.

After the adenylation of the 3′ ends of DNA fragments, the NEBNext adaptor with a hairpin loop structure was ligated in preparation for hybridization. The AMPure XP system (Beckman Coulter, Beverly, USA) was used to purify library fragments. Phusion high-fidelity DNA polymerase, universal PCR primers, and index (X) primer were used in the PCR progress. Finally, the AMPure XP system was used to purify the PCR products and the Agilent Bioanalyzer 2100 system (Agilent Technologies, Santa Clara, California, USA) was used to confirm the library quality. After cluster generation, library preparations were sequenced on an Illumina Hiseq platform, and 125 bp-150 bp paired-end reads were generated. The differential expression of lncRNAs with statistical significance was performed using the EdgeR package on R, and cut-offs were established at a fold change > 1.3 and a p value < 0.05.

Table 1: Sets of primers used in qPCR.

Gene name		5′-3′	Size (bp)	Gene bank number
GAPDH	F	CCGTCTTGAGAAACCTGCCA	139	NM_001115114.1
	R	GGATGAACGGCAATCCCCAT		
ALP	F	CTCCATACCTGGGATTTCCGC	299	NM_000478.6
	R	GGCCCCAGTTTGTCCTTCTT		
DSPP	F	GGAATGGCTCTAAGTGGGCA	284	NM_014208.3
	R	CTCATTGTGACCTGCATCGC		
DMP1	F	GAGTGGCTTCATTGGGCATAG	260	NM_004407.4
	R	GACTCACTGCTCTCCAAGGG		
OCN	F	TCACACTCCTCGCCCTATTG	133	NM_199173.6
	R	CTCTTCACTACCTCGCTGCC		
BMP2	F	ACTCGAAATTCCCCGTGACC	144	NM_001200.4
	R	CCACTTCCACCACGAATCCA		
ENST00000333145	F	GGCTTTGGGTATGGCTAAT	89	NR_015377.2
	R	AAGGTTCTGGAAGGTTGC		
ENST00000508328	F	GGTTGGGTTCATGTGCTAC	121	NR_034081.1
	R	AGAATTGCTTCCTGCTAATCT		
ENST00000524152	F	GGCAACAACAGTCTTCTATCC	105	NR_033651.1
	R	TGCTGCCCTTTATTGTGCTA		
ENST00000590622	F	TTTCTCATCCGTCCACCG	94	NR_038278.1
	R	CGTACCTTTAATCTGGAGACAA		

2.6. GO Analysis. Gene Ontology (GO) enrichment analysis of differentially expressed genes was implemented using the clusterProfiler R package (Yu, Wang, Han, and He, 2012), in which gene length bias was corrected. GO terms with corrected p values < 0.05 associated with differential expressed genes were considered significantly enriched.

2.7. RNA Oligoribonucleotides and Cell Transfection. RNA oligoribonucleotides (e.g., small-interfering RNAs (siRNAs) targeting lncRNA IGFBP7-AS1 and siRNA control (siNC)) were purchased from GenePharma (Shanghai, China). The sequences are listed in Table S1. SHED were cultured in 12-well plates prior to transfection. After reaching 60% confluence, the cells were transfected with siRNAs using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA).

2.8. Lentivirus Infection. EF-1aF/GFP and puromycin lentiviruses were created by GenePharma (Shanghai, China) to induce lncRNA IGFBP7-AS1 overexpression. SHED were transfected with lentiviruses (MOI: 20) to upregulate the level of lncRNA IGFBP7-AS1. Polybrene (5 mg/mL) was used in the lentivirus medium to improve the infection efficiency. In addition, the medium was changed after 8 h. After three days, the infection efficiency was detected using an inverted fluorescence microscope (Olympus, Japan) and verified using real-time PCR.

2.9. Western Blot. The protein lysis buffer containing a phosphatase inhibitor (Applygen Technologies Inc., Beijing, China) was used to harvested cells. The cell suspensions were centrifuged at 4°C for 30 min with a speed of 12,000 × g. The BCA Protein Assay (CWBIO, Beijing, China) was used to determine the protein concentration, and each lane was loaded with equal aliquots of the total protein (20 μg). The sample lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA), blocked in blocking sodium (Beyotime, Shanghai, China) for 1 h, and probed with the following antibodies at 4°C overnight: DSPP (1 : 1000; Santa Cruz Technology, Santa Cruz, CA), DMP1 (1 : 1000; Bioss, Beijing, China), p38, p-p38, and β-actin (1 : 10000; Cell Signaling Technology, Beverly, MA, USA). The membrane was incubated at room temperature for 1 h with horseradish peroxidase- (HRP-) conjugated antirabbit immunoglobulin. The protein expression was detected using a Western enhanced chemiluminescence blotting kit (ECL, SOLIBRO, Beijing, China).

2.10. Subcutaneous Transplantation. SHED cultured with α-minimum essential medium (MEM) supplemented with 10% fetal bovine serum (negative control (NC) group) or with upregulated lncRNA IGFBP7-AS1 (IGFBP7-AS1 group) were detached using trypsin EDTA and resuspended

in PBS. 5×10^5 cells were used and mixed with 1 mg hydroxyapatite (HA), then, incubated at 37°C under 5% CO_2 for 4 h.

For the implantation procedure, six-week-old SCID mice (males, CB17) were used as subcutaneous transplant recipients. Operations were performed using anesthesia achieved by intraperitoneal injection of pelltobarbitalum natricum. Incisions approximately 0.5 cm in length on each side were made on the dorsal surface of each animal. Subcutaneous pockets were created by blunt dissection, and the cells and HA (1 mg) were placed into the pockets. Incisions were closed with surgical sutures. Animals were sacrificed 8 weeks after implantation. For histology, the tissues were stained with hematoxylin and eosin (H&E) or Masson trichrome (Trichrome Stain (Masson) Kit, Sigma-Aldrich). For the immunohistochemistry staining of DSPP and DMP1, a 1 : 100 dilution of antibody was applied.

2.11. Statistical Analysis. SPSS21.0 statistical software (IBM Corp., Armonk, NY, USA) was used to perform the statistical calculations. Comparisons between two groups were analyzed using an independent two-tailed Student's *t*-test, and comparisons between more than two groups were analyzed using a one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. All data were expressed as the mean ± standard deviation (SD) of three experiments per group, and $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Odontogenic Differentiation of SHED. Odontogenic differentiation of SHED induced by OM was measured by qPCR, ALP staining, and ARS staining. The intensity of ALP staining on day 7 and ARS staining on day 14 is significantly increased (Figure 1(a)). Alkaline phosphatase (ALP) expression is significantly increased on day 7 and day 14 compared with day 0. In addition, the expressions of odontogenic markers, dentin sialophosphoprotein (DSPP) and dentin matrix acid phosphoprotein 1 (DMP1), are significantly increased on day 14 (Figure 1(b)). The total RNA of successfully differentiated cells was subsequently used for RNA-seq.

3.2. Expression Profiles of lncRNA and mRNA during Odontogenic Differentiation and Validation. RNA-seq analysis identified 234,832 annotated transcripts of lncRNAs expressed during SHED odontogenic differentiation, of which 136,996 were detected on day 0, 136,832 on day 7, and 138,111 on day 14. There are 1138 lncRNAs differentially expressed on day 7 compared to day 0, whereas 569 lncRNAs are upregulated and 569 lncRNAs are significantly downregulated. In addition, 1358 lncRNAs are differentially expressed on day 14 compared to day 0, among which 767 lncRNAs are significantly upregulated and 591 lncRNAs are significantly downregulated. A Venn diagram analysis reveals that 466 lncRNAs are simultaneously altered on both day 7 and day 14 during odontogenesis (Figure 2(a)).

Subsequent mRNA analysis identifies 1034 mRNAs, among which 343 are upregulated and 219 are significantly downregulated on day 7 compared with day 0, while 437 mRNAs are upregulated and 323 are significantly downregulated on day 14 compared with day 0. Venn diagram analysis reveals that 289 mRNAs are simultaneously altered during odontogenesis (Figure 2(b)).

GO analyses at biological process (BP), cellular component (CC), and molecular function (MF) levels were performed on mRNAs differentially expressed during odontogenic differentiation of SHED. GO analysis of day 7 vs. day 0 data shows that the highest enrichment scores for BP are related to "DNA packaging," with 27 differentially expressed genes. For CC, the three highest enrichment scores are "DNA packaging complex," "nucleosome," and "protein-DNA complex," associated with 22, 20, and 22 differentially expressed genes, respectively. For MF, "protein heterodimerization activity" has the highest enrichment score and is associated with 29 differentially expressed genes (Figure 2(c)).

GO analysis of day 14 vs. day 0 data reveals that the three highest enrichment scores for BP are "leukocyte migration," "extracellular structure organisation," and "extracellular matrix organisation," which are functionally associated with bone regeneration. For CC, the two highest enrichment scores are "extracellular matrix" and "proteinaceous extracellular matrix," associated with 43 and 39 differentially expressed genes, respectively. For MF, "glycosaminoglycan binding" has the highest enrichment score (Figure 2(d)).

The characteristics of the top five differently expressed lncRNAs on day 7 and day 14 are listed in Tables 2 and 3. Four candidate lncRNAs (ENST00000333145, ENST00000508328, ENST00000524152, and ENST00000590622) were selected to validate the RNA-seq results, and they exhibit a significant fold change subjected to qRT-PCR analysis (Figure 2(e)). All results are consistent with the normalized RNA-seq data (Figure 2(f)).

3.3. The Odontogenic Differentiation of SHED Was Promoted by IGFBP7-AS1. Through RNA sequencing, lncRNA IGFBP7-AS1 is upregulated on both days 7 and 14 during SHED odontogenic differentiation. This result was also confirmed using qRT-PCR. To determine the effect of IGFBP7-AS1 on SHED odontogenic differentiation, lentiviruses were used to overexpress IGFBP7-AS1, and siRNA targeting IGFBP7-AS1 was used to knockdown its expression. The transfection efficiency was observed by a fluorescence microscope, and the expression levels were confirmed using qRT-PCR (Figures 3(a) and 4(a)). SHED successfully transfected were cultured in OM and harvested on days 7 and 14.

The ALP staining results show that the level of ALP expression is decreased by the IGFBP7-AS1 knockdown and increased by IGFBP7-AS1 overexpression after seven days of induction. The ARS staining results reveal that matrix mineralization is reduced in the IGFBP7-AS1 knockdown group and enhanced in the IGFBP7-AS1 overexpression group (Figures 3(b) and 4(b)).

ALP, DSPP, and DMP1 are odontogenic markers, and the expression of them was assessed in SHED cultured in

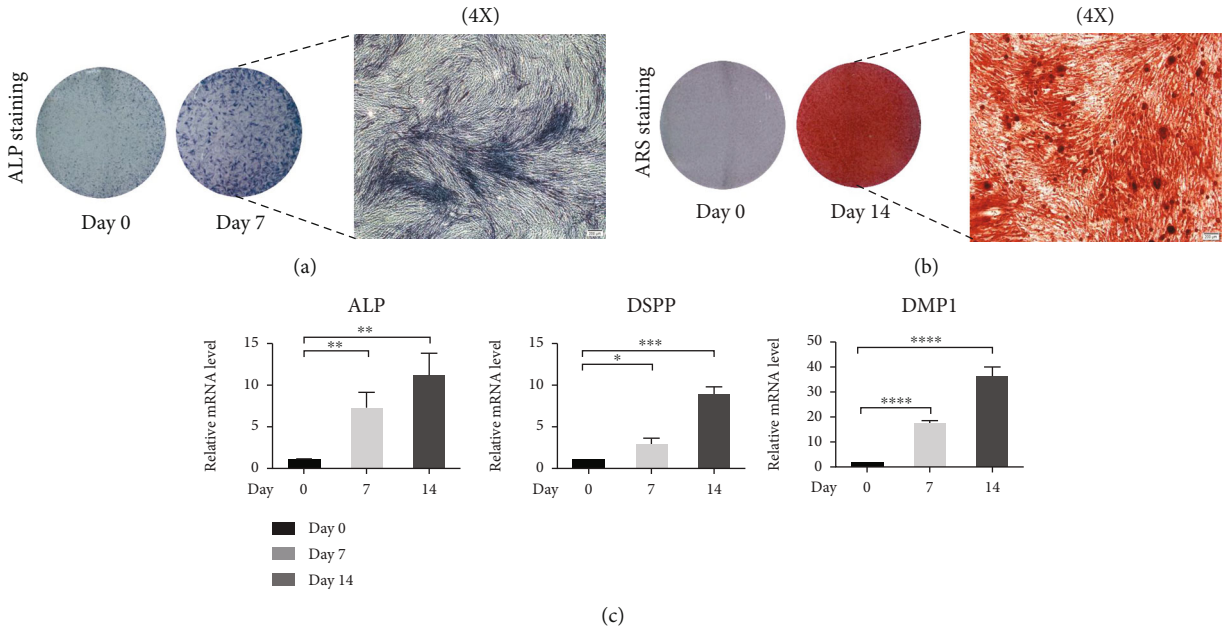


Figure 1: Odontogenic potential of stem cells from human exfoliated deciduous teeth (SHED) during differentiation on day 0, day 7, and day 14. (a) ALP staining of SHED on day 0 and day 7. (b) ARS staining of SHED on day 0 and day 14. Scale bar = 200 μm. ALP: alkaline phosphatase staining; ARS: alizarin red S staining. (c) Expression levels of odontogenic markers ALP, DSPP, and DMP1 are significantly increased during differentiation on day 7 and day 14. Data were expressed as the mean ± standard deviation (SD) of three experiments per group, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

OM for 14 days. IGFBP7-AS1 knockdown significantly downregulates the mRNA level of ALP, DSPP, and DMP1 (Figure 3(c)); however, IGFBP7-AS1 overexpression markedly upregulates the expression of these genes (Figure 4(c)). IGFBP7-AS1 is further confirmed to regulate DSPP and DMP1 at the protein level (Figures 3(d) and 4(d)). Furthermore, the expression of osteogenic differentiation-related markers, BMP2 and OCN, is not significantly altered during this process (Figures 3(c) and 4(c)).

For the *in vivo* experiments, the H&E results (Figure 5(a)) show that there are more odontoblast-like cells around the materials for the IGFBP7-AS1 group than for the control group. Immunohistochemistry staining of DSPP and DMP1 is consistent with these results (Figure 5(c)). New collagen formation, as revealed by Masson's trichrome staining (Figure 5(b)), is observed for both the NC and IGFBP7-AS1 groups.

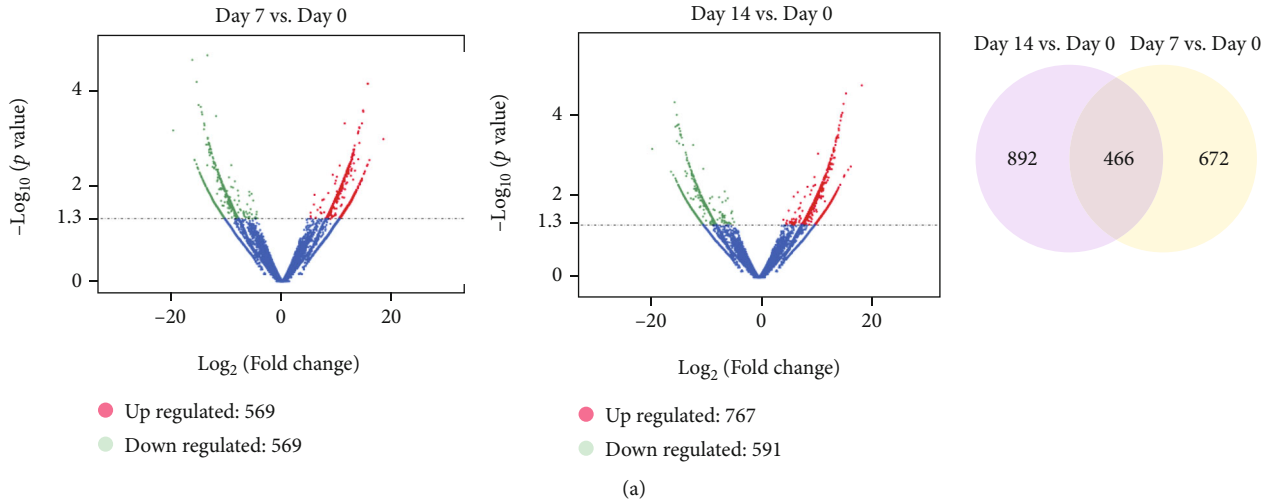
3.4. IGFBP7-AS1 Regulates the Odontogenic Differentiation of SHED via the MAPK Pathway. Several studies have reported that the MAPK family is a classic pathway that plays a crucial role in the differentiation, mineralization, and proliferation of mesenchymal stem cells [19, 20]. Therefore, in this study, we examined the effects of IGFBP7-AS1 on SHED activation mediated by the p38 MAPK signaling pathway. The level of p-p38/p38 is markedly decreased when IGFBP7-AS1 is knocked down (Figure 6(a)). Importantly, these inhibitory effects are reversed when IGFBP7-AS1 are overexpressed (Figure 6(b)). To validate this result, we also added SB203580, an inhibitor of the p38 MAPK signaling pathway, and the level of p-p38/p38 in the IGFBP7-AS1 overexpressed group is decreased (Figure 6(c)). The ALP staining and ARS staining as well as the expression of odontogenic markers are inhibited by SB203580 in the IGFBP7-AS1 overexpressed group (Figures 6(d) and 6(e)).

4. Discussion

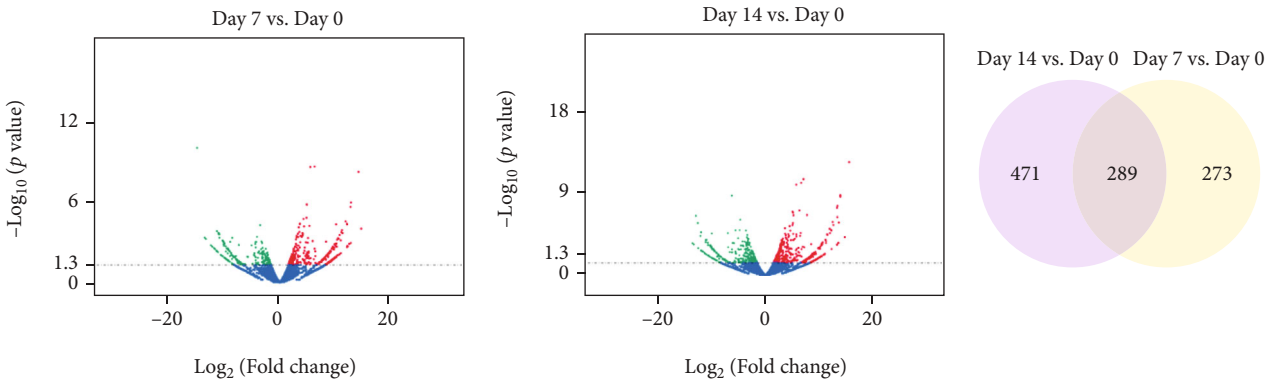
Pulp necrosis or pulpitis is a common disease in the dental clinic, which may lead to a loss of dental hard tissue and even tooth loss. These conditions represent a large financial burden to patients and present huge challenges to dentists. Recently, dental tissue engineering has been found to play an important role in resolving these challenges.

Since SHED have the potential for multidirectional differentiation [21], as such, they are considered to be favorable seed cells for tissue engineering due to their ability to develop into neural cells, adipocytes, and osteoblasts [9]. Moreover, they express odontogenesis-related markers (e.g., DSPP and DMP1), and they have been reported to differentiate into odontoblast-like cells *in vivo* [22]. In the present study, we found that SHED possessed the potential to form calcified tissue and to express DSPP and DMP1 induced by osteo/odontogenetic media, similar to the results of previous studies [8]. This finding indicates that SHED may represent a good choice of seed cells for the regeneration of dental hard tissue.

lncRNAs are an important part of epigenetic regulation. Emerging evidence indicates that lncRNAs may play a



(a)



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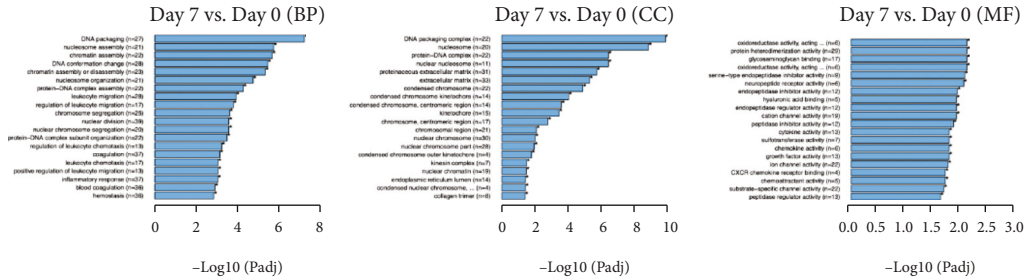

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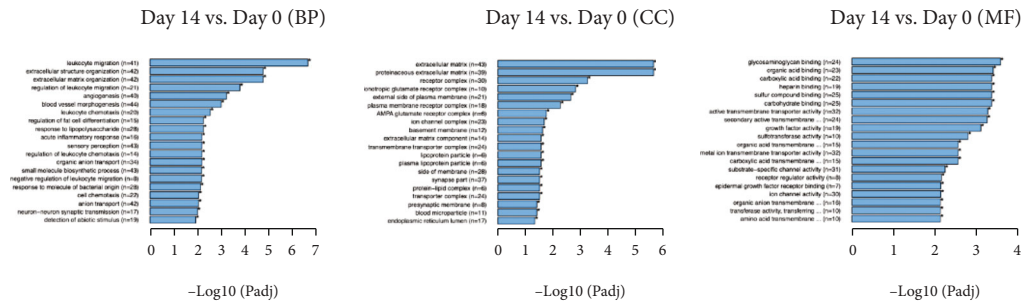

(d)

Figure 2: Continued.

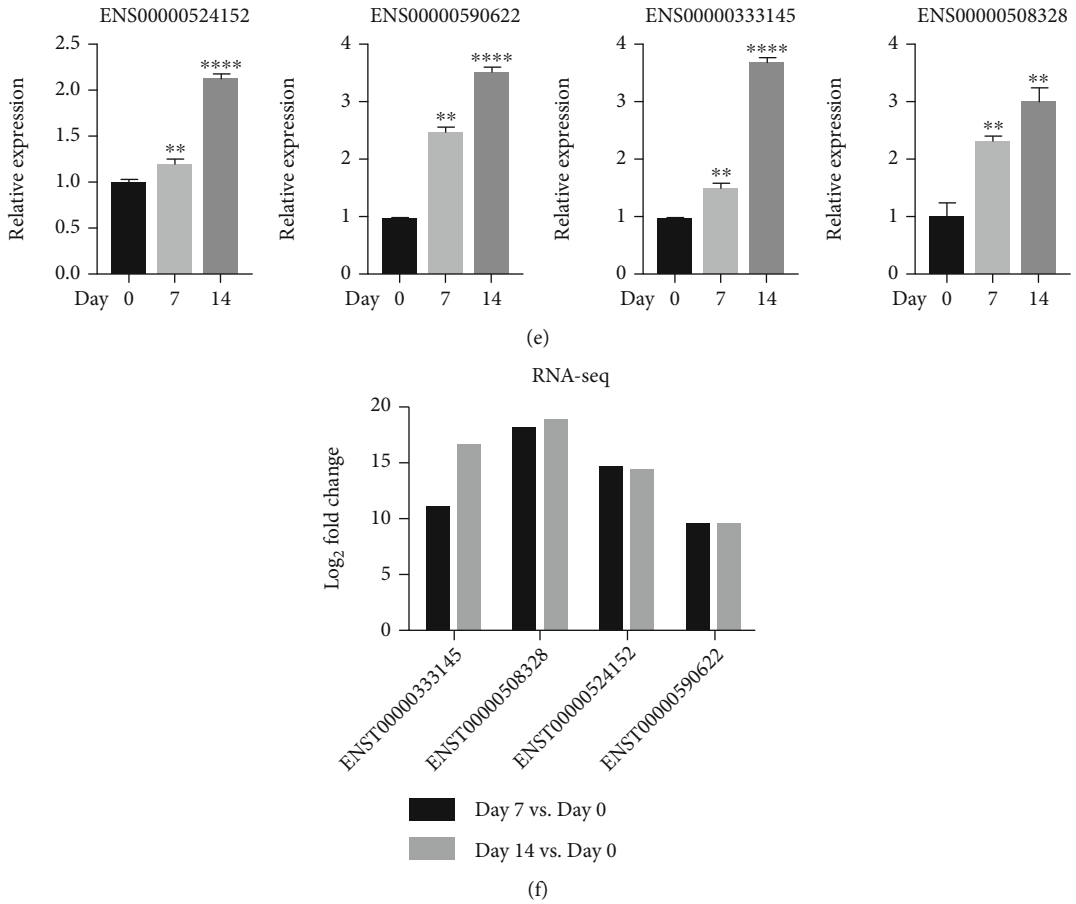

Figure 2: lncRNA and mRNA profiles of stem cells from human exfoliated deciduous teeth (SHED) during odontogenic differentiation. (a) Volcano plot showing 569 lncRNAs upregulated and 569 lncRNAs significantly downregulated on day 7, and 767 lncRNAs upregulated and 591 lncRNAs significantly downregulated on day 14 (Log2 fold change (FC) > 1.3 or ≤-1.3, $p < 0.05$). The Venn diagram shows differentially expressed lncRNAs during odontogenic differentiation of SHED. (b) Volcano plot showing 343 mRNAs upregulated and 219 mRNAs downregulated on day 7, and 437 mRNAs upregulated and 323 mRNAs downregulated on day 14 (Log2 fold change (FC) > 1.3 or ≤-1.3, $p < 0.05$) on gene level. The Venn diagram shows differentially expressed mRNAs during odontogenic differentiation of SHED. (c) Gene Ontology (GO) analysis of mRNAs differentially expressed during odontogenic differentiation in SHED on day 7. (d) GO analysis of mRNAs differentially expressed during odontogenic differentiation in SHED on day 14. (e) qRT-RCR validation of selected lncRNAs differentially expressed during odontogenic differentiation: ENST00000333145, ENST00000508328, ENST00000524152, and ENST0000 0590622. (f) Results of RNA-sequencing data for selected lncRNAs. Data were expressed as the mean ± standard deviation (SD) of three experiments per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

Table 2: Characteristics of the top five differently expressed lncRNAs on day 7.

Gene symbol	Sequence name	Regulation	Chromosome	Log2 fold change	p value
IGFBP7-AS1	ENST00000508328	Up	Chr4	18.1404343	0.00109902
ZBTB16	ENST00000541602	Up	Chr11	16.6554103	$1.27E-05$
RPL4	ENST00000561775	Down	Chr15	-19.561437	0.00072486
ADAMTS9-AS2	ENST00000481312	Down	Chr3	-16.138993	$2.46E-05$
LINC00511	ENST00000649793	Down	Chr17	-15.747108	0.00295472

crucial role in the regulation of differentiation and stem cell biology, and they may also be key regulators involved in human hard tissue regeneration [23, 24]. Previous studies have determined the expression profile of lncRNAs and mRNAs in different types of mesenchymal stem cells during the osteogenic process under various conditions [25–27] and confirmed that some lncRNAs including MALAT1, DANCR, H19, and MIR31HG can regulate osteogenic

Table 3: Characteristics of the top five differently expressed lncRNAs on day 14.

Gene symbol	Sequence name	Regulation	Chromosome	Log2 fold change	p value
IGFBP7-AS1	ENST00000508328	Up	Chr4	18.76836388	$1.69E-05$
ZBTB16	ENST00000541602	Up	Chr11	18.17336972	$1.75E-06$
PAX8-AS1	ENST00000333145	Up	Chr2	16.7385569	0.00176779
SH3BGRL2-OT1	TCONS_00271625	Up	Chr6	16.27896439	0.002127
RPL4	ENST00000561775	Down	Chr15	-19.531867	0.00064332

differentiation through classic osteogenic signaling pathways, including the Wnt/β-catenin, mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF-κB), and bone morphogenetic protein-2 (BMP2) pathways [28–32]. Osteogenesis shares many similarities with odontogenesis, such as extracellular matrix synthesis. One study explored the differential expression of lncRNAs during the osteogenic/odontogenic differentiation of DPSCs and found that lncRNA SNHG7 may represent a potential target for the osteo/odontoblast differentiation of DPSCs [14]. Furthermore, lncRNA H19 has been reported to promote the odontogenic differentiation and lncRNA DANCR has been reported to inhibit the odontogenic differentiation epigenetically. However, lncRNAs tend to exhibit both strong cell- and tissue-specific expression [33]. Thus, in the present study, we measured the level of lncRNA expression in SHED during odontogenic differentiation using high-throughput sequencing. The expressions of lncRNAs and mRNAs are significantly changed during odontogenic differentiation. These remarkable differences during differentiation indicate that lncRNAs might play a key role in the odontogenic differentiation of SHED, consistent with the findings in other types of mesenchymal stem cells [14]. GO analysis on day 7 reveals that the highest enrichment scores of BP, CC, and MF categories are "DNA packaging," "DNA packaging complex," and "protein heterodimerisation activity," which are all related to epigenetic regulation and indicate epigenetically process may exert a crucial role in this stage. Meanwhile, on day 14, the highest enrichment scores for BP, CC, and MF categories are all related to ECM. Interactions between cells and ECM control various cellular activities including adhesion, differentiation, proliferation, and apoptosis, all of which are associated with odontogenesis [34]. The changes in expression patterns of mRNAs and lncRNAs will help us explore the relationship between them in the future.

The RNA-sequencing results reveal that lncRNA IGFBP7-AS1 was significantly upregulated on both day 7 and day 14. lncRNA IGFBP7-AS1 is an antisense transcript of insulin-like growth factor-binding protein 7 (IGFBP7) and has been reported to be relevant to cancer [18]; however, no study has reported the role of IGFBP7-AS1 on odontogenic differentiation of SHED. Thus, qPCR was used to confirm the reliability of the RNA-sequencing results. The level of IGFBP7-AS1 expression is significantly upregulated during the odontogenic differentiation of SHED, which is consistent with the RNA-sequencing results. By overexpressing IGFBP7-AS1, the levels of ALP activity and mineralized matrix deposition are abnormally increased in the SHED. In contrast, the downregulation of IGFBP7-AS1 generates the opposite results. DSPP and DMP1 are specific markers of odontogenic differentiation used to distinguish osteogenesis [35, 36]. In the present study, the expression of DSPP and DMP1 is upregulated at both the gene and protein level after overexpressing IGFBP7-AS1 and downregulated after IGFBP7-AS1 is knocked down. Interestingly, the levels of osteogenesis-related markers, BMP2 and OCN, remain remarkably unchanged when IGFBP7-AS1 was altered. The effect of IGFBP7-AS1 *in vivo* was also confirmed by subcutaneous transplantation into SCID mice. The results are consistent with our hypothesis that IGFBP7-AS1 plays a key role in the promotion of odontogenesis in SHED. Thus, we confirm the positive effect of IGFBP7-AS1 on the directed odontogenic differentiation of SHED, which may provide a new target for dental tissue regeneration.

Osteogenesis shares many similarities with odontogenesis, such as the secretion of type I collagen and noncollagen proteins and the extracellular matrix mineralization, and they may overlap with a signaling pathway, such as Wnt/β-catenin, mitogen-activated protein kinase (MAPK), and bone morphogenetic protein-2 (BMP2) pathways. From our RNA-sequencing results, we found that on day 14, the highest enrichment scores of GO analysis are all related to extracellular matrix, which is important in various cellular activities including adhesion, differentiation, and proliferation. The MAPK signaling pathway is important to the osteo/odontogenic differentiation of mesenchymal stem cells, which includes the p38, JNK, and ERK1/2 pathways. Previous studies have reported that the MAPK pathway regulates the extracellular matrix mineralization and plays a crucial role in triggering osteo/odontogenic differentiation [37–39]. Thus, we used the MAPK pathway as a candidate pathway. In the present study, the level of p-JNK and p-ERK1/2 is not influenced by IGFBP7-AS1 (data was not shown), whereas the level of p-p38 protein expression is eliminated when IGFBP7-AS1 is knocked down and reversed by the overexpression of IGFBP7-AS1. When adding the inhibitor of the p38 MAPK signaling pathway, the increase effect of IGFBP7-AS1 in odontogenesis of SHED is impaired. These findings revealed that IGFBP7-AS1 regulates the odontogenic differentiation of SHED by promoting p38 activation to p-p38 and promoting the expression of odontogenic differentiation-related markers: ALP, DSPP, and DMP1.

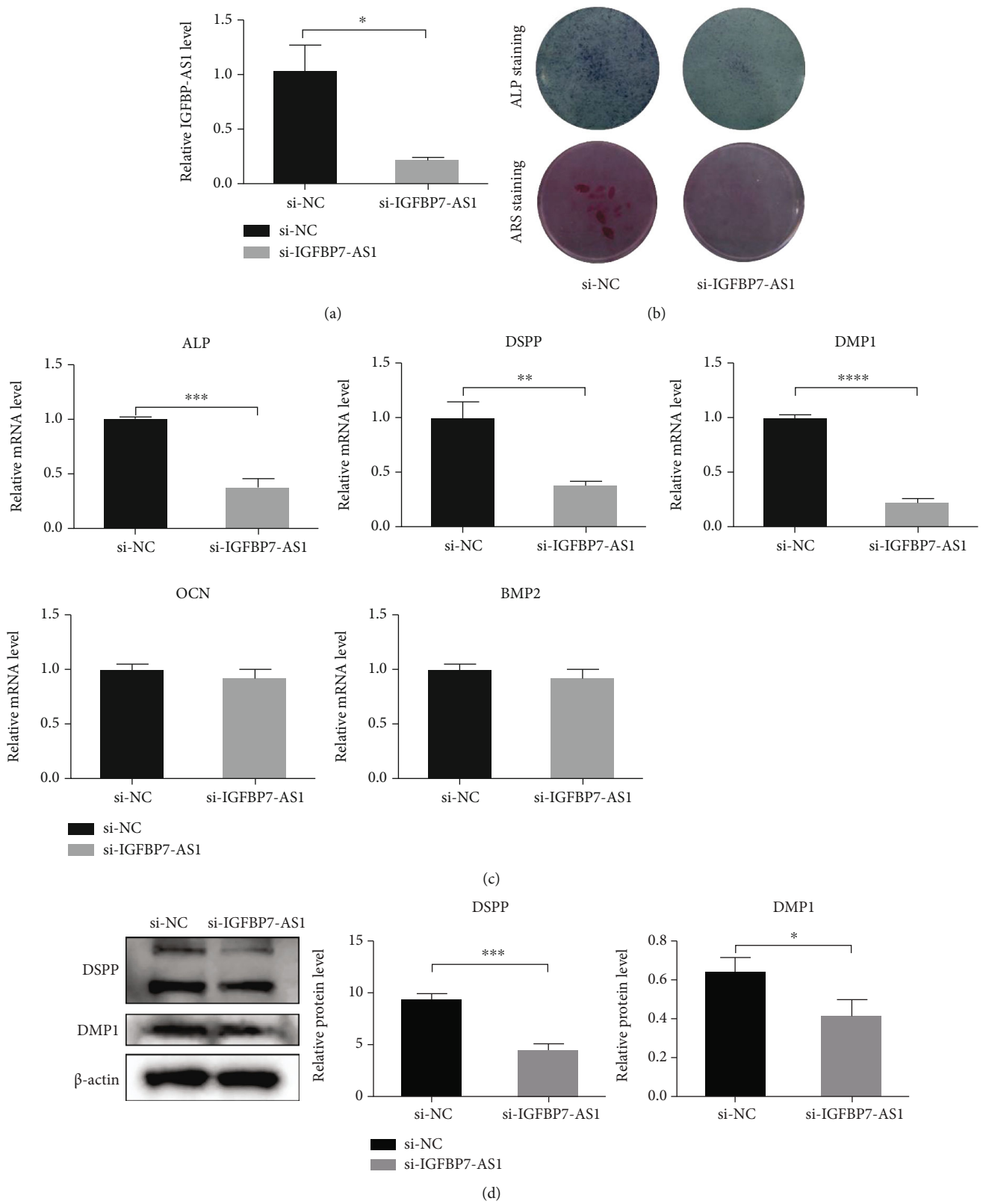

Figure 3: IGFBP7-AS1 knockdown suppresses the odontogenic differentiation of SHED. (a) qRT-PCR detected lncRNA IGFBP7-AS1 levels in the si-NC and si-IGFBP7-AS1 groups. (b) Images of ALP staining of SHED on day 7 after odontogenic differentiation and ARS staining of SHED on day 14 after odontogenic differentiation in the si-NC and si-IGFBP7-AS1 groups. (c) qRT-PCR detected osteo/odontogenic differentiation-related markers: ALP, DSPP, DMP1, OCN, and BMP2 in the si-NC and si-IGFBP7-AS1 groups. (d) Western blot detected odontogenic differentiation-specific markers: DSPP and DMP1 in the si-NC and si-IGFBP7-AS1 groups. Data were expressed as the mean ± standard deviation (SD) of three experiments per group, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

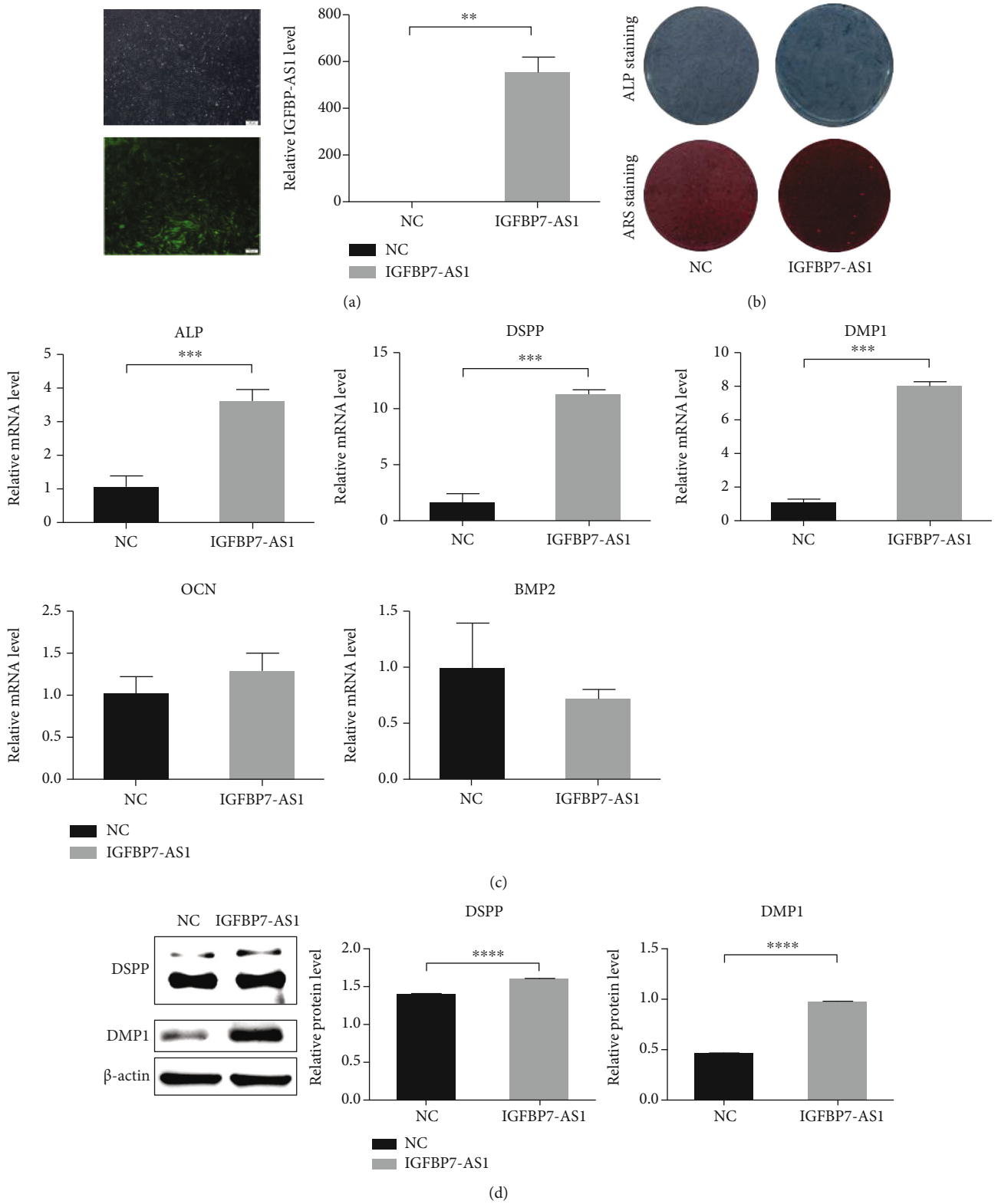

Figure 4: IGFBP7-AS1 overexpression increases the odontogenic differentiation of SHED. (a) Fluorescence microscope and qRT-PCR detected the transfection effect of IGFBP7-AS1 overexpression lentiviruses in the NC and IGFBP7-AS1 groups. Scale bar = 100 μm. (b) Images of ALP staining of SHED on day 7 after odontogenic differentiation and ARS staining of SHED on day 14 after odontogenic differentiation in the NC and IGFBP7-AS1 groups. (c) qRT-PCR detected osteo/odontogenic differentiation-related markers: ALP, DSPP, DMP1, OCN, and BMP2 in the NC and IGFBP7-AS1 groups. (d) Western blot detected odontogenic differentiation-specific markers: DSPP and DMP1 in the NC and IGFBP7-AS1 groups. Data were expressed as the mean ± standard deviation (SD) of three experiments per group, $^{**}p < 0.01$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

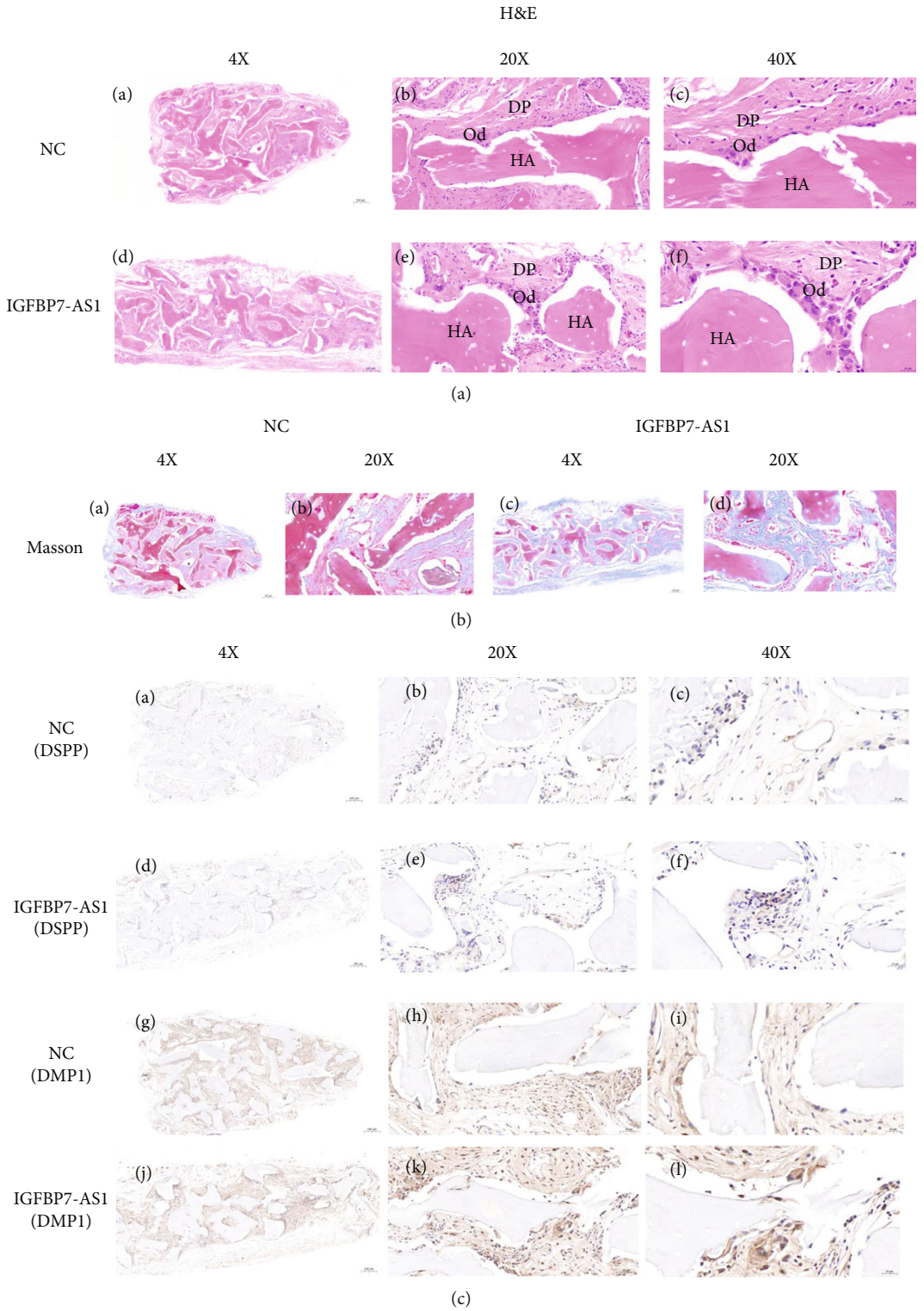

Figure 5: Subcutaneous transplantation results on 8 weeks showing that IGFBP7-AS1 promotes the odontogenic differentiation of SHED *in vivo*. (a) H&E staining, (a–c) NC group, and (d–f) IGFBP7-AS1 group. (b) Masson staining. (c) Immunohistochemistry staining, (a–f) immunohistochemistry staining of DSPP, and (g–l) immunohistochemistry staining of DMP1. Scale bar = 200 μm (4x); scale bar = 50 μm (20x); scale bar = 20 μm (40x) (HA: hydroxyapatite; DP: dental pulp; od: odontoblast).

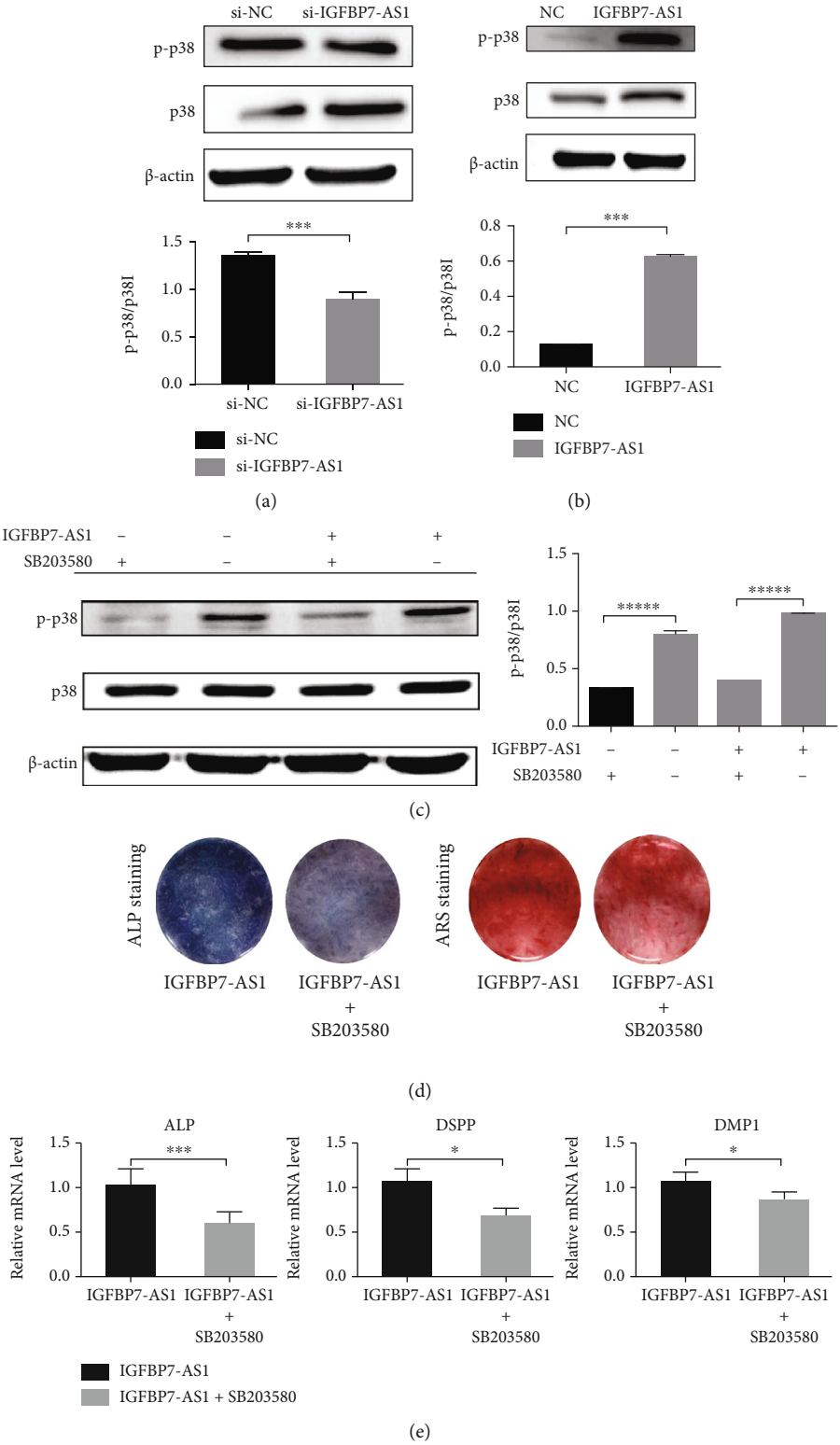

Figure 6: IGFBP7-AS1 regulates the odontogenic differentiation of SHED via the p38 MAPK pathway. (a) Western blot detected the expression of p-p38 and p38 in the si-NC and si-IGFBP7-AS1 groups. The ratio of p-p38 and p38 was calculated. (b) Western blot detected the expression of p-p38 and p38 in the NC and IGFBP7-AS1 groups. (c) Western blot detected the expression of p-p38 and p38 in the NC and IGFBP7-AS1 groups after adding the inhibitor of the p38 MAPK signaling pathway. The ratio of p-p38 and p38 was calculated. (d) Images of ALP staining and ARS staining of IGFBP7-AS1 overexpressed SHED odontogenic differentiation with or without SB203580. (e) qRT-PCR detected odontogenic differentiation-related markers: ALP, DSPP, and DMP1 of IGFBP7-AS1 overexpressed SHED odontogenic differentiation with or without SB203580. Data were expressed as the mean ± standard deviation (SD) of three experiments per group, $^{*}p < 0.05$, $^{***}p < 0.001$, and $^{****}p < 0.0001$.

5. Conclusion

In conclusion, we determined the lncRNA and mRNA expression profiles of SHED during odontogenesis and the role of IGFBP7-AS1 in this process. The results indicate that lncRNAs may represent important regulators of odontogenic differentiation in SHED and the expression profiles help us to further understand the epigenetic mechanism in odontogenesis. In addition, IGFBP7-AS1 may promote the odontogenic differentiation of SHED through the MAPK pathway. Although these findings provide new potential targets for dental tissue engineering, the specific molecular mechanism of IGFBP7-AS1 is needed in the future.

Data Availability

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

Conflicts of Interest

The authors declare that there is no conflict of interest.

Acknowledgments

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Supplementary Materials

Table S1 Sequences of siRNA targeting lncRNA IGFBP7-AS1. *(Supplementary Materials)*

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Review Article

IGFs in Dentin Formation and Regeneration: Progress and Remaining Challenges

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Tertiary dentin results from the interplay between the host defense and dental injury or infection. Modern endodontics aiming vital pulp treatment take the tertiary dentin formation as the interim step, with the final goal of a physiological pulp-dentin like tissue regeneration. Dental pulp stem cells have been nominated for contributing to differentiating into odontoblast-like cells who are responsible for reparative dentin formation. Understanding the original dentin formation mechanism provides us a blueprint while exploring the reparative dentin formation mechanism builds bridge to bonafide pulp-dentin tissue regeneration. Among all the regulators, growth factors have long been revealed under the spotlight. The insulin-like growth factor (IGF) family has been implicated in critical events of inducing dentin formation, which is essential for pulp treatment. The expression of IGF family members including IGF1, IGF1R, IGF2, and IGF2R has been well characterized in dental papilla cells, dental pulp stem cells, and periodontal ligament cells. Recent studies indicated IGF binding to the receptors activated pathways, including MAPK pathway, and AKT pathway, orchestrated proliferation, and differentiation, and finally, contributed to dentin formation. This review summarizes the role of IGF family in dentin formation during tooth development and tertiary dentin formation during dentin-pulp repair and sheds light on key parts of research for future treatment improvements.

1. Introduction

Both trauma and caries can lead to dentin-pulp injuries. Pulp diseases or injuries are not fatal diseases, however, markedly affect the quality of life. Over the last decades, improvements in the performance of capping materials have substantially decreased the pulp loss associated with caries and dental trauma. Although dental pulp diseases are common, and capping materials are more advanced, our ability to preserve dental pulps and recover their functions remains limited and challenged.

When the dentin-pulp is impaired, the interaction between direct tissue injury, infection, inflammation, and the host defense responses determines the final outcomes [1]. While the mild injury can induce early inflammatory defense responses, contributing to odontoblast activation and reactionary dentin deposition, long-term and more severe injury takes the lead of outcome to be out-of-balance and away from dentin regeneration [2]. Tertiary dentin formation plays an important role in injury repairing and promoting pulp healing. Understanding the mechanism beneath will underpin and drive biologically based healing strategies ahead.

Growth factors (GFs) are a kind of proteinic bioactive agents who are essential to tissue growth and regulate cell fate [3]. GFs are usually stored in the extracellular matrix, where the proteases/enzymes often induce their cleavages. GFs are divided into different families, including translate growth factors (TGFs), bone morphogenetic proteins (BMPs), connective tissue growth factors (CTGFs), vascular endothelial growth factors (VEGFs), and insulin-like growth factors (IGFs) [4]. Growth factors can be secreted through

autocrine, paracrine, or incretion and then bind to specific transmembrane receptors to trigger the subsequent bioreaction [5]. Each growth factor can only interact with one exclusive receptor among the others on the cells' superficies. The bioeffects induced by GF-receptor binding possess following features: (1) multitargeting: one singular growth factor can act on multiple target cells inducing multiple bioeffect; (2) overlapping: multiple growth factors can act on one target cell inducing similar bioeffect; (3) antagonism: one growth factor may trigger the bioeffect against another one; (4) synergism: one growth factor may trigger the bioeffect enhancing another one; (5) crosslinking: growth factors act synergistically or antagonistically with each other.

To achieve the injury repairing and promote pulp healing, multiple GFs reveal their essential roles in the formation of odontoblasts and odontoblast-like cells, subsequently leading to enhancement of dentin formation and tertiary dentin formation. Among these factors, the IGF family stands out in the spotlight and shows great potential for future bioactive pulp treatment.

2. Dentin Formation

The original dentin formation during tooth development exhibits us the biological process which we expect to work during dentin regeneration. Tooth development is initiated by the interaction between epithelium and mesenchyme [6]. Dentin formation starts at the interface of epithelium and mesenchyme, beginning at late bell stage [7]. In the bell stage, with the maturation of ameloblasts near the basement membrane, the inner enamel epithelium produces signaling molecules inducing dental papilla cells turning into odontoblasts. As the odontoblast maturation starts, odontoblasts' bodies are elongated with one end towards the dental papilla, while the other attaching to the basement membrane, and then develop into the odontoblastic processes extending into dentinal tubule. Odontoblasts are responsible for secreting organic matrix including collagen, noncollagenous protein, proteoglycan, and glycoprotein. When minerals complete deposition in the dentin matrix, dentin proceeds to mineralize and is ultimately matured. The mature dentin protecting the pulp with enamel lying in the outer layer can be divided into three parts—dentinal tubules, odontoblastic processes, and extracellular matrix. Dentinal tubules are filled with tissue fluid and odontoblastic processes. Odontoblastic processes are very sensitive to stimuli and play crucial roles in host defense. Extracellular matrix comprises peritubular dentin, intertubular dentin, interglobular dentin, Tomer's granular layer, and predentin [8].

Upon injury, would healing process in dentin-pulp initiates. It is a pathological event reflecting the extent and severity the dentin-pulp complex has suffered, and alongside the tertiary dentin formation, reactionary, and reparative dentinogenesis included. When the injury is mild enough for primary odontoblasts to survive, the following formed dentin can contain a completely regular tubular structure, which is defined as reactionary dentin. However, more intense injury strikes the dentin-pulp complex causing local death of the primary odontoblasts at the injury site and triggering a cascade of progenitors, most possibly dental pulp stem cells, recruited to the specific site, and ultimately forming reparative dentin with atubular morphology.

Zooming in to the cellular level, following tooth injury, cell death and cell replacement take place. In recreationary dentinogenesis, primary odontoblasts survive and, upon mild stimuli, keep secreting regular tubular dentin. While in reparative dentinogenesis, primary odontoblast death is followed by new generation of odontoblast-like cells, lining the pulp interface with dentin and secreting atubular dentin-like matrix. However, lacking unique molecular or morphological markers and unclear morphological evidence makes the identification of odontoblast-like cells and its origination difficult [9]. Several mesenchymal stem cell (MSC) populations, largely dental pulp stem cells, and fibroblasts are reported to contributing to the odontoblast-like cell formation, but evidence is limited.

Regenerative endodontics including vital pulp therapy, pulp revitalization, and cell homing treatments aims at healing the pulp injury with natural manners [10]. Bonafide bioactive pulp-dentin tissue is the desired result. Yet, due to limitations of current technique and materials, activation of wound healing responses to preserve the rest alive dentin-pulp complex with tertiary dentin and odontoblast-like cells formation has been taken as an interim step. Regenerative endodontics must fully consider the ability losing of tissue repairing, cell replacing, and dentin-pulp regeneration due to aging, injury, or genetic defects to rebuild physiological function. To achieve this, efforts have been exerted on studies including differentiation and formation of odontoblast-like cells and the underlying mechanism pathways. Among all the investigated biological factors, growth factors have shown its vital and irreplaceable role in the mist.

3. Growth Factors in Dentin Formation

Dentin formation is a complex process involving sequential and ordered deposition of an extracellular matrix, followed by its mineralization [11]. The formation of odontoblasts or odontoblast-like cells and the dentin formation is a continuous process. Growth factors affect the whole process directly or indirectly. Up till now, multiple factors including the IGFs, TGFs, and BMPs have been proved to play an irreplaceable role in dentin formation.

BMP family regulates various biological processes, such as cell proliferation, differentiation, migration, and extracellular matrix remodeling [12]. BMP-2 and BMP-7 could serve as odontogenic and osteogenic differentiation enhancers of human tooth germ cells (hTGSCs) [13]. Deficient in BMP-2 and BMP-4 caused dentin reduction and enlarged pulp chambers during tooth development [14]. BMP-2/FGF-9 signaling and TGF-β/BMP signaling were proved to be vital for odontoblast maturation and dentin formation in both temporal and spatial manner [15, 16]. BMP family contributes to dentin formation by promoting odontogenic differentiation and subsequently dentin quantity.

Among TGF family, TGF-βs has long been proven indispensable in dentin formation [17]. TGF-β1 increased the

mineralization and ALP activity of dental pulp cells which marked the enhancement of odontogenic differentiation by regulating transcription of two critical noncollagenous proteins, dentin sialophosphoprotein (DSPP), and dentin matrix acidic phosphoprotein 1 (DMP1), in odontoblasts [18]. TGF-β2 strongly upregulated when pulp cells differentiated into odontoblasts in vitro [19]. In addition, elevated TGF-β2 signaling in dentin resulted in sex-related enamel defects [20]. Upregulated TGF-β3 enhanced odontogenic differentiation and the formation of ectopic dentin [21]. TGF family act mainly on the formation and function of odontoblasts.

IGFs have also been investigated and have shown great potential in regulating dentin formation.

4. The IGF Axis: IGFs, IGF-1R, IGFBPs, and Postreceptor Signaling

IGF family composes of IGF-1 and IGF-2 participant in massive physiological activity, including development, growth, organogenesis, metabolism, cell proliferation, and cell differentiation [22]. IGF-1 and IGF-2 contains 70 and 67 amino acids, respectively. Both IGF-1 and IGF-2 are composed of one single polypeptide chain and four distinct domains-B, C, A, and D domains from N-terminal to C-terminal. IGF-1 has a 62% amino acid similarity with IGF-2 and 60% similarity with insulin. This is the origin of nomenclature of insulin-like growth factors [23, 24].

IGF receptors have different affinities for different ligands. Both IGF-1 and IGF-2 can act via the type 1 IGF receptor (IGF-1R), which is a dimeric transmembrane glycoprotein expressed ubiquitously both pre- and postnatally. IGF-1R contains two separate IGF-contacting sites, the high affinity-binding site made up with L1 and αCT domains and the low affinity-binding site composed of the Fn-III-1 domain. The type 2 IGF receptor (IGF-2R) is a single-chain type I intramembrane protein, which entraps the extracellular IGF-2 exclusively.

IGF-binding proteins (IGFBPs) are a group of proteins who regulate IGF ligand function. IGFBP1-6 share 50% homology and have high affinities for IGF-1 and IGF-2. 80% of the total IGF-1 are bound by IGFBP-3, who is the most important subtypes of IGFBPs for IGF-1, while IGFBP-6 is essentially IGF-2 specific. Most IGFBPs compete for activity of IGFs at the receptor level and antagonize IGF function, while some (e.g., IGFBP2) appear to amplify IGF signaling. IGFBP7-10 also play a role in IGF axis. They share conserved N-terminal domain of the conventional IGFBPs. But unlike IGFBP1-6, IGFBP7-10 show low affinity for IGFs. IGFBP7 is proved to be at least 5-fold to 25-fold lower than conventional IGFBP1-6 in binding IGFs with its IGF-dependent and IGF-independent cell growth regulating function [25, 26]. All these IGFBPs have been proven to exert irreplaceable effects on IGF ligand-receptor binding process and to be thus essential in post-binding cell events. It is widely believed that tissue specific effects of IGFs were largely related with the tissue specific expression of IGFBPs [27].

Once binding to the cognate receptor, their inner kinase domain starts to autophosphorylate, leading to activation of two main downstream pathways, phosphatidylinositol 3-kinase (PI3K)-AKT/mammalian target of rapamycin (mTOR) pathway, and Ras-mitogen-activated protein kinase (RAS-MAPK) pathway. PI3K-AKT/mTOR pathway activated by insulin receptor substrate (IRS) predominantly leads to metabolic outcomes. The SHC-initiated RAS-MAPK pathway controls mitogenic outcomes, both play vital roles in the host defense of dental tissue injury, wound healing, and pulp regeneration [28, 29].

5. IGFs in Dentin Formation and Regeneration

IGFs have been revealed to drive cell proliferation and differentiation during the tooth development. Recent studies showed that IGF-1 had an essential role in cellular migration and cell proliferation in many dental tissues [30]. Immunohistochemical localization proved the occurrence of IGF axis in human deciduous teeth and human permanent teeth from root to enamel, implicating its key role in tooth development [31, 32]. When root development started, IGF-1 expression soared in dental pulp [33]. Fujiwara et al. proved that the presence of IGF-1 resulted in elongation of Hertwig's epithelial root sheath (HERS) and increased cell proliferation in its outer layer compared with the control. This indicates its regulatory effect on early root formation through HERS [34]. Furthermore, the IGF axis can also regulate the formation of amelogenin, ameloblastin [35], and dentin matrix through paracrine/autocrine, hence confirmed to be crucial for amelogenesis and dentinogenesis [36, 37]. IGFBP5-7 were found to be upregulated during tooth germ mineralization in vivo and to be differentially localized in ameloblasts and odontoblasts, showing their potential role in downregulating mineralization [38]. IGF axis also reaches the periodontal ligament. Konermann et al. found a temporal increase of IGF-2 and IGFBP-6 in periodontal ligament cells (PDLCs) in vivo, as opposed to a general decrease at protein level, arousing the researchers' interest for further investigation. They reported the peculiar enhancement of IGF-2 and IGFBP-6 led to the reduced differentiation and proliferation ability of PDLCs [39].

Dental papilla cells turning into odontoblasts through cell fate determination is a pivotal step for dentinogenesis, where multiple signaling pathways and factors are involved, and IGF axis has a place. Similarly, the odontogenic differentiation of stem cells originated from dental tissues is also governed by IGF axis. In stem cells from dental papilla (SCAPs), IGF-1 were investigated to bind to IGF-1R, activating phosphorylation of MAPK pathways, leading to enhance the differentiation and mineralization of SCAPs by increasing key markers' expression, including Alp, Runx2, Osx, Ocn, Col-I, Dspp, and Dmp-1. This effect could be enhanced by microRNA hsa-let-7c depression whilst reversed by the overexpression [40]. Evidence accumulates that IGF-1 also contributes to the proliferation and differentiation of dental pulp stem cells (DPSCs). Magnucki et al. studied how IGF-1, IGFBP-3, and IGF-1R expressed in STRO-1-positive DPSCs of fully impacted wisdom teeth. Protein levels of IGF-1,

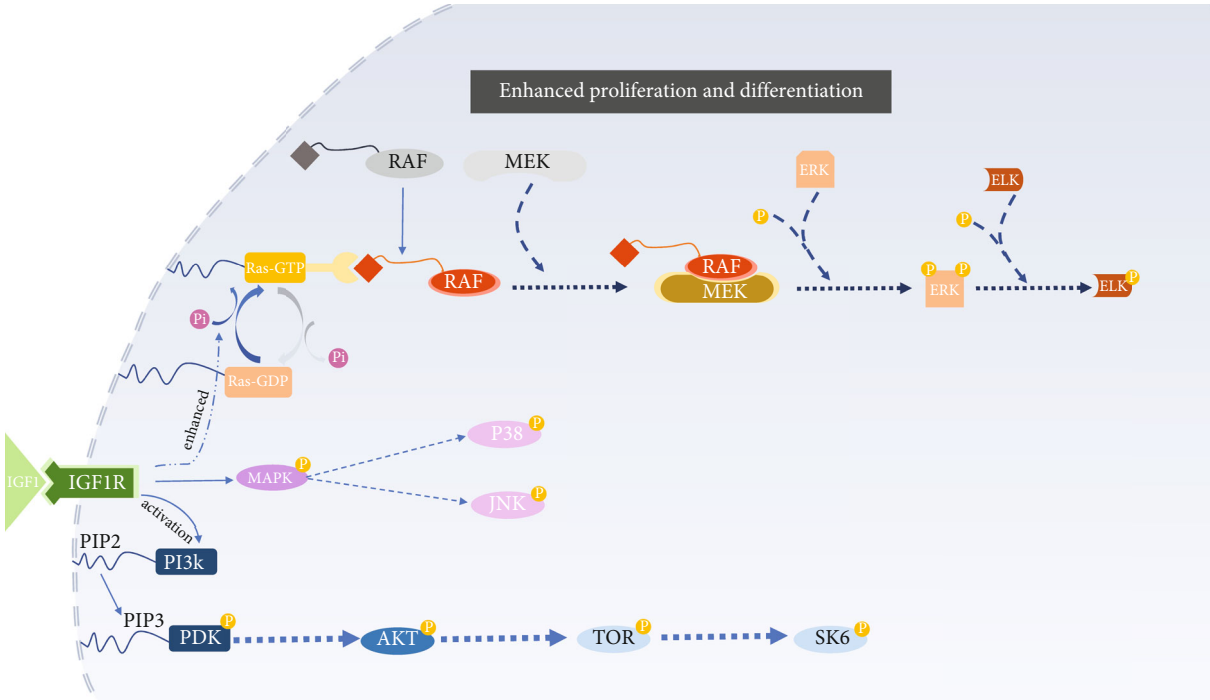

Figure 1: The postevent of IGF1 and IGF1R binding in cells. After IGF1 binds to IGF1R, two main pathways are activated. Ras-GTP expression is enhanced, leading to the activation RAF pathway, thus activating ELK and MAPK pathway. PI3K is activated, then boost AKT and mTOR expression.

IGFBP-3, and IGF-1R were shown to be particularly higher in young third molars with ongoing development and especially the STRO-1-positive DPSCs. It can be speculated that IGF-1 axis governed a variety of underneath mechanism in the final period of tooth development and the pulp cell differentiation [41]. Under the high glucose conditions, DPSCs showed low proliferation and differentiation ability with reduced ALP activity and mineralization. However, adding IGF-1 can significantly reverse the effects. The reduced ability of DPSC proliferation, differentiation, and mineralization induced by high GLU could be rescued by IGF-1 [42]. Besides, IGF-2 is also involved. Khan et al. found that IGF-2 expression is active in both epithelial and mesenchymal area, in developing cusp mesenchyme, and in newly formed enamel layer and dentin tubules. Methylation of cytosine-phosphate-guanine (CpG) islands in Igf2 underwent a time-dependent increase with correlated decreased levels of DLK1 and IGF-2 proteins in the tooth germ. Thus, IGF-2 expression reduced during tooth development regulated by epigenetic factors [43]. By binding to IGF-1R, IGF-2 could enhance the cell mitogenic activity leading to cell proliferation. In contrast, when IGF-2 binding to IGF-2R, it was subsequently trafficked to the lysosome, where IGF-2 was degraded, and thus, the cell mitogenic activity was largely weakened [44, 45]. The SCAPs showed stronger osteo-/dentinogenic differentiation potentials with the rhIGF2 compared with the control group indicating its positive effect towards odontogenic differentiation [46].

When pulp injury happens, pathological events emerge [47, 48]. IGF axis demonstrated to play an important role in the formation of dental mineralized tissue [49]. Alkharobi et al. found that DPSCs originated from the teeth suffered from superficial caries showed higher potential to differentiate into odontoblasts and/or into osteoblasts, indicating the exposure to the mild inflammatory condition may be one positive factor. Along with the mild inflammatory, the intracellular IGF axis components of DPSCs dramatically changed with IGF-2 and IGFBP-2 upregulated and IGFBP-3 downregulated. It implicated that IGF axis-oriented mineralization might occur in DPSCs from carious teeth [50]. In vitro studies showed the cell volume mitotic index and cell differentiation of DPSCs from mouse mandibular molars were both increased by improving IGFs, suggesting IGFs' role in optimizing odontogenic differentiation of DPSCs [36, 51]. Deposition of dentin extracellular matrix could only proceed with the presence IGF-1 rather IGF-2. The expression of DSPP mRNA can be decreased both by IGF1 and IGF2, while IGF2 is more talent in it [35]. EphrinB1-EphB2 interaction has been proved to regulate odontogenic/osteogenic differentiation from dental pulp cells in vitro [52]. Matsumura et al. found that ephrinB1 was strongly expressed in odontoblasts 4 weeks postinjury in a pulp exposure model. They also found that with the inhibition of IGF-1 receptor signaling inducing the block of both Ras/Raf-1/MAPK pathway and the PI3K/Akt/mTOR pathway, specifically inhibited EphB2 expression and ephrinB1 gene expression, respectively. Hence, the IGF-1/ephrinB1 axis involves in the early period of tooth injury events [53].

With the roles of growth factors in dentin formation and regeneration getting more and more defined, proper carriers facilitating clinic application are gaining more and more attention. Exosomes, small double-lipid layer particles

containing proteins, mRNA, and miRNA, can convey a multiple of bioinformation and stimuli. Exosomes secreted by MSCs showed strong regenerative potential and immunomodulation [54]. Cai et al. successfully promoted neurite outgrowth in vitro and enhanced regeneration after sciatic nerve injury in vivo by applying exosomes containing natural growth factors from adipose-derived MSCs [55]. In addition, exosomes from the human periapical cyst mesenchymal stem cells (hPCy-MSCs) were proved to be effective in the pathogenesis of Parkinson's disease [56]. For its excellent biocompatibility, biological safety, and bioeffect, exosomes might also be the ideal carrier of IGFs, waiting for scientists to further investigate.

6. Summary and Future Directions

The underneath mechanism of dental wound healing and repair sheds light on advanced healing strategies inducing pulp regeneration processes. IGFs orchestrating crucial cell events in dentin formation makes it one of the potential candidates for dentin regeneration. Yet, there are still gaps between our current knowledge about dentin formation and what is really going to there. Previous research proved spatio-temporal expression of IGF axis in dentin development, arousing interests in probing into the role of IGFs in tooth development. Further research revealed IGF axis' involvement in dentin and dentin-like tissue formation. Additionally, Ras/Raf-1/MAPK and PI3K/AKT/mTOR pathways were found activated by IGF-axis during this biological process. Yet, the adequate release and spatio-temporal expression of IGF-axis in an active form and the manual control release of IGF-axis imitating bioprocess have not yet been investigated. The gaps mentioned above also reminds us of potential barriers within the IGFs' application in the future. Moving forward, it will be critical to further investigate growth factors both at the genomics level in biologically relevant cell types and in the potential therapeutic application (Figure 1).

Data Availability

Data sharing is not applicable.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Review Article

Metabolic Remodeling Impacts the Epigenetic Landscape of Dental Mesenchymal Stem Cells

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Epigenetic regulation can dynamically adjust the gene expression program of cell fate decision according to the cellular microenvironment. Emerging studies have shown that metabolic activities provide fundamental components for epigenetic modifications and these metabolic-sensitive epigenetic events dramatically impact the cellular function of stem cells. Dental mesenchymal stem cells are promising adult stem cell resource for *in situ* injury repair and tissue engineering. In this review, we discuss the impact of metabolic fluctuations on epigenetic modifications in the oral and maxillofacial regions. The principles of the metabolic link to epigenetic modifications and the interaction between metabolite substrates and canonical epigenetic events in dental mesenchymal stem cells are summarized. The coordination between metabolic pathways and epigenetic events plays an important role in cellular progresses including differentiation, inflammatory responses, and aging. The metabolic-epigenetic network is critical for expanding our current understanding of tissue homeostasis and cell fate decision and for guiding potential therapeutic approaches in dental regeneration and infectious diseases.

1. Introduction

Adult mesenchymal stem cells (MSCs) residing in various tissues still have the capacity to undergo differentiation and self-renewal. Most MSCs stay quiescent in local tissues and can differentiate into certain cell types upon specific signaling, which plays a critical role in tissue homeostasis maintenance and regenerative need preservation [1]. Multiple adult stem cell types with diverse biological properties have been reported in many tissues and organs, including the bone marrow, blood, muscle, skin, and teeth. Stem cells derived from dental tissue include dental pulp stem cells (DPSCs), periodontal ligament stem cells (PDLSCs), stem cells from the apical papilla, and stem cells from human exfoliated deciduous teeth. Dental mesenchymal stem cells can undergo odontogenic, osteogenic, neurogenic, and chondrogenic differentiation, which contribute to injury repair of oral and maxillofacial tissues [2]. As a promising cell resource in tissue engineering, dental mesenchymal stem cells possess superior pluripotency and regenerative potential compared with other MSCs and can be easily obtained from extracted teeth [3, 4]. Clarifying the cellular mechanism in the cell fate decision of dental mesenchymal stem cells will greatly benefit therapeutic approaches in local injury repair and stem cell-based tissue engineering.

Cell fate decision is driven by a highly coordinated transcriptomic program and epigenetic events. There are multiple regulators and crucial features that contribute to the mechanism by which cells make choice and determination. During cell fate decision, a cell transition state is an intermediate stage with mixed identity, which could be kind of unstable and reversible [5]. Emerging evidence has suggested that dynamic epigenetic modifications play an important role in the cellular function of stem cells, including differentiation, self-renewal, and apoptosis [6]. Genomic DNA can be expressed differently due to alterations in chromatin structure constructed by nucleosome core particles. For each nucleosome, 147 base pairs of DNA were wrapped around a histone octamer (including histone proteins H2A, H2B, H3, and H4). The methylation modification in the cytosine-

guanine (CpG) islands of the DNA promoter regions leads to the inhibition of transcription [7]. Histones can be dynamically modified with various chemical groups and form methylation, acetylation, ubiquitylation, or other modifications [8, 9]. Histone modifications play a critical role in regulating the chromatin organization and accessibility of DNA to proteins [10]. Histone acetylation mainly occurs on lysine residues of histones that would weaken the charge attraction between histones and DNA and loosen the chromatin architecture, thereby facilitating gene transcription [11, 12]. Histone methylation also alters chromatin structure and has diverse effects on transcription control depending on the specific residues and number of methyl groups. For example, methylation of H3K9 and H3K27 is known as a signal of chromatin compaction and silencing [13], while trimethylation of H3K4 is primarily associated with promotor and transcription activation [14, 15]. After transcription, modified reactions can also occur on the RNA. As the most prevalent RNA modification, N^6-methyladenosine (m^6A) is an abundant methylation on adenosine residues that modulates every step of RNA metabolism, including processing, degradation, and translation [16]. Reversible modifications in DNA, histones, and RNA are involved in the temporal and spatial control of gene programs without alterations in nucleotide sequences.

Epigenetic regulation of the coordinated transcriptomic profile at the posttranscriptional level is required for cellular reprogramming, tissue homeostasis, and regeneration in response to environmental cues and metabolic signaling [17, 18]. For a long time, metabolic states have been recognized as consequences defined by cellular inputs and demands in specific stages. Cellular function and tissue homeostasis are supported by the cooperation of several metabolic pathways, such as glycolysis, mitochondrial oxidative metabolism, and fatty acid catabolism [19, 20]. Metabolic remodeling during cell fate transition is accompanied by fluctuations of various metabolites [21, 22]. Beyond energy production and biomass synthesis, metabolic fluctuations are now found to be essential cues in the regulation of self-renewal and differentiation, with technological advancements. The dynamic metabolic pattern is coordinated with the stage-specific requirements and nutrition availability to different cells in the microenvironment [23]. Metabolic intermediates such as methyl groups, α-ketoglutarate (α-KG), and acetyl coenzyme A (acetyl-CoA) are key substrates and cofactors for enzyme activities in epigenetic events that act as the bridge between the intracellular microenvironment and cell biology [23, 24]. Great strides have been made in the transcriptional pattern and epigenetic regulatory network during cell fate transition and decision [25]. Recent studies have addressed the metabolic cues in epigenetic regulation that overcome the knowledge gaps in understanding cell fate decisions [26–28]. The metabolic-epigenetic network could shed light on the cellular mechanism of dental mesenchymal stem cell fate decision.

In the present study, we review the conceptual foundation of the link from metabolism to epigenomics and the regulatory effect of epigenetic modifications on dental mesenchymal stem cells. Furthermore, we summarize the current knowledge of metabolic pathways and explore the potential role of metabolism as an epigenetic regulator in the oral and maxillofacial regions.

2. The Link from Metabolism to Epigenomics in Dental Mesenchymal Stem Cells

In addition to the well-known modifications of methylation, acetylation, and ubiquitination, there are many other less-known modifications, such as acylation and glycosylation, which comprise more than one hundred modifications in the epigenetic region [23]. Although the establishment of different modifications is complex and specific, there are some basic principles in this process that will help us understand the interaction between metabolic pathways and epigenetic events. Epigenetic reactions rely on the availability of corresponding chemical groups, cofactors, or antagonists derived from cellular metabolism [23, 29]. Chromatin-modifying enzymes such as methyltransferase and acetyltransferase are capable of utilizing metabolite substrates to install chemical marks, which can also be removed by specific enzymes. Epigenetic dynamics depend on the balance between the thermodynamic (K_d value) and kinetics (K_m value) of enzymes and the intracellular concentrations of corresponding metabolic substrates [29, 30]. In this way, metabolism plays an essential regulatory role in enzyme activity and subsequently defines the modifications in chromatin and RNA. Given the differences in the kinetic and thermodynamic properties of epigenetic enzymes and the availability of metabolite intermediates, chemical reactions display diverse sensitivities with metabolic states. Epigenetic modifications such as methylation and acetylation are canonical reactions in which enzyme activities are sensitive to the intracellular concentrations of metabolite substrates. Some other modifications seem to be less sensitive and not responsive to metabolic fluctuations. For example, ubiquitination reactions require ATP metabolites to form modifications, while the intracellular level of ATP is far more than the requirement [31]. Moreover, there are other mechanisms that link metabolic pathways to epigenetic events, permitting the complexity and plasticity of the metabolism-epigenetic network. Epigenetic modifications can be established without enzyme activity in certain situations, even for typical modifications such as methylation and acetylation [32]. Cell fate decision can be induced by altering the epigenetic sets of critical regulatory factors [10]. Global chromatin modification preferentially impacts the transcriptional dynamics of specific genes that are highly associated with cellular identity and function [33–35]. Metabolic remodeling reshapes the epigenetic landscape by inducing new epigenetic states or changing specific modification reactions. These metabolic-related epigenetic alterations further impact the transcriptional program and participate in the cell fate decision [27, 28]. Current studies regarding epigenetic regulation in the oral and maxillofacial regions have mainly focused on the functional effects of epigenetic modifying enzymes, while the regulatory role of metabolism remains elusive. In the following sections, we discuss the interaction of metabolic fluctuations and context-dependent

epigenetic events during the cell fate decision of dental mesenchymal stem cells.

2.1. The Impact of Metabolism on Methylation. Methylation conducted by methyltransferases can occur on DNA, histones, and RNA. DNA and histone methylation in chromatin are characterized by methyl group transfer conducted by DNA methyltransferases (DNMTs) and histone methyltransferases (HMTs) [7, 9]. RNA m^6A methylation is catalyzed by the methyltransferase-like 3 (METTL3)/METTL14 complex [36]. DNA methylation profiles in whole genomes of odontogenic cell populations show that the differences in DNA methylation are correlated with the osteogenic capacity of dental mesenchymal stem cells [37]. DNA methylation also participates in the immune and inflammatory responses of dental mesenchymal stem cells by regulating inflammatory cytokine secretion and signaling pathways [38, 39]. For histone methylation, the trimethylation of histone 3 lysine 27 (H3K27me3) regulates DPSC differentiation by suppressing the expression of odontogenic-related genes and activation of the Wnt/β-catenin pathway [40]. Other histone methylations such as H3K9me2, H3K9me3, and H3K4me3 also participate in the differentiation processes of DPSCs and PDLSCs [41–43]. Additionally, high levels of H3K27me3 and H3K4me3 inhibit cytokine secretion and corresponding pathway activation during the inflammatory response [44, 45]. METTL3-mediated RNA m^6A methylation modulates tooth root development [46] and regulates DPSC apoptosis via cell cycle progression [47]. METTL3 is also involved in the inflammatory response of DPSCs by modulating the alternative splicing of RNA [48].

The methyl donor S-adenosylmethionine (SAM) in these methylation reactions is primarily provided by one-carbon metabolism, which generates one-carbon units (methyl groups) for biosynthetic processes [49, 50]. One-carbon metabolism is a network of interconnected metabolic pathways, including the methionine cycle, folate cycle, and transsulfuration pathway [50]. SAM is an important metabolite substrate catalyzed by methionine adenosyltransferase in methionine metabolism. Other amino acids, such as threonine, serine, choline, and glycine, in the folate cycle can also interact with methionine metabolism and support SAM generation (Figure 1). Then, DNMTs, HMTs, and RNA methyltransferase complex can convert the SAM to S-adenosylhomocysteine (SAH), which supplies the methyl groups for DNA/histone and RNA methylation [51, 52]. The ratio of SAM/SAH is recognized as the intracellular methylated potential that impacts enzymatic activities.

The methyl donors and intermediates generated from one-carbon metabolism drive epigenetic reprogramming for the cell fate decision [49]. Accumulating studies have suggested a regulatory effect of one-carbon metabolism on global DNA methylation levels, and nutrient deficiency can lead to DNA hypomethylation [53]. Methionine and threonine support DNA and histone methylation in embryonic stem cells, which are essential for self-renewal and pluripotency maintenance [54]. The reduction in SAM accumulation due to threonine depletion leads to cell differentiation by reducing H3K4me3 levels [55]. Serine metabolism also regulates neural stem cell differentiation by modulating the methylation state of H3K4 [56]. In LPS-induced macrophages, activation of one-carbon metabolism promotes SAM generation and supports the trimethylation level of H3K36 on interleukin-1β [57].

In dental mesenchymal stem cells, amino acid levels change in response to physiological stimulation, which leads to the alteration of metabolite substrates in one-carbon metabolism [58, 59]. Yang et al. demonstrated that the differences in one-carbon metabolism of young DPSCs and aging DPSCs contribute to the alteration of regenerative capacity [59]. Serine, glycine, and threonine are significantly suppressed in aging DPSCs which restrict the SAM supply, resulting in low DNA methylation of the senescence marker p16 [59].

2.2. The Impact of Metabolism on Demethylation. Methylation marks can be reversibly turned over by demethylases in coordination with the metabolic state. Demethylases are capable of removing the methyl groups from DNA, histones, and RNA in a context-dependent manner. Active removal of DNA methylation can be catalyzed by the ten-eleven translocation proteins (TETs) including TET1, TET2, and TET3, which are α-KG-dependent dioxygenases [23, 60]. TET1 is upregulated during DPSC mineralization and facilitates odontogenic differentiation [61]. TET1 and TET2 are also involved in modulating the immunomodulatory response of PDLSCs [62]. For histone demethylation, Jumonji domain-containing demethylase 3 (JDJM3) and another demethylase, lysine demethylase 5A (KDM5A), regulate the odontogenic differentiation by inhibiting the trimethylation levels of H3K27 and H3K4 on odontogenic-related genes, respectively [63–65].

The catalytic activities of some critical demethylases such as TETs and JMJDs depend on the metabolite substrate, α-KG and oxygen. α-KG is an important intermetabolite generated from the tricarboxylic acid cycle (TCA cycle) of mitochondrial metabolism and deamination of glutamate. In the TCA cycle, α-KG is produced from isocitrate upon the activity of isocitrate dehydrogenase and then decarboxylated into isocitrate dehydrogenase by α-KG dehydrogenase [66]. α-KG in the TCA cycle can also be anaplerotically generated from glutamate by glutamate dehydrogenase or glutamine transaminase (Figure 1). The enzyme activities of the TETs and JMJD families also require other cofactors from metabolic pathways, such as oxygen, ascorbate, and ferrous iron [67, 68]. Meanwhile, analogues of α-KG, such as succinate, citrate, and fumarate and 2-hydroxyglutarate (2HG), can inhibit the catalysis of demethylases [67, 69]. Additionally, hypoxia also suppresses enzymatic activities, which leads to high levels of DNA and histone methylation by limiting the oxygen supply [70, 71]. The balance between α-KG and its antagonists in intracellular pools is essential for defining the functional activity of α-KG-dependent demethylases.

Glucose metabolism alteration leads to changes in energy production and metabolite generation, which are essential for the cell fate decision. The differentiation initiation of hematopoietic stem cells presents a shift from

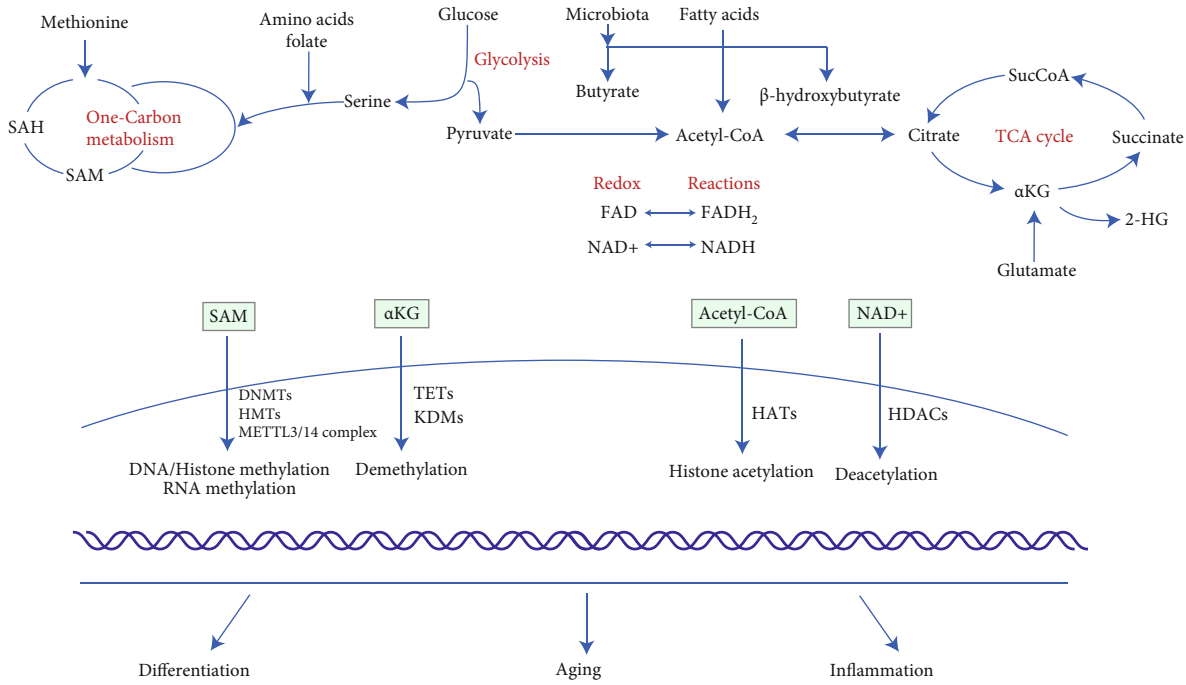

Figure 1: Amino acids, glucose, and fatty acids are utilized by metabolic pathways including one carbon metabolism, glycolysis, TCA cycle, and fatty acid catabolism. Metabolic intermediates generated from cellular metabolic pathways are substrates, cofactors, or antagonists for enzyme activity in epigenetic events including methylation, demethylation, acetylation, and deacetylation. These metabolic-sensitive epigenetic events are involved in the transcriptional program alteration and drive the dental mesenchymal stem cells into differentiation, aging, or inflammatory responses. SAM: S-adenosylmethionine; SAH: S-adenosylhomocysteine; acetyl-CoA: acetyl coenzyme A; SucCoA: succinyl-CoA; 2-HG: 2-hydroxyglutarate; αKG: α-ketoglutarate; TCA cycle: tricarboxylic acid cycle; NAD: nicotinamide adenine dinucleotide; FAD: flavin adenine dinucleotide; DNMTs: DNA methyltransferases; HMTs: histone methyltransferases; METTL3: methyltransferase-like 3; TETs: ten-eleven translocations; KDMs: histone lysine demethylases; HATs: histone acetyltransferases; HDACs: histone deacetylases.

glycolysis toward mitochondrial oxidative metabolism [72]. Conversely, a marked switch to predominantly glycolytic activity in energy supply is required for neural stem cell differentiation [73]. The metabolic substrates generated from glycolysis and mitochondrial oxidative metabolism are essential for epigenetic regulation of gene expression [19]. In naïve embryonic stem cells, a high level of intracellular α-KG produced by both glucose and glutamine metabolism is critical for maintaining pluripotency [74]. α-KG accumulation promotes the demethylation of H3K27me3 and TET-related DNA demethylation, which in turn regulates target gene expression [74]. During adipocyte differentiation, α-KG suppresses the adipogenic gene expression and brown adipogenesis by reducing the H3K4me3 methylation level [75]. The switch between glycolysis and mitochondrial oxidative metabolism contributes to intracellular α-KG alterations that orchestrate the requirement of different stages in a cell type-dependent manner [76].

Glucose metabolism remodeling also occurs during the differentiation processes of dental mesenchymal stem cells. In the differentiation initiation (for 0-3 days), mitochondrial ATP production increases with glycolytic activity, which might result from the rapid upregulation of energy demand [77]. Another study suggests that a high level of phosphofructokinase-dependent aerobic glycolysis is essential for odontoblast differentiation after induction for 6 days [78]. Maity et al. examined the whole differentiation process of DPSCs (from 0 to 14 days) and found that lactate production increased while metabolic activity was reduced [43]. The shift from mitochondrial oxidative metabolism to the glycolytic pathway corresponded with the upregulation of H3K4me3 on autophagy-related genes [43]. Metabolic pathway alteration from mitochondrial oxidative metabolism to glycolysis also participates in inflammatory responses. PDLSCs underwent a switch from oxidative phosphorylation to glycolysis after *Porphyromonas gingivalis* infection, characterized by succinate accumulation, ROS upregulation, and HIF-1 pathway activation [79]. These metabolite alterations in the inflammatory state might enhance the global methylation level by suppressing the enzyme reactions conducted by demethylases.

2.3. The Impact of Metabolism on Histone Acetylation. Histone acetylation is another well-known epigenetic modification that is established by histone acetyltransferases (HATs) with acetyl donors. During osteo/odontogenic differentiation of PDLSCs and DPSCs, the acetylation of histone 3 lysine 9 (H3K9ac) and H3K27ac is significantly increased [80–82]. Histone lysine acetyltransferase 2A (KAT2A) enhances the osteogenic differentiation of PDLSCs by increasing the acetylation levels of H3K9 and H3K14 [83]. Histone acetyltransferase p300 conducts H3K9ac of

odontogenic-related genes and modulates DPSC differentiation and proliferation [82]. p300-related H3K9 acetylation also impacts the inflammatory response of periodontitis [84].

The key acetyl group in histone acetylation is acetyl-CoA, which is generated in diverse metabolic pathways [85]. In glycolytic activity, acetyl-CoA is generated from pyruvate catalyzed by pyruvate dehydrogenase and subsequently forms citrate, which is acquired by the TCA cycle in mitochondrial metabolism (Figure 1). Citrate can also be lysed and reverted back to acetyl-CoA by ATP-citrate lyase [86]. Additionally, acetate produced from fatty lipid catabolism and ethanol metabolism can be converted into acetyl-CoA by acetyl-CoA synthetase, which is another important source of acetyl-CoA. The nutrition supply and enzyme activity cooperate to define the availability of acetyl-CoA for histone acetylation [30].

The glucose metabolism alteration in dental mesenchymal stem cell fate transition not only impacts the generation of α-KG and acetyl-CoA; high levels of glycolysis are essential for pluripotency maintenance of embryonic stem cells by supplying acetyl-CoA for histone acetylation. After differentiation initiation, acetyl-CoA is rapidly consumed in the TCA cycle, leading to a reduction in the acetylation of histones [87]. Meanwhile, aerobic glycolysis in active T cells sustains the acetyl-CoA supply and enhances the H3K9ac of interferon gamma, which is essential for cell differentiation [88]. ATP-citrate lyase promotes acetylation of histone H3 and skeletal muscle cell differentiation by regulating the availability of acetyl groups [89].

As mentioned before, the differentiation process of DPSCs is characterized by enhanced aerobic glycolysis levels, and this alteration fuels sufficient acetyl-CoA that can be utilized for histone acetylation [43, 90]. DPSCs with faster aging rates display a reduction in the ability to use glucose and fatty acids as energy resources [91]. The lack of acetyl-CoA might contribute to cell senescence and aging via histone acetylation inhibition, which needs to be verified. Epigenetic reactions are also involved in the translation of lipid metabolic cues into the developmental setting. Fatty acid metabolism is an important resource of acetyl groups that is essential for cell differentiation and tooth development [92]. Dyslipidemia induced by a high-fat diet leads to an elevation in dentin thickness and a reduction in pulp cavity diameter and dentin formation width [92, 93]. Short-chain fatty acids (SCFAs) derived from microbiota promote the murine incisor regeneration [94]. SCFA supplementation facilitates dental mesenchymal stem cell differentiation by inducing acetylation of histone 3 in BMP signaling, which might result from increasing intracellular acetyl-CoA concentration and HDAC inhibition [94].

2.4. The Impact of Metabolism on Histone Deacetylation. As DNA and histone methylation, the acetyl groups installed on histones can be removed by the functional activities of histone deacetylases (HDACs) in a context-dependent manner. HDACs can be classified into classes I, II, and IV, which are zinc-dependent enzymes, and class III (also referred to as sirtuins), which are nicotinamide adenine dinucleotide- (NAD-) dependent enzymes. HDACs have essential effects on the biological processes of odontoblast, osteoblast, and cementoblast differentiation [95–97]. Sirtuin1 (SIRT1) facilitates the cellular differentiation of DPSCs in tissue regeneration and participates in inflammatory response [97–99]. The histone acetylation level in the differentiation process of dental mesenchymal stem cells is a consequence of the balance between HATs and HDACs.

NAD+ serves as the key coenzyme in multiple metabolic pathways and the cosubstrate for three classes of enzymes, including the sirtuin family. NAD is generated from vitamin B3 in the forms of nicotinamide, nicotinic acid, nicotinamide riboside, and amino acid tryptophan via de novo synthesis [100]. As an electron receptor in redox reactions, NAD+ can be converted into its reduced form, NADH, for oxidative phosphorylation and ATP synthesis, which mainly occurs in mitochondrial pathways (Figure 1). The intracellular NAD+ concentration modulates the enzyme activity of sirtuins which further impacts transcription and chromatic stability by histone deacetylation. On the other hand, the metabolic substrates from fatty acid catabolism, such as butyrate and β-hydroxybutyrate, are capable of inhibiting the enzymatic function of class I and some class II HDACs. Butyrate is a kind of SCFA produced by microbiota, and β-hydroxybutyrate is generated in the process of fatty acid oxidation or ketogenesis [101, 102].

Metabolic fluctuations due to glucose metabolism remodeling and fatty acid oxidation modulate histone deacetylase function during cell fate decision [103]. Skeletal muscle stem cell activation is characterized by increased glycolysis metabolism and reduced intracellular NAD+ and NAD+-dependent SIRT1 activity, which induce histone acetylation and gene transcription [104]. Sirtuins are downregulated during the cellular process of senescence and aging, and increasing mitochondrial NAD+ levels can delay this process [105]. Sodium butyrate, which is widely used as an HDAC inhibitor, can induce neurogenic effects and suppress the inflammatory response via histone deacetylation [103, 106].

The cellular differentiation of dental mesenchymal stem cells is accompanied by a high level of glycolysis that results in a decreased NAD+/NADH ratio and reactive oxygen production [43, 107]. The histone acetyltransferase SIRT1 is slightly upregulated on day 1 and then decreases over time during DPSC differentiation [97, 108]. The expression pattern of sirtuins might be related to the decreased NAD +/NADH ratio, which remains to be clarified. Periodontal microbiota, such as *Porphyromonas* and *Fusobacterium*, can produce SCFAs, including butyrate and isobutyrate, which are known as resources for HDAC inhibitors [109, 110]. High concentrations of butyric acid derived from *Porphyromonas gingivalis* can suppress the expression levels of HDACs while inducing the acetylation of histone 3, which further promotes bone absorption in periodontitis [110, 111]. Butyric-induced histone acetylation is also involved in cell vitality, differentiation, and extracellular matrix remodeling of osteoblastic cells [109].

2.5. The Impact of Metabolism on Other Modifications. Beyond methylation and acetylation, there are also a

growing number of modifications that add functional diversity to epigenetic regulation. Histone acylation is similar to histone acetylation in function and activity and relies on histone acetyltransferases to utilize acyl-CoA [112, 113]. Acyl-CoA molecules are metabolite substrates generated from short-chain acyl group-containing molecules, including SCFAs [114]. RNA acetylation relies on the same metabolite acetyl-CoA as histone acetylation to form N^4-acetylcytidine and impacts RNA stability and translation efficiency [115]. Histone acylation and RNA acetylation exhibit some similarities with histone acetylation in enzyme activity or metabolite substrates; however, their biological function remains unknown in dental mesenchymal stem cells. Metabolite-derived modifications, such as histone homocysteinylation, histone monoaminylation, and histone ADP-ribosylation, also need to be explored in future studies [9, 116, 117].

3. Metabolism-Epigenetic Network in Dental Mesenchymal Stem Cells

The epigenetic landscape alteration driven by metabolic remodeling participates in a highly coordinated transcriptional program in a stage- and cell type-specific manner. On the other hand, cellular progress can also be partially reversed by the metabolism-epigenetic network. Dental mesenchymal stem cells retain plasticity and flexibility to adjust their cell biology toward the surrounding microenvironment. The present study discusses the potential impact of metabolic remodeling on epigenetic events during biological processes of dental mesenchymal stem cells, including differentiation, aging, and inflammation.

3.1. Differentiation. Metabolic remodeling in the cellular differentiation process orchestrates the emerging changes in energy supply and metabolite demand. According to previous studies, an increasing level of aerobic glycolysis was identified in DPSC differentiation based on indicators such as the oxygen consumption rate and extracellular acidification rate [43, 77, 78]. Metabolic remodeling could result in changes in the corresponding metabolites such as acetyl-CoA, α-KG, and the NAD+/NADH ratio. However, the concentrations of metabolic intermediates have not been quantified in these studies and need to be confirmed in the future. Furthermore, epigenetic alterations of certain transcripts were detected in a previous study [43]. Whether and how metabolic remodeling contributes to global changes in epigenetic events remain elusive. Modulating metabolic enzymes and nutrient availability could impact the transcriptional program of dental mesenchymal stem cell differentiation through epigenetic regulation. These metabolite-sensitive epigenetic events in differentiation processes will expand our regenerative strategies in clinical practice, such as vital pulp therapy and periodontal regeneration.

3.2. Aging. A long lifespan and persistence of adult stem cells contribute to a reduction in regenerative capacity [118]. The reprogramming and self-renewal ability of dental mesenchymal stem cells peaks in immature teeth and then declines with age in older teeth [91]. DPSC senescence and aging also involve metabolic remodeling characterized by a reduction in glucose, fatty acid utilization, and one-carbon metabolism-related amino acids (glycine, serine, and methionine) [59, 91]. The metabolic pathway alterations can impact the intracellular concentrations of SAM, acetyl-CoA, α-KG, and NAD+ and subsequently change the epigenomic states. Alterations in serine metabolism were reported to be responsible for decreased methyl donor (SAM) and enzyme activity of DNMT1 [59]. The potential role of other metabolic pathways and corresponding metabolites in epigenetic events still needs to be examined. Considering the role of metabolic signaling in senescence and aging, nutrient reservoirs can be taken as a therapeutic intervention to improve the regenerative potential of adult stem cells.

3.3. Inflammation. Periodontitis and pulpitis are chronic inflammatory diseases mainly caused by bacterial invasion that induce local inflammatory and immune responses. The presence of pathogens and inflammatory factors leads to metabolic remodeling that is capable of impacting the epigenetic landscape of dental mesenchymal stem cells. SCFAs derived from periodontal microbiota contribute to the intracellular acetyl-CoA generation and enzyme activity of HDACs. SCFAs play an important role in murine incisor development by facilitating the acetylation of histone H3 [94]. Meanwhile, histone acetylation induced by sodium butyrate promotes bone absorption and impairs PDLSC differentiation [110]. SCFAs are also a source of crotonyl-CoA for histone crotonylation that subsequently induces the cytokine secretion by activated macrophages [119]. These studies suggest an important role of the metabolic-epigenetic network in inflammatory disease, which provides new insight into the therapeutic strategy development in periodontitis and pulpitis.

4. Future Perspectives

Accumulating evidence has made it clear that the coordination of metabolic pathways and epigenetic events is essential for the transcriptomic program in cell fate decision. Metabolite-sensitive epigenetic events are highly specific, depending on different cell types and stages. Integrating metabolic cues into epigenetic events and transcriptomic programs will advance our knowledge of epitranscriptional mechanisms and provide new insight into therapeutic approaches in the oral and maxillofacial regions. Taking advantage of metabolic signaling in epigenetic regulation might help us harness a greater regenerative capacity of dental mesenchymal stem cells in vital pulp therapy and endodontic regeneration. For infectious diseases such as pulpitis and periodontitis, therapeutic inventions targeting metabolite-sensitive epigenetic events might drive inflammatory responses toward a preferable shift to injury repair. Some researchers have begun to explore metabolic remodeling in the cell fate transition of dental mesenchymal stem cells. To date, direct evidence of the metabolism-dependent epigenetic regulation of dental mesenchymal stem cells is somehow limited, and more research is needed to elucidate the exact mechanisms in the future. The metabolism-

epigenetic network in dental mesenchymal cell fate specification sheds light on the therapeutic opportunity to manipulate nutrient availability and metabolite substrates and subsequently drive dental mesenchymal stem cell fate determination by epigenetic regulation.

Conflicts of Interest

The authors declare no potential conflicts of interest with respect to the authorship and/or publication of this article.

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Research Article

EZH2 Might Affect Macrophage Chemotaxis and Anti-Inflammatory Factors by Regulating CCL2 in Dental Pulp Inflammation

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Objectives. We aimed to evaluate the effects of Enhancer of Zeste Homolog 2 (EZH2) on regulation of macrophage migration and expression of anti-inflammatory genes in pulpitis. *Methods*. Dental pulp inflammation was verified by histology in rat pulpitis model induced by lipopolysaccharide (LPS). Immunohistochemistry staining was used to detect changes of the expression of EZH2 and tumor necrosis factor alpha (TNF-α) in dental pulp inflammation. The expression of EZH2, CCL2, and cluster of differentiation 68 (CD68: macrophage surface marker) was measured by immunofluorescence staining. The effect of EZH2 on microphage migration was assessed by cell migration assay. The expressions of anti-inflammatory cytokine interleukins (IL-4 and IL-10) and transforming growth factor-β (TGF-β) in HDPCs which were treated by EZH2 complex, CCL2 complex, and CCL2 antibody were examined by quantitative real-time polymerase chain reaction (q-PCR). *Results*. The expression of TNF-α gradually increased in dental pulp inflammation. The expression of EZH2 in dental pulp decreased in 8 hours after LPS stimulation. However, the expression of EZH2 gradually increased in dental pulp after 1 day stimulation by LPS. The results of immunofluorescence staining showed that the expressions of EZH2, CCL2, and CD68 were significantly upregulated in dental pulp inflammation of rats. EZH2 could enhance macrophage migration. And the chemotactic activity of macrophages exposed to supernatants of EZH2-treated HDPCs could be inhibited by CCL2 inhibition. In addition, EZH2 suppressed the expression of anti-inflammatory genes, but CCL2 inhibition reversed the downregulation of anti-inflammatory factors, including IL-4 and TGF-β in HDPCs. *Conclusions*. EZH2 might affect chemotaxis of macrophages and the expression of anti-inflammatory factors by regulating CCL2. EZH2 plays an important role in the development of dental pulp inflammation, and it might be as a target for treatment of pulpitis.

1. Introduction

Pulpitis is a multifactorial disease that could be mainly caused by dental caries, as well as mechanical and chemical irritations. These events, such as dental caries, can irritate dental pulp healing process if the infection is not too severe [1]. The mechanisms regulating pulpitis and repair were complicated. Dental pulp inflammation usually could persist in the dental pulp despite treatment, reducing innate repair capacities [2].

Studies have found that epigenetic regulation plays an important role in the progress of dental pulp inflammation [3]. Epigenetics is defined as a heritable change in gene function without a change in the DNA sequence, which ultimately leads to a change in phenotype [4]. Epigenetics includes DNA methylation, histone modification, and noncoding RNA. The role of histone modification in inflammation and repair has gradually attracted attention [5–7]. Histone H3 on lysine residue 27 (H3K27me) is a common site for histone modification. Some studies have confirmed that demethylation of H3K27me3 can promote the repair reaction of dental pulp. And Enhancer of Zeste Homolog (EZH2) is a trimethylation transferase of H3K27. It is the catalytic subunit of polycomb

repressive complex 2 (PRC2). And EZH2 has been shown to play an important role in a variety of inflammatory diseases, such as nervous system inflammation and enteritis [8–10]. However, the mechanism of EZH2 in pulpitis is still unclear. Previous studies have shown that EZH2 promoted the progress of dental pulp inflammation [6]. EZH2 could promote the proliferation of human dental pulp cells and inhibit osteogenic differentiation [6]. In addition, EZH2 can directly combine with the promoters of IL-6, IL-8, and CCL2 to regulate the histone modification and increase expressions of the genes. Among these changing inflammatory factors, the expression of CCL2 changed most [3].

CCL2 is a chemokine of mononuclear macrophage. CCL2 could promote the chemotaxis of a large number of macrophages to accumulate at the site of the inflammatory area [11, 12]. Then, the chemokine-cytokine network is activated, which could result in the amplification and persistence of the inflammatory response [13, 14]. In dental pulp inflammation, HDPCs express chemokines including CCL2, which could be induced by LPS or TNF-α stimulation [15]. DPSCs exhibit their immunomodulatory effects on macrophage phenotype in inflammatory diseases [16]. HDPCs are the most numerous cells in the dental pulp and maintain the collagen matrix of the pulpal tissue, and a population of immune cells, such as macrophages, hold themselves ready to respond to microbial incursion [17]. Macrophages and neutrophils are important mediators of the innate inflammatory response in the dental pulp [18]. Macrophages stimulated with IL-10 and TGF-β could decrease the production of inflammatory cytokines, such as TNF-α in dental pulp [19]. We speculated that macrophages might play an important role in pulpitis and modulate the pulp regenerative environment. According to the current research, epigenetic reprogramming has been involved in macrophages activation [20]. It is speculated that the effect of EZH2 on dental pulp inflammation might include microphage chemotaxis. EZH2 could affect the production of inflammatory/chemokines, immune regulatory functions, and process of the pulpitis [3]. However, the regulatory mechanism of EZH2 in the process of dental pulp inflammation and immune response remains to be further studied.

In our study, we explored the chemotactic effects of EZH2 on macrophages and anti-inflammatory genes in dental pulp inflammation.

2. Materials and Methods

2.1. Construction of Dental Pulp Inflammatory Models in Rats. All animal manipulations were approved by the local ethical committee at Peking University (LA2018044). A well-characterized rat experimental pulp inflammation model was established as described previously [3]. Rats (6 weeks of age) were used and divided into 2 groups with three rats each. LPS from E.coli (10 g/L, Sigma, United States) for inflammation group (with different time points, including 2 hours, 8 hours, 1 day, 3 days, and 7 days). The control group did not have any treatment, and the number of experimental teeth in each group was not less than 5. LPSs were applied to the amputated pulp using Spongel (Astellas Pharma) and sealed with glass ionomer cement (GIC) (Fuji IX, Ketac Molar, and d-tech). Rats were sacrificed 2 hours, 8 hours, 1 day, 3 days, and 7 days after operation by decapitation. And the jaws were fixed promptly in 4% formaldehyde and then decalcified for 1 month and embedded in paraffin. Sections of 5 μm thickness were stained with H-E and immunohistochemistry.

2.2. Histopathologic. Three consecutive sections per teeth were stained with hematoxylin and eosin (H&E) and selected for the morphometric analysis under the microscope (BX51 Olympus Micro Imaging System, Japan).

2.3. Immunohistochemical Analysis. The paraffin sections were baked at 65°C overnight. A standard immunohistochemistry kit (Zhongshanjinqiao, Beijing, China) was used for immunohistochemistry according to the manufacturer's instructions. Primary antibody, 1 : 50 dilution of EZH2 (Cell Signaling Technology, #5246, United States) and 1 : 100 dilution of TNF-α (Abcam, ab199013, UK), was added, and the slides were incubated at 4°C overnight. 3,3′-Diaminobenzidine kit (Zhongshanjinqiao) was used for coloration according to the manufacturer's instructions. Results were obtained using an Olympus BX51 upright microscope (Olympus Optical, Tokyo, Japan).

2.4. Immunofluorescence. For immunofluorescence staining, serial sections of 4 μm thickness were incubated with antibody against EZH2 (1 : 200; Cell Signaling Technology, #5246), antibody against CCL2(1 : 200; Abcam, ab25124), and antibody against CD68 (1 : 200, Abcam, ab31630) overnight at 4°C. Bound primary antibodies were detected with Alexa Fluor 488 preadsorbed anti-rabbit IgG secondary antibody (1 : 200; Abcam, ab150117) and Alexa Fluor 647 preadsorbed anti-mouse IgG secondary antibody (1 : 200; Abcam, ab150083), following a 1 h incubation at room temperature. The sections were then counterstained with DAPI (Zhongshanjinqiao, Beijing, China); then, images were taken with a fluorescence microscope (BX51; Olympus).

2.5. Cell Culture. The study was approved by the ethics committee of the Peking University of Stomatology (PKUSSIRB-201732003). THP-1 cells were purchased EK-Bioscience were incubated in a medium containing 15 μg/L 12-myristate 13-acetate (phorbol ester, phorbol 12-myristate 13-acetate, PMA, Sigma) for 48 hours to induce differentiating into macrophages [21]. Primary HDPCs were cultured as described previously [22], and passages 3–5 were used. Cells were treated with recombinant EZH2 (20 ng/ml, Abnova, Taiwan), CCL2 (20 ng/ml, Abnova, Taiwan), EZH2+CCL2, and EZH2+anti-CCL2 (100 μg/L, Abcam, Cambridge, MA) for 24 hours.

2.6. Cell Migration Assay. The migration capacity of macrophages was measured in transwell chambers (3 μm pore, Corning). HDPC monolayers were incubated with serum-free DMEM with or without EZH2 (20 ng/ml), CCL2 (10 μg/L), EZH2+CCL2, and EZH2+anti-CCL2 (100 μg/L) treatment for 48 hours in a 5% CO_2 incubator at 37°C before collection of supernatants. After overnight culture in serum-free RPMI1640 medium, 200 μl of macrophages was resuspended

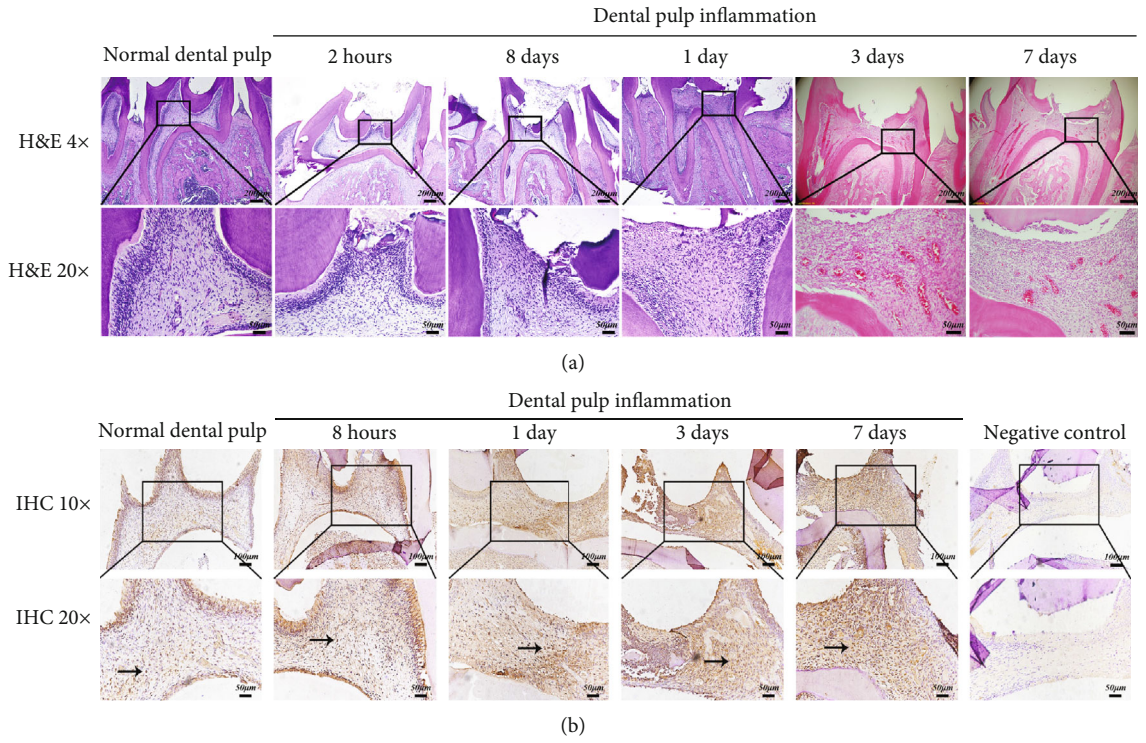


Figure 1: The effects of LPS on dental pulp tissue and TNF-α expression. (a) Hematoxylin-eosin analyses of nontreated pulp (control) and experimental rat pulpitis induced by LPS treatment observed after 2 hours, 8 hours, 1 day, 3 days, and 7 days. Higher magnifications are shown in boxed areas. Scale bar: 200 μm in low magnifications and 50 μm in higher magnifications. (b) Immunohistochemical analyses of TNF-α expression of dental pulp with or without LPS treatment after 8 hours, 1 day, 3 days, and 7 days. Scale bar: 100 μm in low magnifications and 50 μm in higher magnifications ($n = 3$).

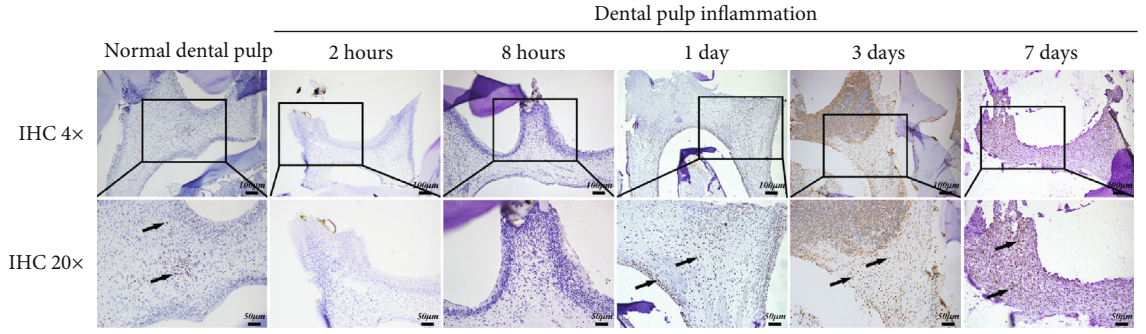
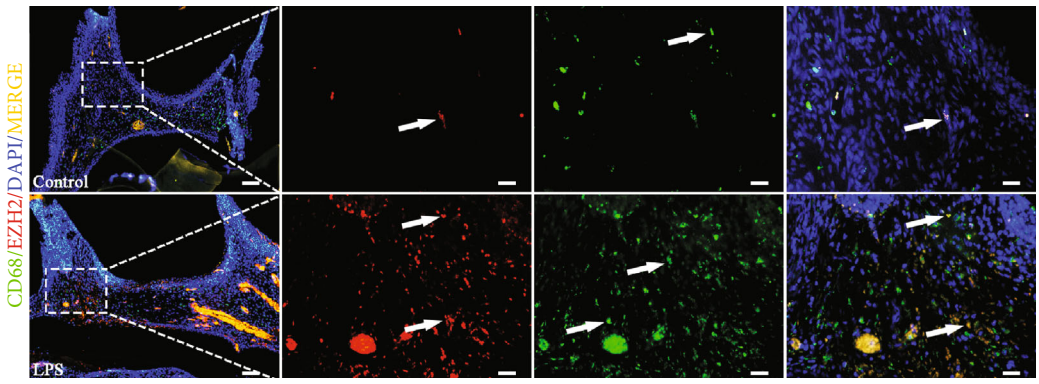


Figure 2: Immunohistochemical staining showed that EZH2 expression was significantly increased after 1 day stimulation. LPS stimulated rat dental pulp at different time points (2 hours, 8 hours, 1 day, 3 days, and 7 days). Black arrows indicate positive EZH2 staining. Higher magnifications are shown in boxed areas. Scale bar: 100 μm in low magnifications and 50 μm in higher magnifications ($n = 3$).

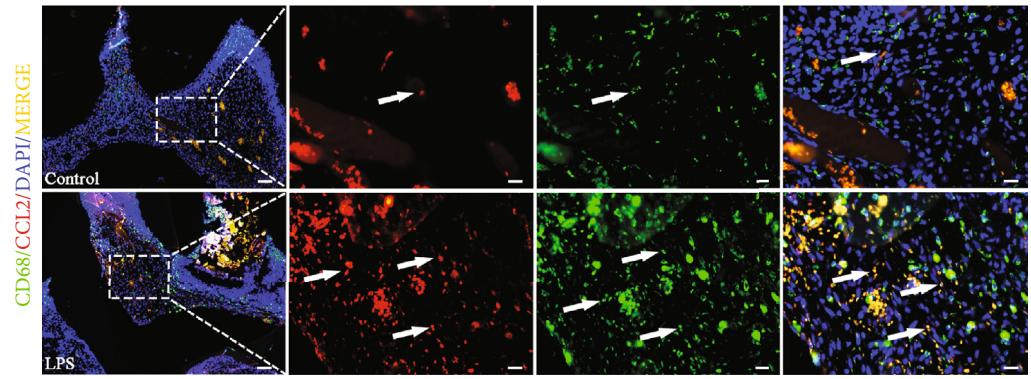
(2×10^6/ml) and added to the upper chamber in serum-free RPMI 1640 medium, and 600 μl of the supernatant of untreated or treated HDPCs was placed into the bottom wells. After 4 hours of incubation, nonmigrating cells on the upper surface of the membrane were removed, and the cells that migrated to the underside of the polycarbonate membrane were fixed with ethanol and stained with 1% crystal violet for 30 min. The mean of triplicate assays for each experimental condition was used for analysis.

2.7. Real-Time Polymerase Chain Reaction Analysis. Total RNA was isolated using the RNeasy mini kit (Qiagen, Valencia, CA). Complementary DNA was synthesized from RNA by using the PrimeScript RT Reagent Kit (Takara, Dalian, China). The produced complementary DNA was prepared as templates for the polymerase chain reaction using SYBR Premix Ex Taq (Takara) according to the manufacturer's instruction. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the control. Primer sequences and conditions for real-time polymerase chain reaction are shown in the supplemental material (available here).

2.8. Statistical Analyses. All data were presented as mean SD and compared by one-way analysis of variance tests were



(a)



(b)

Figure 3: Immunofluorescence staining of rats' pulp tissues with LPS stimulation and control. Immunopositivity of (a) EZH2, (b) CCL2, and (a, b) CD68 in healthy and inflamed pulp tissues. Healthy and inflamed pulp tissues were stained with antibody to EZH2, CCL2 (red), CD68 (green), and colocalization (yellow). White arrows indicate positive staining. Higher magnifications are shown in boxed areas. Scale bar: 100 μm in low magnifications and 20 μm in higher magnifications ($n = 3$).

calculated for statistical analysis of differences by SPSS23.0 (SPSS Inc., Chicago, IL, USA). All the experiments were independently repeated at least in triplicate. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Dental Pulp Inflammatory Model with LPS Stimulation in the Rats Was Established and TNF-α Expression Was Detected. To observe the effect of LPS on dental pulp tissue, histologic observations were carried out at different time points after treatment with LPS (Figure 1(a)). In LPS-treated dental pulp tissue, the areas of inflammatory cell infiltration were larger than controls. Then, the expression of TNF-α was detected (Figure 1(b)). The immunohistochemical staining results indicated that the expression of TNF-α was increased in response to LPS treatment. These results showed success of construction of dental pulp inflammatory models in rats.

3.2. Changes of EZH2 Expression in Dental Pulp of Rats with LPS Treatment. We investigated the involvement of EZH2 in dental pulp inflammation using immunohistochemistry staining. The EZH2 protein was lowly expressed in the nucleus of normal dental pulp but increased in inflamed dental pulp tissue (Figure 2). EZH2 has a lower expression in 8 hours compared with control. After 1 day, 3 days, and 7 days, EZH2 expression was increased obviously in inflamed dental pulp tissue. These results suggested that EZH2 might play a role in dental pulp inflammation.

3.3. Macrophages Are Involved in Dental Pulp Inflammation. To reveal further relationship between the macrophages and pulp inflammation, CD68, a common surface marker for macrophages was detected as the target protein. Compared with the control group, the expression of EZH2 and CD68 was significantly upregulated in inflamed pulp tissue (Figure 3(a)). Then, we found the expression of CCL2 and CD68 was increased with LPS stimulation as compared with the control group (Figure 3(b)). These results indicated that the macrophages might be involved in inflamed dental pulp tissue. And EZH2 might be involved in macrophages in dental pulp.

3.4. EZH2-Treated HDPCs Enhanced Macrophage Migration via Regulating CCL2. We investigated the effects of EZH2 and CCL2 on macrophage migration, which has been as a hallmark of the immune response. We examined the chemotactic migration of human macrophages in response to supernatants of HDPCs that were treated with EZH2, CCL2, EZH2+CCL2, and EZH2+anti-CCL2 by a transwell migration assay. The migration of macrophages toward the

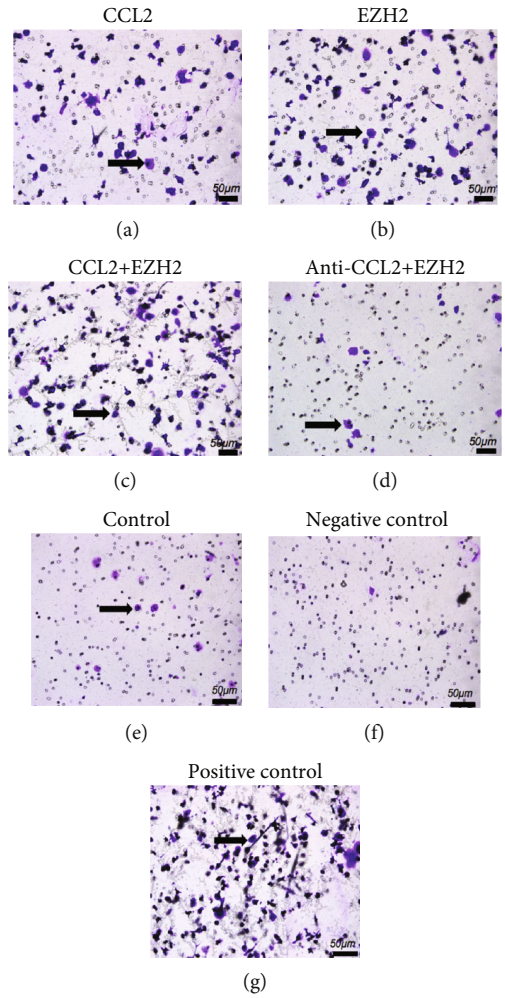


Figure 4: Chemotactic activity of EZH2 and CCL2-treated HDPCs on macrophages. (a) Transwell assay showing that CCL2 promotes macrophage migration. (b) Transwell assay showing macrophage migration in response to treatment with supernatants of EZH2-treated HDPCs. (c) Cell migration was induced by treatment with CCL2 plus EZH2. (d) Cell migration was suppressed by treatment with anti-CCL2 protein. (e) Cell migration of macrophages in response to HDPCs without any treatment, as control group. (f) Negative control group. (g) Positive control group. Scale bar: 50 μm ($n = 3$).

supernatant of CCL2-treated HDPCs was enhanced compared with untreated HDPC supernatants (Figure 4(a)). The chemotactic activity of the supernatant of EZH2-treated HDPCs was increased when compared with control (Figure 4(b)). The chemotactic activity of the supernatant of CCL2 plus EZH2-treated HDPCs was increased when compared with control (Figure 4(c)). As shown in Figure 4, pretreatment of anti-CCL2 could inhibit the chemotaxis of macrophages that was induced by EZH2 (Figure 4(d)). These data indicate that the chemotactic activity of macrophages exposed to supernatants of EZH2-treated HDPCs could be inhibited by CCL2 inhibition.

3.5. EZH2 Might Affect the Expression of Anti-Inflammatory Factors in HDPCs via CCL2. We confirmed the effects of EZH2 and CCL2 on anti-inflammatory cytokines in HDPCs by qPCR (Figure 5). The results showed that expression of anti-inflammatory factors IL-4 and TGF-β decreased when the cells were stimulated with EZH2 complex for 24 hours (Figures 5(a) and 5(b)). But the expression of IL-10 increased (Figure 5(c)). We found the similar changes when the cells were stimulated with CCL2 complex and EZH2 plus CCL2 complex. In order to identify whether CCL2 inhibition could reverse the expression changes of anti-inflammatory factors induced by EZH2, we used EZH2 plus anti-CCL2 to treat HDPCs for 24 hours. EZH2 mediated decreasing of anti-inflammatory factors IL-4 and TGF-β could be upregulated by CCL2 inhibition (Figures 5(a) and 5(b)). The expression of IL-10 decreased in group of EZH2 plus anti-CCL2 compared with group of EZH2 (Figure 5(c)).

4. Discussion

Previous studies have confirmed that EZH2 can promote the progress of dental pulp inflammation by regulating the expression of cytokines *in vitro*. EZH2 can directly bind to the promoter of CCL2 and affect the transcription of CCL2

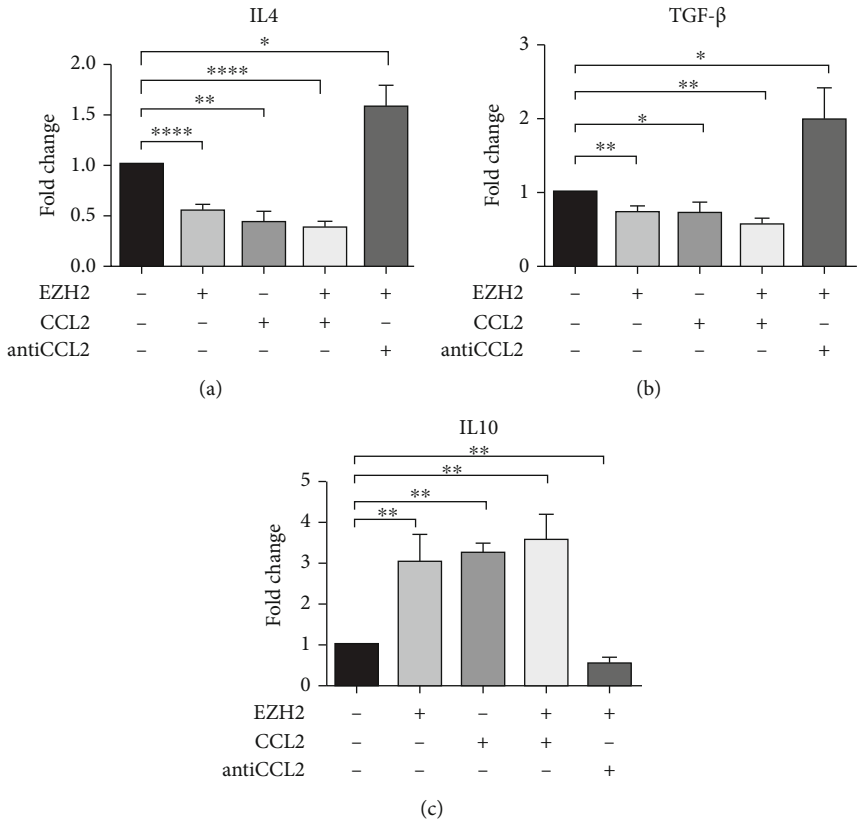


Figure 5: Interleukins were influenced by EZH2, CCL2, and anti-CCL2 in HDPCs. Relative mRNA expression levels of (a) IL-4, (b) TGF-β, and (c) IL-10 were assessed by q-PCR in HDPCs treated by EZH2, CCL2 complex, EZH2 plus CCL2 complex, and EZH2 plus anti-CCL2 for 24 hours. mRNA expression levels were normalized to GAPDH ($n = 3$).

and promoting the progress of dental pulp inflammation [3]. However, the regulatory mechanism of EZH2 and CCL2 in the process of pulp inflammation and immune response remains to be studied. This study further confirmed that EZH2 participates in the development of dental pulp inflammation through *in vivo* and *in vitro* experiments. The expression of TNF-α was significantly upregulated in LPS-induced inflammatory pulp of rats. We found that the expression of EZH2 decreased within 8 hours of LPS-induced pulp inflammation. But after 1 day of stimulation, the expression of EZH2 gradually increased in time-dependent manner. The reduced expression of EZH2 in the early period of dental pulp inflammation may be related to the mechanism of repair in the early stage of dental pulp inflammation. This is consistent with our previous research [6]. And it might indicate that EZH2 might have important roles in regulating pulpitis. In the progress of pulpitis, there are lots of immune cells have been activated, such as macrophages and dendritic cells (DCs). Macrophages are one of the main cells involved in dental pulp inflammation. It plays an immunomodulatory role in dental pulp tissue due to strong phagocytic ability. A large number of macrophages are mainly distributed in the central region of dental pulp [19, 23]. In our study, a large number of CD68-positive cells infiltrated in the inflammatory pulp. Macrophages play a key role in maintaining tissue homeostasis and regulating immunity. It could be migrated to the injured sites of dental pulp by CCL2 chemotaxis [24]. In our previous study, we found that expression of CCL2 has been induced significantly by EZH2 in HDPCs [3]. So, we hypothesize that EZH2 might modulate the migration of macrophages in dental pulp inflammation. According to the results of immunofluorescence staining, we speculated that EZH2 might be able to regulate macrophages in dental pulp. And we found that EZH2 may promote the chemotaxis of macrophages by regulating CCL2. Therefore, it can be speculated that EZH2 in dental pulp can regulate the chemotaxis of macrophages via CCL2.

Studies have confirmed that dental pulp cells could participate in the immune response and regulate the inflammatory process of dental pulp [25]. Dental pulp cells also have certain immunomodulatory capabilities and can secrete inflammatory factors and anti-inflammatory factors [26]. Hui et al. confirmed that the stimulation of EZH2 complex could upregulate the expression of inflammatory factors of HDPCs, and the inhibitor of EZH2 could inhibit the inflammatory process of dental pulp [3]. However, the effect of EZH2 on the anti-inflammatory factors in dental pulp needs further research. In our study, the expression of anti-inflammatory factors IL-4 and TGF-β decreased when the HDPCs were stimulated with EZH2 and CCL2 complex compared with control, while the expression of IL-4 and TGF-β has been increased when the HDPCs were stimulated with EZH2 plus anti-CCL2, compared with EZH2 or CCL2

stimulation group. We speculated that EZH2 could promote pulp inflammation by inhibiting expression of anti-inflammatory factors. CCL2 inhibition can alleviate the reduced expression of anti-inflammatory factors affected by EZH2. However, the expression of anti-inflammatory factor IL-10 was contrary to IL-4 and TGF-β. It is reasonable to envisage that organism systems exist to modulate the response to microbial antigens by secreting immunoregulatory cytokines in order to prevent excessive inflammation. One of them might involve IL-10. Indeed, IL-10 is an immunosuppressive cytokine synthesized by many cell types, which decreases the production of several proinflammatory cytokines including IL-6 and CXCL8, thereby suppressing inflammation-associated immune responses and limiting damage to the host [27]. IL-10 could promote the differentiation of regulatory T cells which control excessive immune responses and then produce higher expression of IL-10. This might provide a positive regulatory loop for IL-10 induction [28]. We speculated that the increased expression of the anti-inflammatory factor IL-10 may be due to the existence of an immune response regulatory system which could prevent excessive inflammation. In recent years, many studies have found that EZH2 can play important roles in various inflammatory diseases such as lupus-like diseases, dental pulp inflammation, and neuropathic pain by promoting the expression of macrophage chemokine CCL2 [6, 29]. This is consistent with the results of our study.

However, there are also reports that intestinal mucosal epithelial EZH2 has a strong protective effect on experimentally induced inflammatory bowel disease [8]. This is diametrically opposed to the results of this study. It may be due to the different effects of EZH2 on different cells. Lim et al. found that EZH2 can regulate the adhesion and chemotaxis of inflammatory cells through methylating the protein talin directly [30]. Therefore, the mechanism of EZH2 promoting macrophage chemotaxis in pulpitis needs further exploration.

5. Conclusion

EZH2 might activate the macrophage chemotaxis and affect the transcription of anti-inflammatory factors via regulating the expression of CCL2 in the process of dental pulp inflammation.

Data Availability

No data were used to support this study.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Supplementary Materials

Supplementary Information: Supplementary Figure S1-5: primer sequences of genes detected by qPCR. Supplementary Figure S1-4: quantitative analyses of IHC/IF and transwell assay. *(Supplementary Materials)*

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