Comparison of long non-coding RNA expression profiles in human dental follicle cells and human periodontal ligament cells

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Abstract. The dental follicle develops into the periodontal ligament, cementum and alveolar bone. Human dental follicle cells (hDFCs) are the precursor cells of periodontal development. Long non-coding RNAs (lncRNAs) have been revealed to be crucial factors that regulate a variety of biological processes; however, whether lncRNAs serve a role in human periodontal development remains unknown. Therefore, the present study used microarrays to detect the differentially expressed lncRNAs and mRNAs between hDFCs and human periodontal ligament cells (hPDLCs). A total of 845 lncRNAs and 1,012 mRNAs were identified to be differentially expressed in hDFCs and hPDLCs (fold change >2.0 or <-2.0; P<0.05). Microarray data were validated by reverse transcription-quantitative polymerase chain reaction. Bioinformatics analyses, including gene ontology, pathway analysis and coding-non-coding gene co-expression network analysis, were performed to determine the functions of the differentially expressed lncRNAs and mRNAs. Bioinformatics analysis identified that a number of pathways may be associated with periodontal development, including the p53 and calcium signaling pathways. This analysis also revealed a number of lncRNAs, including NR_033932, T152410, ENST00000512129, ENST00000540293, uc021sxs.1 and ENST00000609146, which may serve important roles in the biological process of hDFCs. In addition, the lncRNA termed maternally expressed 3 (MEG3) was identified to be differentially expressed in hDFCs by reverse transcription-quantitative polymerase chain reaction. The knockdown of MEG3 was associated with a reduction of pluripotency makers in hDFCs. In conclusion, for the first time, to the best of our knowledge, the current study determined the different expression profiles of lncRNAs and mRNAs between hDFCs and hPDLCs. The observations made may provide a solid foundation for further research into the molecular mechanisms of lncRNAs in human periodontal development.

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Introduction

The dental follicle, originating from the cranial neural crest, is a loose ectomesenchyme-derived tissue, which surrounds the dental papilla and enamel organ during the development of teeth. The dental follicle develops into periodontal supporting tissues, which serve important functions, including in support, buffering, rebuilding and regeneration (1). hDFCs exhibit a high capacity for proliferation, self-renewal and multi-directional differentiation, and form structures, including the cementum and periodontal ligament (PDL), when subcutaneously transplanted into immunodeficient mice (2,3). hDFCs share a similar phenotype with human PDLCs (hPDLCs), including a mineralization ability, and they also exhibit apparent embryonic characteristics, including pluripotency, heterogeneity and a higher potential for cementum formation *in vivo* (4-6). It has been suggested that hDFCs may provide a new source of seed cells for stem cell therapy and periodontal tissue engineering. Therefore, understanding the key target genes and underlying molecular mechanisms of hDFC differentiation is required for promoting periodontal development and regeneration.

Long non-coding RNAs (lncRNAs) are defined as non-protein coding RNA molecules that are >200 nucleotides long (7). lncRNAs perform their biological effects through a number of mechanisms, including genetic imprinting, chromatin remodeling, cell cycle regulation, splicing and mRNA inactivation. lncRNAs control the pluripotency and stemness of embryonic stem cells and induced pluripotent stem cells, or promote the differentiation of pluripotent cells in the opposite manner. Additionally, lncRNAs may transcriptionally or post-transcriptionally regulate gene expression via different molecular mechanisms (8,9).

An increasing number of studies indicate that lncRNAs serve critical roles in the development of organs, including the brain (10), heart (11), liver (12), lungs (13) and bone (14). lncRNAs also serve significant roles in tooth development. For example, the lncRNA differentiation antagonizing non-protein coding RNA (DANCR) serves a role in reparative dentin formation and regenerative endodontics (15). DANCR is an essential mediator in the proliferation and differentiation of dental tissue-derived stem cells, including dental pulp stem cells, stem cells from the apical papilla and periodontal ligament stem cells (PDLSCs) (16). hDFCs and hPDLCs are essential cells in different stages of periodontal development. However, it remains unclear what potential roles lncRNAs serve in periodontal development and whether lncRNAs are involved in specific activities in different cells. Therefore the current study used microarrays to obtain the different expression profiles of lncRNAs and mRNAs between hDFCs and hPDLCs. Furthermore, the microarray data were validated by reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Bioinformatics analyses were performed to predict the possible roles of the differentially expressed lncRNAs and mRNAs in periodontal development. The results demonstrated that lncRNAs may serve critical roles in periodontal development, and provided a solid foundation for further research.

Materials and methods

Cell culture. Human dental follicle and periodontal ligament samples were obtained from four adolescents (2 males and 2 females) between 12 and 16 years old following premolar and immature impacted third molar (roots developed to <2/3 their full size) extraction for orthodontic reasons. No significant differences were identified in age or sex. Participants included in the study had no history of systemic disease, smoking or specific medication. Tooth extraction was performed at the Hospital of Stomatology, Sun Yat-Sen University (Guangzhou, China) between June 2017 and July 2017. All experimental protocols were conducted under the guidelines set by the Sun Yat-Sen University Ethics Committee and written informed consent was obtained from all patients and their parents. hDFCs and hPDLCs were isolated as previously described (17,18). Briefly, dental follicle tissues were gently removed with a scalpel from where they attached to the root dentin and were digested in a solution containing 1 U/ml collagenase type I (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), hyaluronidase (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1 U/ml DNase I (Roche Applied Science, Mannheim, Germany). Periodontal ligament tissues were isolated from the middle one-third of the root surface and digested in a solution of 3 mg/ml collagenase type I (Sigma-Aldrich; Merck KGaA) and 4 mg/ml dispase (Sigma-Aldrich; Merck KGaA). hDFCs and hPDLCs were cultured in Dulbecco's modified Eagle's medium supplemented with 20% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich; Merck KGaA) at 37˚C in 5% CO_2. Every 2-3 days, the medium was replaced. When cells reached 80% confluence, they were passaged at a 1:3 ratio using 0.25% trypsin/EDTA (Gibco; Thermo Fisher Scientific, Inc.). Third-generation cells were used for the subsequent experiments.

RNA extraction. Total RNA was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). Quantification and quality checks were conducted using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA integrity and genomic DNA contamination were determined by denaturing agarose gel electrophoresis.

RNA microarray. The total RNA was purified using an RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) and labeled with a Quick Amp Labeling kit (Agilent Technologies, Inc., Santa Clara, CA, USA). Labeled RNA was purified again using the RNeasy Mini kit. The RNA was subsequently hybridized onto an Arraystar Human lncRNA Expression Microarray (version 4.0; Arraystar, Inc., Rockville, MA, USA), which was designed for 30,586 lncRNAs and 26,109 coding genes based on the RefSeq (https://ncbi.nlm.nih.gov/refseq/), UCSC Known Genes and Gencode (https://genome.ucsc.edu/) and Ensembl databases (http://ensemblgenomes.org/). Agilent Feature Extraction software (version 11.0.1.1; Agilent Technologies, Santa Clara, CA, US) was used to analyze the acquired array images. The microarray data were deposited in the Gene Expression Omnibus (GEO; https://www.ncbi.nlm.nih.gov/geo/) and are accessible through GEO Series accession no. GSE124352. Differentially expressed lncRNAs and mRNAs were identified to be statistically significant (fold change >2.0 or <-2.0; P<0.05) using a paired t-test. The microarray was performed by Kangchen BioTech Co., Ltd. (Shanghai, China). Subgroup analysis was conducted to classify the differentially expressed lncRNAs according to their expression levels.

RT-qPCR analysis. Total RNA of hDFCs and hPDLCs was reverse transcribed into complementary DNA using an RT kit (Takara Bio, Inc., Otsu, Japan), conducted at 37˚C for 15 min and 85˚C for 5 sec. In total, six lncRNAs and six mRNAs were randomly selected for RT-qPCR analysis using the RANDBETWEEN function in Microsoft Excel (version 2010, Microsoft Corporation, Redmond, WA, USA). RT-qPCR was performed using SYBR Green Real-Time PCR Master mix (Invitrogen; Thermo Fisher Scientific, Inc.), and was run at 95˚C for 5 min, followed by 40 cycles at 95˚C for 10 sec, 65˚C for 20 sec and 72˚C for 30 sec. Primers were synthesized by Invitrogen; Thermo Fisher Scientific, Inc. (Table I). The characteristics of the lncRNAs are presented in Table II. Data were normalized to GAPDH and the relative level of gene expression was calculated using the $2^{-\Delta\Delta Cq}$ method (19).

Bioinformatics analyses. Gene ontology (GO; http://geneontology.org/) was conducted to analyze the functions of the differentially expressed genes. GO analysis examines gene regulatory networks based on biological processes, cellular components and molecular functions (20). A χ^2 test and a two-sided Fisher's exact test were used to classify the GO category. Enrichment of the GO category was determined by the significance of the functions. Kyoto Encyclopedia

Table I. Primers used for the reverse transcription-quantitative polymerase chain reaction.

Gene name	Forward (5'-3')	Reverse (5'-3')
NR_033917	TCACTGTCATGCACTAGCGG	CAGCCCTTGAGGTTGTCCTT
NR_038367	CCCACCGTTCAATGAAAG	GTTTCAAACACCCACATTTC
NR_026861	ACTCGCCTTTTGCGAGAAGA	TCGCAGTTTCCTAGTGGGAC
NR_102703	AGCTGTGAACGGTAGCAGTG	AGGCAGTTTTGTGGGTCAGT
NR_110162	GGCCCATTCCTGTGAATCGT	GTGACTCAAAGCAGCAGAAGC
ENST00000430859	CCCCTTAGCCTGCTTGTGAT	CCAGTGAAAACTGCACAGAGC
KCNK12	CATGTACACCAGCGTGGAGG	AGCGAGTAAATGCAGCACAC
CCL11	TCCCTGGAATCTCCCACACT	CACTCAGGCTCTGGTTTGGT
MDK	TCAGACCGGTTCTGGAGACA	TTTGCTTTGGTCTTGGGGGT
SAA2	GAGTGGCAGAGACCCCAATC	TCAGCTTCTCTGGACATAGACC
MGST1	CGGCCTCACCATTCCAGAC	CAAGGTCATTCAGGTGGGCT
HIST1H2BG	GCAGTGTCCGAAGGTACCAA	TGTGAGACTTGAGTGGCTCTG
GAPDH	CTGGGCTACACTGAGCACC	AAGTGGTCGTTGAGGGCAATG

KCNK12, potassium two pore domain channel subfamily K member 12; CCL11, C-C motif chemokine ligand 11; MDK, midkine; SAA2, serum amyloid A2; MGST1, microsomal glutathione S-transferase 1; HIST1H2BG, histone cluster 1 H2B family member g.

Table II. Characteristics of lncRNAs for validation.

lncRNA	Expression	Chromosome	Strand	Start	End	Class
NR_033917	Down	Chr20	+	4173736	4176600	Intergenic
NR_038367	Down	Chr7	+	27135712	27139877	Antisense
NR_026861	Down	Chr6	-	166337535	166401527	Intergenic
NR_102703	Up	Chrx	-	149007562	149025779	Undefined
NR_110162	Up	Chr7	-	112594689	112635698	Undefined
ENST00000430859	Up	Chr7	+	20257208	20261315	Bidirectional

lncRNA, long non-coding RNA; Chr, chromosome.

of Genes and Genomes (https://www.genome.jp/kegg/) pathway analysis of the differentially expressed mRNAs was performed to identify the associated pathways. Connections between the genes were revealed based on their associations in the identified pathways. In addition, a coding-non-coding gene co-expression (CNC) network was constructed to investigate the interactions between the differentially expressed lncRNAs and mRNAs. A number of differentially expressed mRNAs were identified to be associated with development. Pearson's correlation coefficient was calculated for each pair of lncRNAs and mRNAs, and the most significantly correlated genes (Pearson's correlation coefficient ≥0.90 or ≤-0.90; P<0.05) were selected to construct the network. The network was generated using Cytoscape software (v2.8.2; https://cytoscape.org/). The default annotation categories were selected in the software and an enrichment score >2.0 was considered significant.

Transient transfection. hDFCs were transfected with 50 nM MEG3-small interfering (si)RNA and NC-siRNA using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) at a density of $1.5x10^5$ cells, according to the manufacturer's protocol. The MEG3-siRNA and negative control (NC)-siRNA were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). The MEG3-siRNA sequences were as follows: GACTTAAACCAATGCCCTA, CCTCTT ACCTAAAGACTTA, CCCTCTTGCTTGTCTTACT. The expression of pluripotency-associated genes was detected by RT-qPCR 72 h post-transfection.

Statistical analysis. Data are presented as the mean ± standard deviation (n=3) and were analyzed using a Student's t-test. All statistical analysis was performed with SPSS 20.0 (IBM Corp., Armonk, NY, USA). A fold-change >2.0 or <-2.0 and P<0.05 was considered to indicate a statistically significant difference.

Results

lncRNA and mRNA expression profiles of hDFCs and hPDLCs. Gene expression patterns of hDFCs and hPDLCs were screened with the high-throughput microarray method to reveal potential molecular mechanisms underlying hDFC development into periodontal tissues. Microarray probes detected 36,430 transcripts in hDFCs and hPDLCs (Fig. 1).

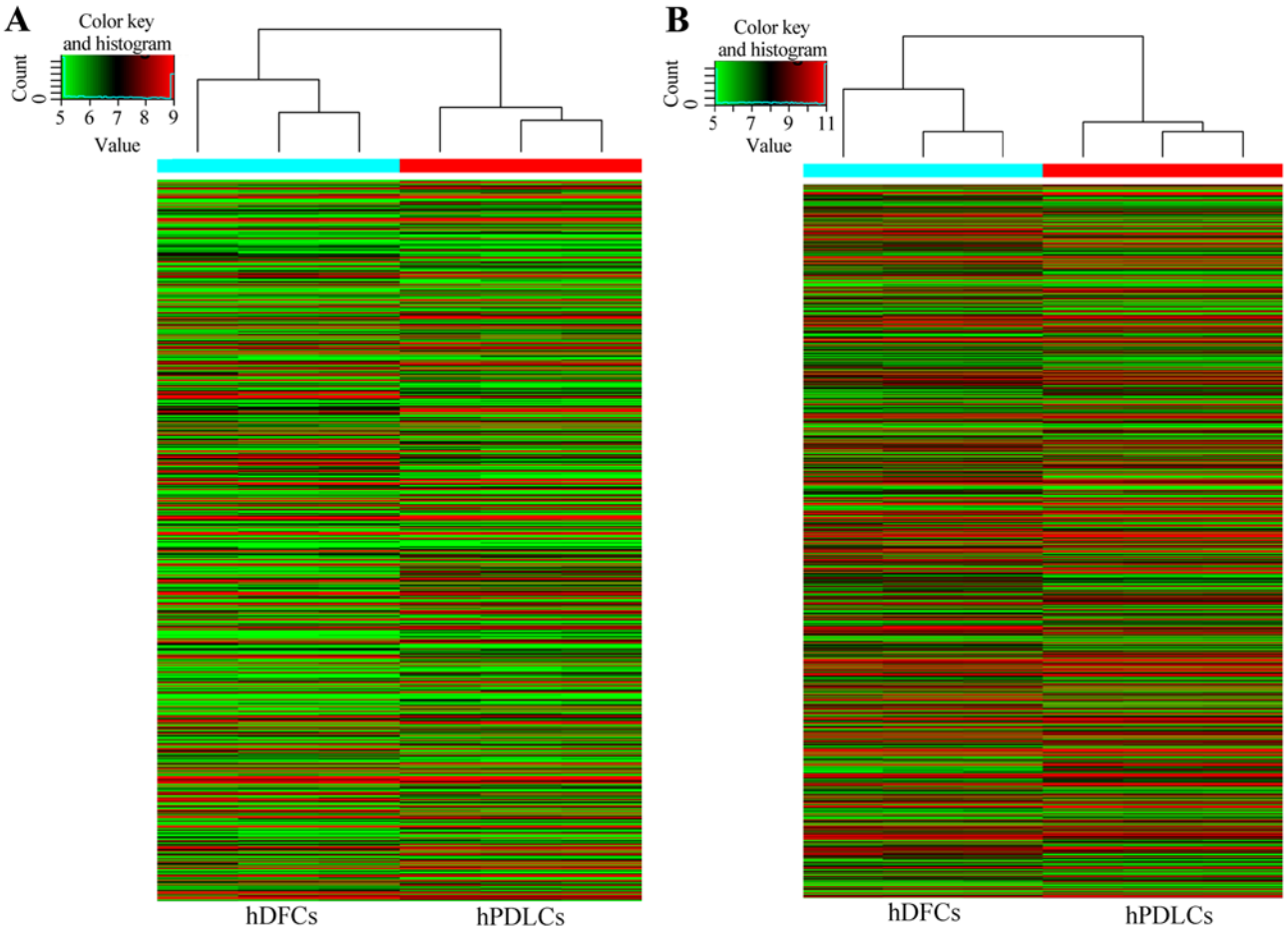

Figure 1. Expression profiles of lncRNAs and mRNAs in hDFCs and hPDLCs. (A) Hierarchical clustered heat maps of differentially expressed lncRNAs. (B) Hierarchical clustered heat maps of differentially expressed mRNAs. Each column represents one sample and 'Value' indicates the gene expression. The intensity of the color indicates the expression level, with red representing a high expression level and blue representing a low expression level. 'Count' refers to the number of genes that are expressed at the same level. lncRNA, long non-coding RNA; hDFC, human dental follicle cell; hPDLC, human periodontal ligament cell.

A total of 845 lncRNAs were identified to be differentially expressed in the hDFCs and hPDLCs, of which 460 were upregulated and 385 were downregulated in the hDFCs compared with the hPDLCs. In addition, a total of 1,012 mRNAs (6.12%) were differentially expressed, 553 mRNAs were upregulated and 459 mRNAs were downregulated in the hDFCs compared with the hPDLCs. A scatter plot was generated to visualize the variation in expression of lncRNAs and mRNAs in the hDFCs and hPDLCs (Fig. 2A and B). Additionally, a volcano plot was used to visualize significantly differentially expressed lncRNAs and mRNAs (Fig. 2C and D). Furthermore, the microarray results revealed the top ten differentially expressed lncRNAs and mRNAs (Tables III and IV).

Subgroup analysis classified the differentially expressed lncRNAs as intergenic lncRNAs (lincRNAs) and antisense lncRNAs (ASlncRNAs). The nearby protein-coding genes were identified to predict potential functions of lncRNAs. A total of 113 lincRNAs and 35 ASlncRNAs, including NR_033932, T152410, ENST00000512129 and ENST00000540293, were revealed to be located near known protein-coding genes.

To verify the reliability of the microarray data, six differentially expressed lncRNAs and six differentially expressed mRNAs were randomly selected for analysis of their expression levels by RT-qPCR. Each differentially expressed gene was numbered and the RANDBETWEEN function in Microsoft Excel was used. Compared with hPDLCs, hDFCs exhibited decreased expression levels of the lncRNAs NR_033917, NR_038367 and NR_026861, and the mRNAs potassium two pore domain channel subfamily K member 12, C-C motif chemokine ligand 11 and midkine. By contrast, hDFCs demonstrated increased expression levels of the lncRNAs NR_102703, NR_110162 and ENST00000430859, and the mRNAs serum amyloid A2, microsomal glutathione S-transferase 1 and histone cluster 1 H2B family member g (Fig. 3). These results were consistent with the microarray data.

GO analysis and pathway analysis. GO analysis revealed functions associated with the differentially expressed genes and provided annotations to describe the genes and gene products (Fig. 4). The top five downregulated GO functions were the following: 'Complex of collagen trimers'; 'endothelial cell morphogenesis'; 'penile erection'; 'positive regulation of renal sodium excretion'; and 'positive regulation of stem cell differentiation' (Fig. 5A). The top five upregulated GO functions were as follows: 'Condensed chromosome outer kinetochore'; 'aldehyde dehydrogenase activity'; 'regulation of mononuclear

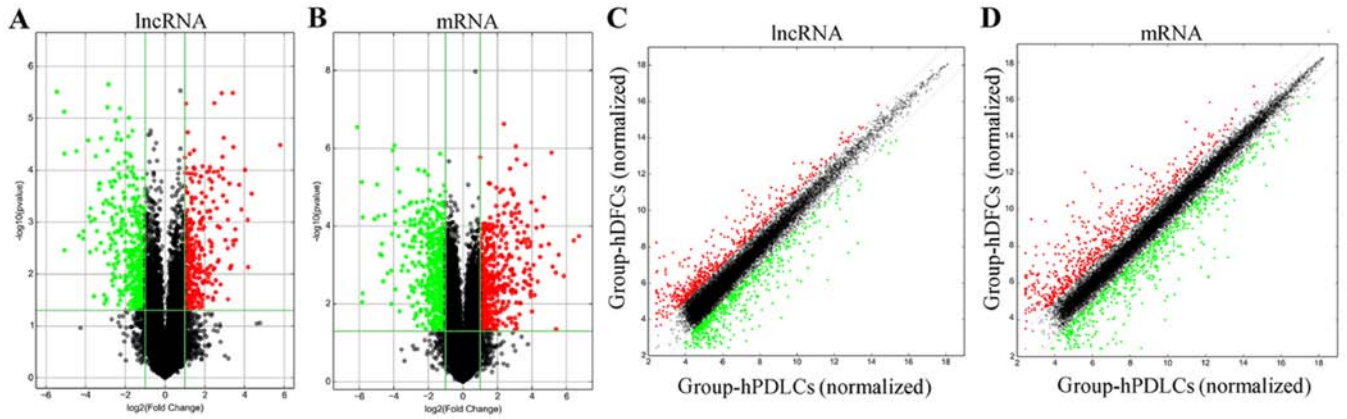

Figure 2. Scatter plots and volcano plots of differentially expressed genes. Scatter plots of significantly differentially expressed (A) lncRNAs and (B) mRNAs. The x-axis represents the mean normalized log2 fold-change for the hPDLCs and the y-axis represents the mean normalized log2 fold-change for the hDFCs. Volcano plots were generated to visualize the differentially expressed (C) lncRNAs and (D) mRNAs. The x-axis indicates the log2 fold-change and the y-axis represents the log2 P-values. The green and red points indicate the upregulated and downregulated lncRNAs and mRNAs with statistical significance, respectively. lncRNA, long non-coding RNA; hDFC, human dental follicle cell; hPDLC, human periodontal ligament cell.

cell migration'; 'mononuclear cell migration'; and 'condensed nuclear chromosome and centromeric region' (Fig. 5B).

Pathway analysis revealed 20 downregulated pathways. The top three enriched networks were 'rheumatoid arthritis', 'ECM-receptor interaction' and 'arrhythmogenic right ventricular cardiomyopathy (ARVC)' (Fig. 6A). In addition, 36 upregulated pathways were identified and the top three enriched networks were 'p53 signaling pathway', 'oocyte meiosis' and 'renin secretion' (Fig. 6B).

CNC network. A total of 615 lncRNAs and 16 mRNAs were selected to construct the CNC network (Fig. 7). The CNC network included 488 positive pairs and 438 negative pairs, and each mRNA could be associated with 1-20 lncRNAs, and vice versa. The CNC network indicated the different molecular mechanisms of hDFCs compared with hPDLCs associated with the inter-regulation of lncRNAs and mRNAs.

MEG3 regulates the pluripotency of hDFCs. MEG3 was reported to serve a crucial role in initiating embryogenesis and development (21). The current study depleted MEG3 expression in hDFCs and identified a reduction in the transcription of pluripotency-associated genes (Fig. 8). In summary, these results may suggest a possible regulatory role of MEG3 in the pluripotency of hDFCs.

Discussion

The dental follicle contains precursor cells that may develop into periodontal ligament, cementum and alveolar bone during periodontal development. hDFCs and hPDLCs are the major cells of the dental follicle and periodontal ligaments, respectively. These cells are key markers that represent different stages of periodontal tissue development; however, hDFCs exhibit more obvious embryonic characteristics, including pluripotency and heterogeneity. Therefore, understanding the mechanisms associated with the differentiation of hDFCs to hPDLCs is crucial for promoting periodontal development and regeneration.

An increasing number of studies have indicated that numerous lncRNAs regulate cell differentiation through epigenetics or in-cis/in-trans gene transcription. Previous studies have demonstrated that differential expression levels of lncRNAs are associated with human diseases and biological processes. lncRNAs serve vital roles in cell development and lineage commitment. Therefore, lncRNAs may serve as key regulators of human tissue development and regeneration (22). lncRNAs are differentially expressed during the differentiation of human bone marrow mesenchymal stem cells and PDLSCs (23,24). To investigate the changes and roles of lncRNAs during the development of hDFCs, the current study used high-throughput microarrays to detect the expression levels of genes. Only the middle one-third of the root surface was taken while obtaining the periodontal ligament from the extracted tooth; the quantity was small, and collagen fibers and blood vessels in the tissues may have affected the array results. The aim of the present study was to compare the differentially expressed genes between hDFCs and hPDLCs with differentiation capability. Cells obtained at P3 have a stronger differentiation capability. A total of 845 lncRNAs and 1,012 mRNAs were detected to be differentially expressed in hDFCs compared with hPDLCs. By comparing the expression of lncRNAs and mRNAs in hDFCs and hPDLCs, the present results identified variation in the expression levels of lncRNAs and mRNAs during the development of hDFCs. The results also indicated that upregulated lncRNAs and mRNAs were predominant during the development of hDFCs, and differentially expressed mRNAs and lncRNAs were used as candidates to screen the key genes associated with the differentiation of hDFCs by bioinformatics analysis. The RT-qPCR results confirmed the reliability of the microarray data. Markedly differential expression levels between the two types of cells indicated that lncRNAs may serve crucial roles in the differentiation and development of hDFCs.

lncRNAs may be categorized as sense, antisense, intronic, intergenic and bidirectional (25). Among them, ASlncRNAs are a type of endogenous lncRNA that complement other transcripts. Antisense transcription is a common phenomenon

Table III. Top 10 downregulated and upregulated lncRNAs of human dental follicle cells compared with human periodontal ligament cells.

A, Downregulated

lncRNA	Log2 fold change
G005378	-55.6292005
BC017988	-20.5510994
FGF13-AS1	-18.1455729
RP11-368I23.4	-17.8927488
LINC00473	-16.2682486
RP11-313F23.4	-14.3828901
RN7SKP240	-13.2776605
AP000619.5	-11.8151652
G075702	-11.6414154
RP11-256I23.3	-10.831092

B, Upregulated

lncRNA	Log2 fold change
LINC00944	43.9000497
AK055386	33.9312365
G048345	33.5797092
LOC101929504	33.5392967
LINC01021	21.9723651
APCDD1L-AS1	20.0893837
RP11-13N12.1	17.314853
LOC100506457	17.1318902
uc.176	14.6428966
G060456	13.6926091

lncRNA, long non-coding RNA; FGF13-AS1, fibroblast growth factor 13-antisense RNA 1; APCDD1L-AS1, APC down-regulated 1 like-antisense RNA 1.

Table IV. Top 10 downregulated and upregulated mRNAs of hDFCs compared with hPDLCs.

A, Downregulated

mRNA	Log2 fold change
KRT5	-105.4128548
SOX11	-85.9063061
LRP1B	-57.6366695
SLC7A3	-46.7530193
CTAG2	-42.1623478
SLITRK6	-41.7426552
SPATA22	-36.689978
SHISA2	-35.1885461
LAMP3	-31.6925565
MMP13	-25.9685676

B, Upregulated

mRNA	Log2 fold change
RPTN	69.3550374
PSG2	57.7064349
ADRA2A	56.7419608
SOST	55.5946956
FLG	55.4287499
KCNB1	34.5671271
SLC14A1	31.308326
MFAP5	29.633329
PSG5	27.7407034
PSG7	25.9549841

KRT5, keratin 5; SOX11, SRY-box 11; LRP1B, LDL receptor related protein 1B; SLC7A3, solute carrier family 7 member 3; CTAG2, cancer/testis antigen 2; SLITRK6, SLIT and NTRK like family member 6; SPATA22, spermatogenesis associated 22; SHISA2, shisa family member 2; LAMP3, lysosomal associated membrane protein 3; MMP13, matrix metallopeptidase 13; RPTN, repetin; PSG2, pregnancy specific β-1-glycoprotein 2; ADRA2A, adrenoceptor α 2A; SOST, sclerostin; FLG, filaggrin; KCNB1, potassium voltage-gated channel subfamily B member 1; SLC14A1, solute carrier family 14 member 1; MFAP5, microfibril associated protein 5; PSG5, pregnancy specific β-1-glycoprotein 5; PSG7, pregnancy specific β-1-glycoprotein 7.

in humans, and is based on whether the antisense RNA acts in cis or in trans. ASlncRNAs regulate gene expression at the transcriptional or post-transcriptional level via a number of biological mechanisms, including RNA-DNA interaction (chromatin remodeling), transcription interference and RNA-RNA interaction in the nucleus/cytoplasm. ASlncRNAs can serve as scaffolds between DNA and proteins, and participate in disease processes by assisting with interactions between ASlncRNAs, DNA and proteins (26). The current microarray results indicated the ASlncRNAs and nearby mRNAs that may regulate important biological processes. For example, the lncRNA NR_033932 was identified as one of the transcripts of lncRNA repulsive guidance molecule b (RGMB)-AS1, which was revealed to be upregulated in hDFCs compared with hPDLCs. lncRNA RGMB-AS1 is located in the antisense region of RGMB, and RGMB carries the exon of the lncRNA RGMB-AS1 gene in the reverse strand and orientation of its intron region. lncRNA RGMB-AS1 silencing suppresses lung adenocarcinoma and hepatocellular carcinoma cell proliferation, migration and invasion, and leads to cell cycle arrest at the G_1/G_0 phase (27,28). lncRNA T152410 was identified to be upregulated in hDFCs compared with hPDLCs. The nearby gene SMAD specific E3 ubiquitin protein ligase 2 (SMURF2) is a member of the HECT family of E3 ubiquitin ligases that regulate the polarity of cells during embryonic development and other signaling pathways for osteoblast migration, proliferation and differentiation. Notably, the anti-proliferative effect of SMURF2 siRNA is mediated by arresting cells in the G_0/G_1 phase, similar to the lncRNA RGMB-AS1 (29,30). This suggests that these two ASlncRNAs may promote hDFC migration, proliferation and differentiation by regulating the cell cycle.

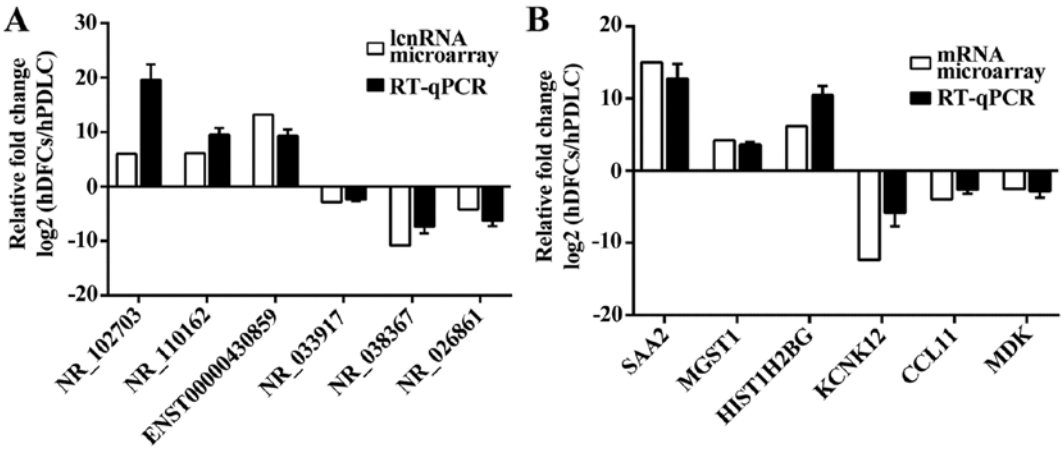

Figure 3. Validation of the microarray results using RT-qPCR. (A) Comparative expression levels of lncRNAs between hDFCs and hPDLCs obtained by microarray and RT-qPCR. (B) Comparative expression levels of mRNA between hDFCs and hPDLCs obtained by microarray and RT-qPCR. The microarray analyses were normalized and the RT-qPCR results were calculated using the $2^{-\Delta\Delta Cq}$ method. SAA2, serum amyloid A2; MGST1, microsomal glutathione S-transferase 1; HIST1H2BG, histone cluster 1 H2B family member g; KCNK12, potassium two pore domain channel subfamily K member 12; CCL11, C-C motif chemokine ligand 11; MDK, midkine; RT-qPCR, reverse transcription-quantitative polymerase chain reaction; lncRNA, long non-coding RNA; hDFC, human dental follicle cell; hPDLC, human periodontal ligament cell.

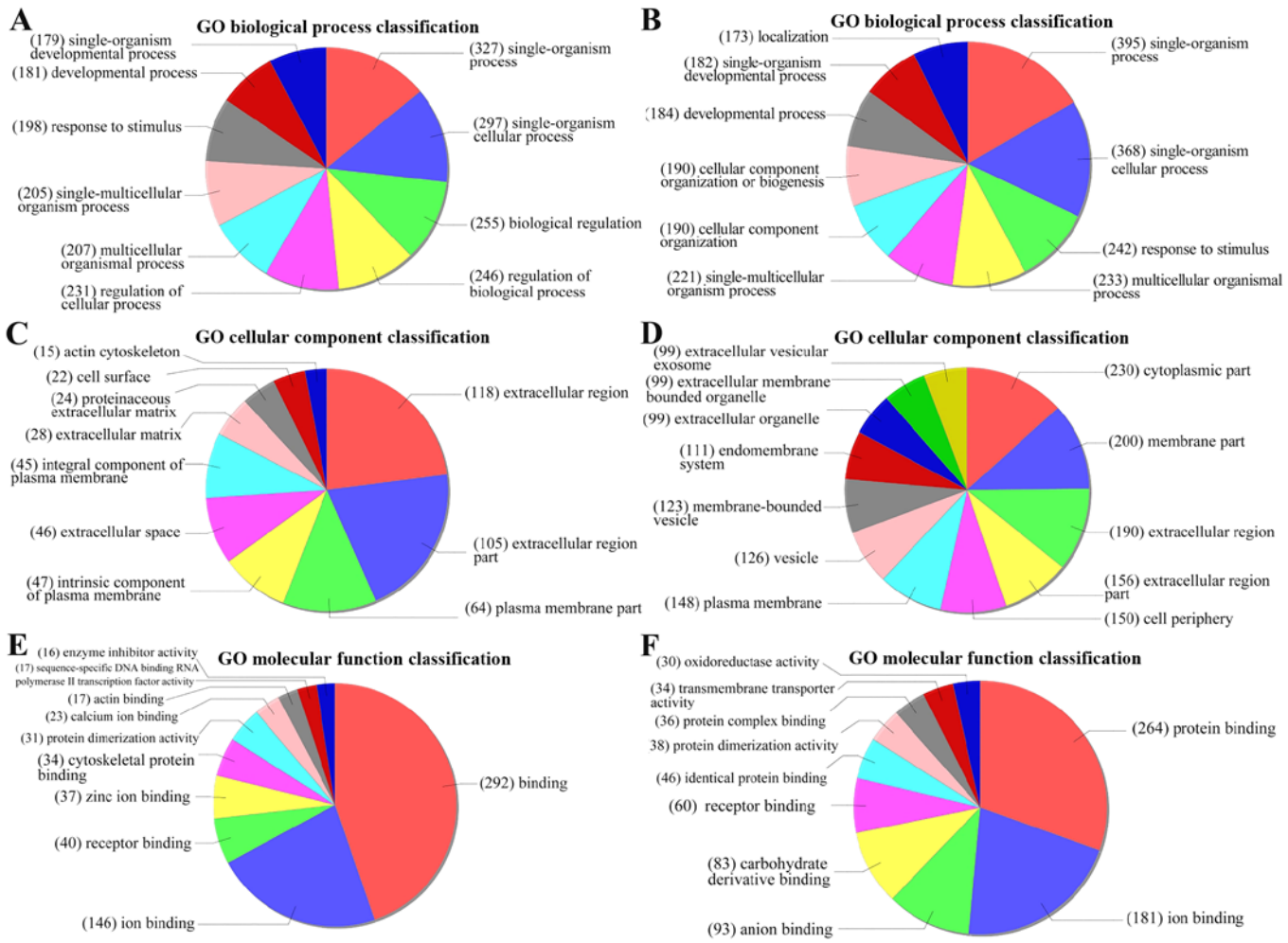

Figure 4. GO analysis of human dental follicle cells compared with human periodontal ligament cells. GO biological process classification of (A) downregulated and (B) upregulated mRNAs. GO cellular component classification of (C) downregulated and (D) upregulated mRNAs. GO molecular function classification of (E) downregulated and (F) upregulated mRNAs. GO, gene ontology.

lncRNAs may exert effects by controlling the transcriptional regulation of nearby coding genes (31). Subgroup analysis of the microarray demonstrated that a number of differentially expressed lincRNAs located near mRNAs

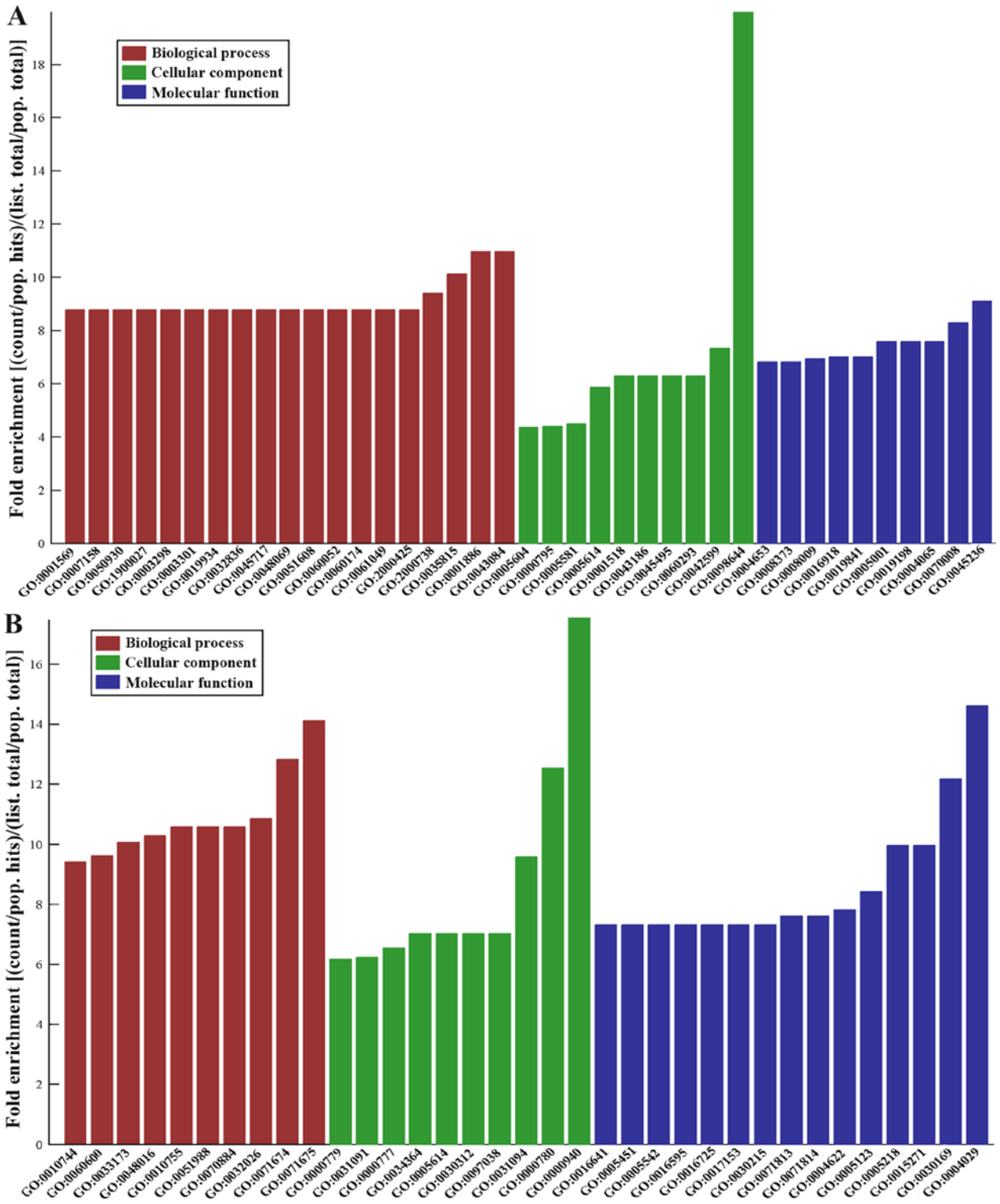

Figure 5. Significant GO terms of differentially expressed mRNAs of hDFCs compared with hPDLCs. (A) Significant GO terms of downregulated mRNAs identified in hDFCs compared with hPDLCs. (B) Significant GO terms of upregulated mRNAs identified in hDFCs compared with hPDLCs. GO, gene ontology; hDFCs, human dental follicle cells; hPDLCs, human periodontal ligament cells.

are associated with tissue development. For example, lncRNA ENST00000512129 and nearby mRNA lymphoid enhancer binding factor 1 (LEF1) were downregulated in hDFCs compared with hPDLCs. As a key mediator of the Wnt/β-catenin signaling pathway and epithelial-mesenchymal interaction, LEF1 has been revealed to regulate incisor development. LEF1 also serves a key role in stem cell maintenance along with SOX2 and paired like homeodomain 2 (32,33). lncRNA ENST00000540293 and some nearby matrix metalloproteinase (MMP) mRNAs were revealed to be downregulated in hDFCs. MMPs are effective proteolytic mediators during ECM remodeling, and regulate the Notch signaling pathway, which is involved in differentiation. MMPs are able to regulate the necessary changes

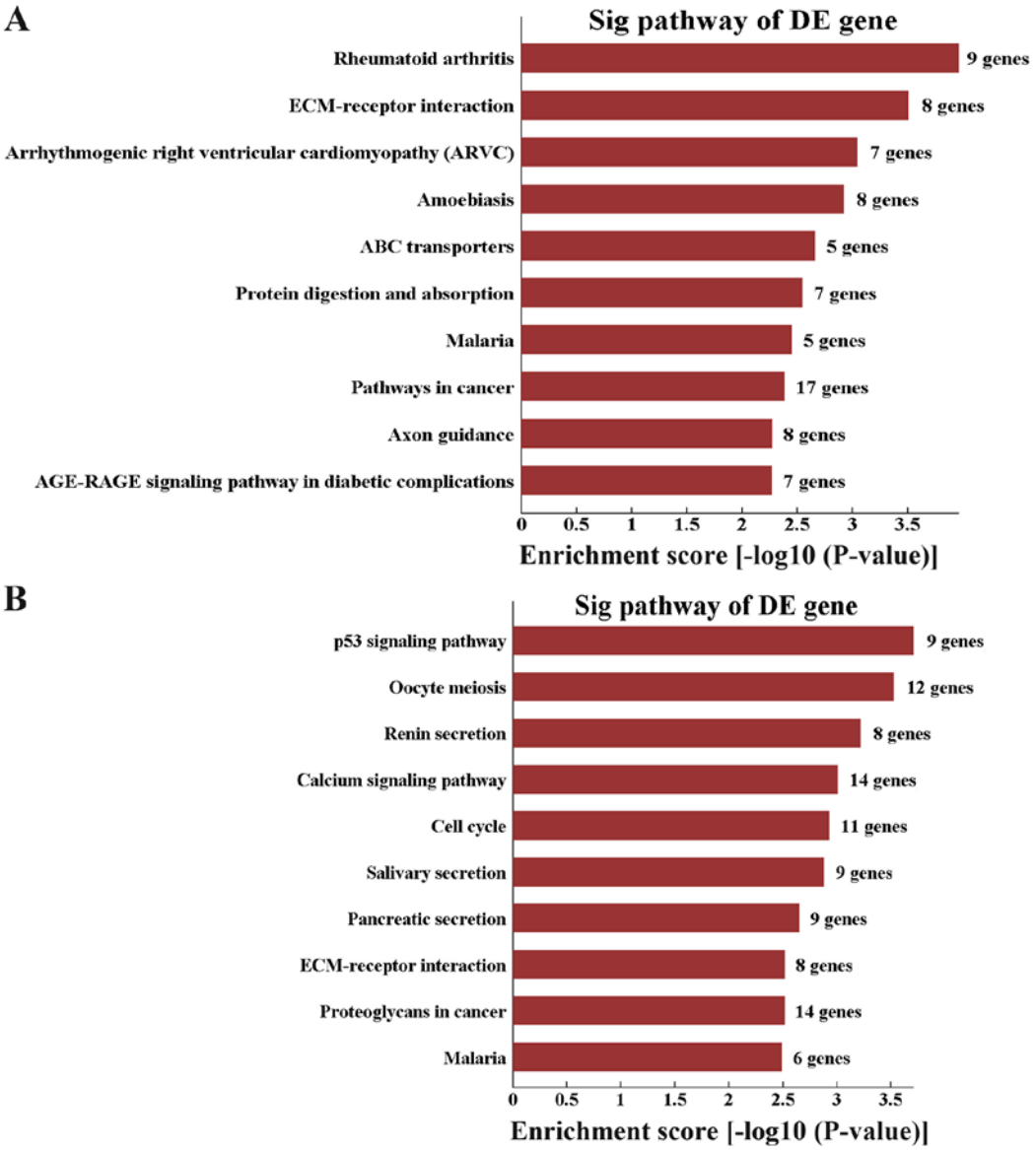

Figure 6. Top 10 pathways of DE mRNAs ranked by enrichment score. (A) Top ten pathways of downregulated mRNAs ranked by enrichment score. (B) Top ten pathways of upregulated mRNAs ranked by enrichment score. Sig, significant; DE, differentially expressed.

in the microenvironment, and overexpression of MMPs increases cell differentiation during development by altering the bioavailability of chemokines and cytokines that affect stem cell function (34). These results could be explained by evidence that lncRNAs regulate the expression of neighboring genes to promote cellular survival and differentiation (35).

Pathway analysis revealed that the top three downregulated pathways were 'rheumatoid arthritis', 'ECM-receptor interaction' and 'arrhythmogenic right ventricular cardiomyopathy (ARVC)'. In addition, the top three upregulated pathways were 'p53 signaling pathway', 'oocyte meiosis' and 'renin secretion'. Among these pathways, the p53 pathway is a vital signaling pathway in tumor biology. The expression level of p53 is high during early embryonic development. p53 serves an important role in self-renewal and human embryonic stem cell differentiation by regulating specific target genes or miRNAs and reactivating developmental pathways for tissue regeneration (36). The calcium pathway is a ubiquitous intracellular signaling pathway that participates in numerous cellular processes, including cell proliferation, differentiation and apoptosis. As an intracellular messenger, calcium serves an important role in the cellular signaling pathways at different stages of stem cell differentiation (37). Upregulation of calcium-mediated signaling is essential for the maintenance of stem cells. The current results indicated that these pathways may be involved in the development of hDFCs.

In addition, CNC analysis revealed a potential regulatory network between lncRNAs and mRNAs, and the mRNAs selected for CNC analysis were closely associated with the pathways in the development process of stem cells (38,39). Among the lncRNA-mRNA pairs, lncRNA uc021sxs.1 was differentially expressed in hDFCs compared with hPDLCs and was correlated with Dickkopf-1 (DKK1). DKK1 serves as an antagonist of the canonical Wnt signaling pathway by binding to the Wnt receptor Lrp5/6, which is central to embryonic and adult bone development (40). The lncRNA ENST00000609146 was upregulated in hDFCs compared with hPDLCs and it was negatively correlated with Wnt5a. Wnt5a regulates a variety of biological processes,

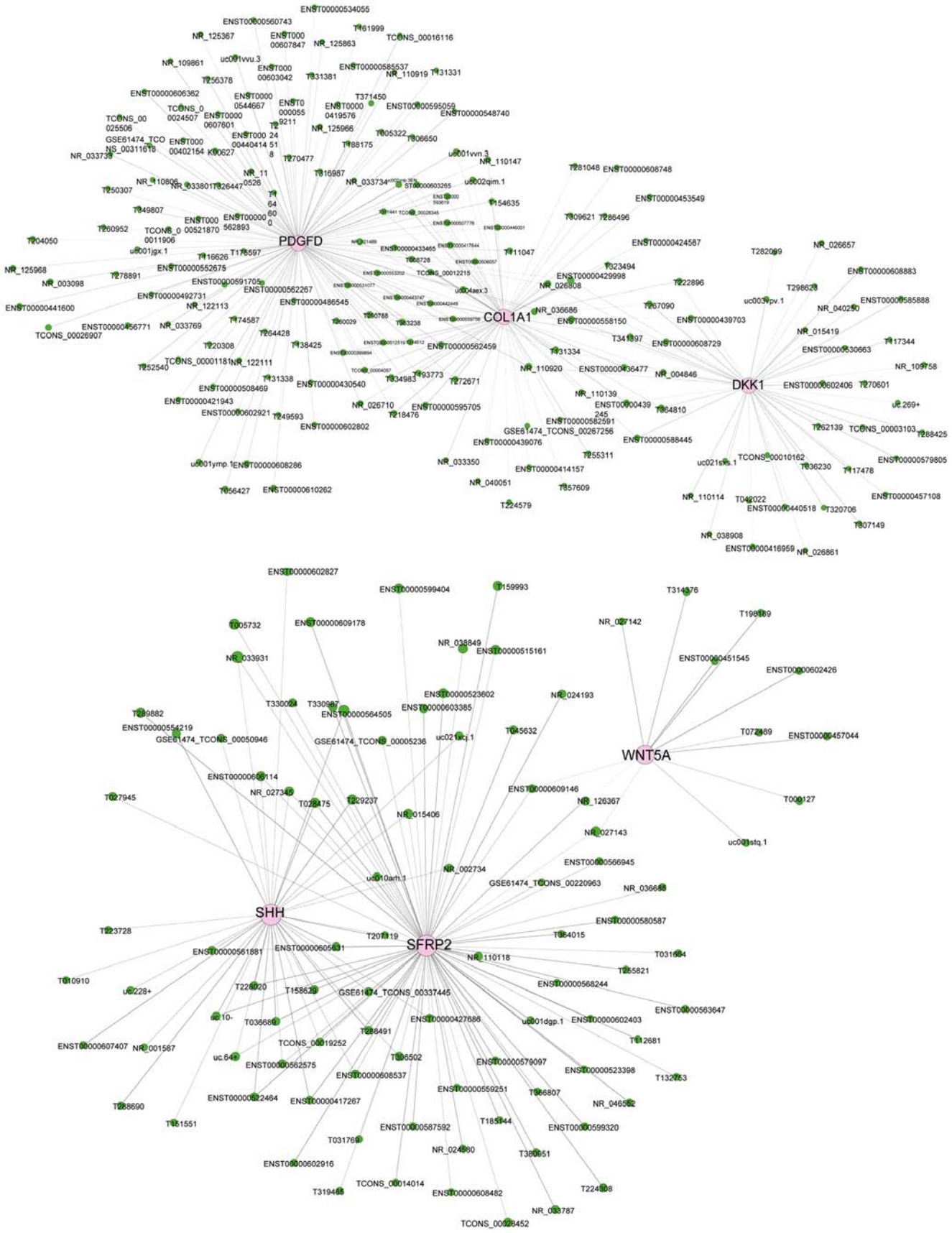

Figure 7. Part of the coding-non-coding gene co-expression network. Pink nodes represent mRNAs and green nodes represent long non-coding RNAs. Solid lines indicate a positive correlation and dashed lines represent a negative correlation.

including proliferation, differentiation, migration, adhesion and polarity (41). Wnt5a appears to serve important roles in the fate of DFSCs in the development and regeneration of the periodontium. Wnt5a can directly or indirectly activate the canonical Wnt

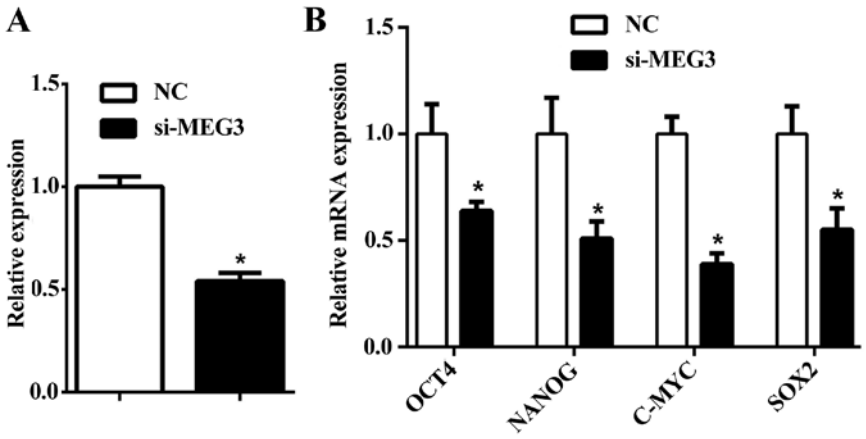

Figure 8. Expression of pluripotency-associated genes detected by RT-qPCR. (A) The transfection efficiency of MEG3-siRNA. (B) Decreased expression levels of pluripotency-associated genes were revealed following knockdown of MEG3 by siRNA for 72 h. Data are presented as the mean ± standard deviation (n=3). *P<0.05 vs. respective NC. MEG3, maternally expressed 3; siRNA, small interfering; NC, negative control; OCT4, POU class 5 homeobox 1; NANOG, Nanog homeobox; SOX2, SRY-box 2.

signaling pathway to promote mesendoderm differentiation (42). The correlations between these lncRNAs and mRNAs indicate that lncRNA uc021sxs.1 and lncRNA ENST00000609146 may be involved in associated signaling pathways by regulating mRNA expression. In addition, the results suggest that the activation of the Wnt signaling pathway may serve a critical role in hDFC development.

Although numerous studies have been conducted to identify cell factors that maintain the pluripotency of periodontal cells (43), potential lncRNAs with similar functions remain unknown. The current results demonstrated that MEG3 may serve a role in the establishment or maintenance of the stem cell state.

A study conducted by Lee *et al* (44) reported that 1.49% of mRNAs in hDFCs were differentially expressed (fold change >2.0 or <-2.0; P<0.05) compared with hPDLCs. The current microarray results indicated that among a total of 16,531 mRNAs, the expression levels of 1,012 mRNAs (6.12%) were significantly different in hDFCs compared with hPDLCs. Differences in the ages of participants and cell state may explain this discrepancy. Compared with the study by Lee *et al* (44), the current analysis predominantly focused on the potential lncRNAs instead of mRNAs, which may serve critical roles in hDFC differentiation.

The present results may promote further studies to investigate the functions of genes and the regulatory mechanisms in hDFCs and periodontal tissue, and may advance the use of stem cell-based therapies in regenerative medicine. Furthermore, more studies should be conducted to investigate the potential functions that differentially expressed lncRNAs serve in multiple biological processes of hDFCs and periodontal tissue.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LW, LD and JL conceived and designed the experiments. LD performed the experiments. LW, LD and HH analyzed the data. LD drafted the manuscript. CP, XZ, and ZC performed the experiments, analyzed the data and revised the manuscript. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

The protocol to acquire human tissues was approved by the Ethical Guidelines of the Ethics Committee of the Hospital of Stomatology, Sun Yat-Sen University (Guangzhou, China). Written informed consent was obtained from all patients and their parents.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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