# Alteration of MicroRNA Expression of Human Dental Pulp Cells during Odontogenic Differentiation

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## Abstract

**Introduction:** MicroRNAs (miRNAs) play momentous roles in various biological processes including cell differentiation. However, little is known about the role of miRNAs in human dental pulp cells (hDPCs) during odontogenic differentiation. The aims of this study were to investigate the expression of miRNAs in the primary culture of hDPCs when incubated in odontogenic medium. **Methods:** The potential characteristics of hDPCs were investigated by miRNA microarray and real-time reverse transcriptase polymerase chain reaction. Bioinformatics (ie, target prediction, Gene Ontology analysis, and Kyoto Encyclopedia of Genes and Genomes mapping tools) were applied for predicting the complementary target genes of miRNAs and their biological functions. **Results:** A total of 22 miRNAs were differentially expressed in which 12 miRNAs up-regulated and 10 miRNAs down-regulated in differentiated hDPCs compared with the control. The target genes of differential miRNAs were predicted to associate with several biological functions and signaling pathways including the mitogen-activated protein kinase (MAPK) and the Wnt signaling pathway. **Conclusions:** The differential expression miRNAs may be involved in governing hDPC odontogenic differentiation, thus contributing to the future investigations of regulatory mechanisms in reparative dentin formation and dental pulp regeneration. *(J Endod 2012;38:1348–1354)*

## Key Words

Differentiation, gene ontology, human dental pulp cells, microRNAs, odontoblasts, pathway analysis

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Dental pulp tissue is a promising stem cell source for the research of bone defect repair and dental tissue engineering (1–3). Human dental pulp cells (hDPCs) isolated from postnatal human dental pulp are a heterogeneous population that can differentiate into a variety of cell types, including odontoblast-like cells, osteoblast-like cells, chondrocytes, adipocytes, and neural-like cells (4, 5). The capacity to differentiate into functional odontoblasts and to produce a mineralized matrix is critical in dentinogenesis, which may contribute to the regeneration of dental pulp. Although numerous mechanisms are involved in the differentiation process (6–8), the underlying molecular pathways during this differentiation program have not been thoroughly elucidated.

MicroRNAs (miRNAs) are a group of small (~22 nt) noncoding RNAs that can bind to the 3′ untranslated region of target messenger RNAs to induce messenger RNA cleavage or translation repression and, consequently, negatively regulate the target genes (9). Several studies have shown that miRNAs are involved in many biological processes, including cell proliferation, stem cell maintenance and differentiation (10), development (11), and tumor initiation (12). A growing body of evidence suggests an important role for miRNAs in osteoblastic differentiation. The miR-26 inhibits osteogenic differentiation of human adipose tissue–derived stem cells by targeting the SMAD1 transcription factor. Mineralization indicators such as alkaline phosphatase (ALP), type I collagen, osteocalcin (OCN), and osteopontin were all down-regulated (13). It has also been shown that miR-125b acts as an inhibitor in the osteoblastic differentiation of mouse mesenchymal stem cells by reducing cell proliferation (14). Meanwhile, miR-133 and miR-135 functionally inhibited bone morphogenetic protein-2–induced differentiation of C2C12 mesenchymal cells by attenuating the Runx2 and Smad5 pathways, which synergistically contribute to bone formation (15). The miR-29 modulates canonical Wnt signaling through a positive feedback loop that can promote osteoblastic differentiation (16). Furthermore, miR-204/211 has been shown to negatively modulate osteoblast differentiation and mineralization in mesenchymal progenitor cells and bone marrow stromal cells by suppressing Runx2, a key transcription factor for osteogenesis (17). All of these suggest that miRNAs play a role in the osteogenic differentiation of various cell types via multiple signaling pathways.

hDPCs can promote reparative dentin formation by differentiating into odontoblasts and then producing mineralized matrix. This differentiation program is similar to the process of osteogenic differentiation and bone formation. Considering the regulatory action of miRNAs in osteogenic differentiation, we hypothesized that miRNAs may participate in odontoblast differentiation of hDPCs. In our previous study, we isolated hDPCs with stem cell characteristics and confirmed their odontoblast-like differentiation potential (5). Herein, we used miRNA microarray and quantitative real-time polymerase chain reaction (qRT-PCR) to investigate and validate the expression profiles of miRNAs in hDPCs during odontogenic differentiation. Furthermore, target prediction, Gene Ontology (GO) analysis, and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway mapping tools were used to systemically analyze the possible biological functions and signaling pathways that are involved in the differentiation program. Studies on miRNA expression profiles may help to elucidate the potential molecular mechanisms of odontoblast differentiation of hDPCs and dentinogenesis and may improve current cell-based therapy for regenerative endodontics.

## Materials and Methods

### Cell Culture and the Induction of Odontogenic Differentiation

Normal human premolars and impacted third molars were extracted from 3 healthy adults (12–25 years) undergoing orthodontic treatment at the Department of Oral and Maxillofacial Surgery at the Affiliate Stomatology Hospital of Sun Yat-sen University, Guangzhou, Guangdong, China. Informed consent was obtained from each patient. The protocols were approved by the University Ethics Committee. hDPCs were isolated and cultured as previously described (5). Briefly, hDPCs from each of the 3 patients were cultured separately in alpha-modified Eagle medium (GIBCO-BRL Life Technologies, Guangzhou Genewindows Biotech Ltd, Guangzhou, China) supplemented with 15% fetal bovine serum (FBS) (GIBCO-BRL Life Technologies), 10U/mL penicillin, and 10 $\mu$g/mL streptomycin (Sigma, St Louis, MO) and incubated at 37°C in 5% $CO_2$.

For odontogenic differentiation, hDPCs at passage 3 were cultured in odontogenic induction medium (15% FBS, 10 mmol/L $\beta$-glycerophosphate, 0.2 mmol/L ascorbic acid, and 100 nmol/L dexamethasone in alpha-modified Eagle medium) for 14 days. Control samples were cultured in 15% FBS medium alone.

As an indication of odontogenic differentiation, mineralization was confirmed through alizarin red S staining and von Kossa staining as previously reported (18). Additionally, the expression of odontoblast-related genes (ie, ALP, dentin sialophosphoprotein, OCN, and bone sialoprotein) during hDPC odontogenic differentiation was monitored using qRT-PCR. The primers used for each gene were shown in Table 1.

### Preparation of RNA Samples

Total RNA was extracted from induced hDPCs and the control with Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Purified DNAase-treated RNA was analyzed on the ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA) to determine the quantity and purity of the samples. RNA samples were used in the microarray analysis only if the 260/280 ratio was above 1.9 and the RNA integrity number was greater than 8.0. Aliquots of the same RNA samples were used for the experiments in both microarray and qRT-PCR assays.

### miRNA Microarray

miRNA microarray was performed using the GeneChip miRNA Array (Affymetrix, Santa Clara, CA). Briefly, 3 $\mu$g total RNA was labeled with the FlashTag Biotin HSR RNA Labeling Kit (Affymetrix). After that, hybridization was performed in the Affymetrix Hybridization Oven 640 (Affymetrix) at 48°C for 16 hours, and then the arrays were washed with GeneChip fluidic station 450. Then, arrays were scanned with an Affymetrix GeneChip Scanner 3000 (Affymetrix), and the images were analyzed with the Affymetrix GeneChip Operating System 1.4. Only miRNAs with a fold change $\geq$1.5 and $P < .05$ (Student's $t$ test) were considered to be significantly differentially expressed, then included for further analysis. The hierarchical cluster analysis was used to classify differentially expressed miRNAs. Principal component analysis (PCA) was performed to assess the overall similarities and differences in miRNA expression profiles between differentiated hDPCs and controls.

**TABLE 1.** Primer Sequences of Odontoblast-related Genes Used in qRT-PCR

| Genes | Sequence | Size |
|---|---|---|
| ALP | Forward: 5′-CCGCTTTAACCAGTGCAACA-3′<br>Reverse: 5′-TCCCACTGACTTCCCTGCTT-3′ | 85 bp |
| DSPP | Forward: 5′-GGGATGTTGGCGATGCA-3′<br>Reverse:5′-CCAGCT ACTTGAGGTCCATCTTC-3′ | 70 bp |
| OCN | Forward: 5′-AGCAAAGGTGCAGCCTTTGT-3′<br>Reverse: 5′-GCGCCTGGGTCTCTTCACT-3′ | 63 bp |
| BSP | Forward:<br>5′-CAGAGGCAG AAA ACG GCA AC-3′<br>Reverse: 5′-TTCCGGTCTCTGTGGTGTCTT-3′ | 105 bp |
| $\beta$-actin | Forward: 5′-GCATGGGTCAGA AGGATTCCT-3′<br>Reverse: 5′-TCGTCCCAGTTGGTGACGAT-3′ | 106 bp |

BSP, bone sialoprotein; DSPP, dentin sialophosphoprotein.

### qRT-PCR

To validate the result of the miRNA microarray, we selected 8 differentially expressed miRNAs (3 were up-regulated: miR-20b, miR-130b, and miR-17 and 5 were down-regulated: miR-382, miR-431, miR-542-5p, miR-203, and miR-135b) for qRT-PCR assay. Primers were synthesized by Funeng Gene Co Ltd(Guangzhou, China), and subsequent qRT-PCR using SYBR Green (Qiagen, Valencia, CA) was performed following the protocols supplied by the manufacturer. The results are presented as the fold change of each miRNA in differentiated cells relative to the control. These data were calculated from 3 different cell samples with the same cell sample in triplicate. The miRNA expression levels were normalized against U6, and the fold change was determined by the $2^{-\Delta\Delta Ct}$ method. To further test the potential changes of miRNAs during the odontogenic differentiation of hDPCs, qRT-PCR was performed to monitor the expression levels of the most prominently regulated miRNAs (miR-135b) in hDPCs at different time points (ie, 0, 7, 14, and 21 days) in odontogenic induction.

### Target Prediction and Functional Analysis

Three types of miRNA target prediction databases, including TargetScan (issue 2009, http://www.targetscan.org/vert_50/), miRanda (issue 2010, http://www.microrna.org/microrna/), and miRDB (issue 2009, http://mirdb.org/miRDB/download.html), were used to predict the target genes of 22 differentially expressed miRNAs. The intersection of these 3 datasets was assayed according to the prediction results. To determine the potential biological functions and pathways, the DAVID database (issue 2008, http://david.abcc.ncifcrf.gov/content.jsp) was used for GO and pathway analysis (linked to the KEGG database, http://www.genome.jp/kegg/pathway.html) of these predicted target genes.

### Statistical Analysis

These data were performed in triplicate on 3 different cell samples. Data are expressed as the mean $\pm$ standard deviation. Statistical analyses were performed by 1-way analysis of variance followed by the least significant differences multiple-comparison test. Statistical significance was set at $P < .05$.

## Results

### Odontogenic Differentiation of hDPCs

The third passage of hDPCs showed fusiform or polygon-sharped monoplast before adding odontogenic medium (Fig. 1*A*). Mineralized matrix deposition, which was positive to von Kossa staining (Fig .1*B*) and alizarin red S staining (Fig. 1*C* and *D*), was evident after 14 days of odontogenic induction. Moreover, the expressions of odontoblast marker genes including ALP, dentin sialophosphoprotein, OCN, and bone sialoprotein in differentiated hDPCs were increased after