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miR-675 promotes odontogenic differentiation of human dental pulp cells by epigenetic regulation of DLX3

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ABSTRACT

In a previous study, we showed that microRNA-675 (miR-675) was significantly down-regulated in patients with tricho-dento-osseous (TDO) syndrome. One of the main features of TDO syndrome is dentin hypoplasia. Thus, we hypothesize that miR-675 plays a role in dentin development. In this study, we determined the role of miR-675 in the odontogenic differentiation of human dental pulp cells (hDPCs). Stable overexpression and knockdown of miR-675 in hDPCs were performed using recombinant lentiviruses containing U6 promoter-driven miR-675 and short hairpin-miR675 expression cassettes, respectively. Alkaline phosphatase (ALP) assay, Alizarin red staining assay, quantitative polymerase chain reaction (qPCR), Western blot analysis, and immunofluorescent staining revealed the promotive effects of miR-675 on the odontogenic differentiation of hDPCs. Further, we found that miR-675 facilitates the odontogenic differentiation process of hDPCs by epigenetic regulation of distal-less homeobox (DLX3). Thus, for the first time, we determined that miR-675 regulates the odontogenic differentiation of hDPCs by inhibiting the DNA methyltransferase 3 beta (DNMT3B)-mediated methylation of DLX3. Our findings uncover an unanticipated regulatory role for miR-675 in the odontogenic differentiation of hDPCs by epigenetic changes in DLX3 and provide novel insight into dentin hypoplasia feature in TDO patients.

1. Introduction

The homeodomain transcription factor distal-less (Dlx) family plays essential roles in vertebrate development [1,2]. Distal-less homeobox 3 (DLX3), a member of Dlx the family, is mapped to chromosome 17q21 and plays essential roles in development [3,4]. Previous studies have shown that DLX3 is required for hair follicle differentiation, bone skeletal formation and development, and tooth development [5]. DLX3 mutation is associated with tricho-dento-osseous syndrome (TDO; OMIM 190320) , a rare autosomal-dominant disorder [6]. One of the main clinical features of TDO syndrome is dentin hypoplasia, which suggests that mutations in DLX3 have abnormal effects on dentin development [6,7]. Interestingly, our research group recently reported a novel *de novo* missense mutation (c.533 A > G; Q178R) in the homeodomain of DLX3 [6]. However, the mechanism by which this novel mutant DLX3 results in dentin hypoplasia remains unknown.

Human dental pulp cells (hDPCs) are composed of a heterogeneous cell population that contains neural crest-derived mesenchymal stem cells with multi-differentiation potential [8,9]. Research on hDPCs has become one of the fastest-growing areas due to their multi-differentiation potential and optimistic therapeutic implications in regenerative medicine [10]. Their multi-differentiation potential allows hDPCs to play an essential role in dentin formation [11]. In TDO syndrome, affected individuals manifest a dentin defect, which indicates that this novel mutant DLX3 is responsible for imperfect dentinogenesis and plays a differential role in disrupting dentin development [5]. While the effects of this novel mutant DLX3 are known (impaired dentinogenesis), its precise regulatory mechanism in hDPCs differentiation remains to be elucidated.

MicroRNAs (miRNAs) are noncoding RNAs approximately 22 nucleotides in length that serve as important regulatory transcripts in cell differentiation [12,13]. Growing evidence suggests that miRNAs play important roles in human disorders [14,15], and previous studies have estimated that more than 30% of human genes are potentially under the control of miRNAs [16,17]. In addition, one class of miRNAs is considered as a positive or negative regulator in hDPCs differentiation

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[18,19], which indicates an important role for miRNAs in the odontogenic differentiation of hDPCs. Thus, we investigated whether miRNAs are involved in hDPCs differentiation in TDO syndrome.

The expression profiles of miRNAs were obtained by comparing hDPCs with novel DLX3 mutation (MU-hDPCs) and wild-type hDPCs (WT-hDPCs). miR-675 was significantly down-regulated in MU-hDPCs compared to WT-hDPCs. miR-675 promotes the growth of hepatocellular carcinoma cells by interacting with the Cdc25A signaling pathway, which indicates that miR-675 plays a critical role in proliferation [20]. Further, miR-675 promotes human bone marrow mesenchymal cells differentiation by associating with TGF-β1/Smad3/HDAC signaling pathway, which indicates a vital role for miR-675 in osteogenic differentiation [21]. However, the function and underlying mechanism of miR-675 in the odontogenic differentiation of hDPCs require further investigation. Therefore, we investigated the function of miR-675 in the odontogenic differentiation of hDPCs and explored the underlying mechanism.

In this report, we describe miR-675 as a mediator in the odontogenic differentiation of hDPCs. First, we obtained the expression profile of miR-675 by qPCR. Then, we overexpressed and knock down of miR-675 in hDPCs and explored its function in odontogenic differentiation of hDPCs. Finally, functional analysis revealed that miR-675 facilitates odontogenic differentiation by epigenetic regulation of DLX3 expression, which sheds light on the regulatory mechanism of dentin hypoplasia in TDO patients.

2. Materials and methods

2.1. Cell culture

The entire study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology. All individuals who participated in this study provided informed consent. Human dental pulp tissues were obtained from healthy premolars going through tooth extraction due to orthodontic treatment. hDPCs isolation was performed according to the informed protocol [22]. Primary isolated hDPCs were cultured in 100 mm dishes with alpha minimum essential medium (Gibco, Grand Islang, NY, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Islang, NY, USA), and 1% penicillin-streptomycin. Cells from the third to sixth passages were used in subsequent trials. For odontogenic differentiation, cells were cultured in odontogenic medium (OM) containing 50 mg/mL ascorbic acid, 100 nmol/L dexamethasone, and 10 mmol/L β-glycerolphosphate (Sigma, St Louis, MO, USA).

2.2. Lentivirus generation and establishment of stably infected cells

For generation of human miR-675 overexpression and knockdown lentiviruses, the human miR-675 sequence was subcloned into the BamH I and EcoR I sites of the pGLV-U6-RFP-T2A-Puro vector, and a short hairpin RNA (shRNA) specific to human miR-675 was subcloned into the BamH I and EcoR I sites of the pGLV-U6-RFP-T2A-Puro vector, thus generating lentiviruses with overexpression of miR-675 (miR-675) or knockdown of miR675 (sh-miR675) expression cassettes. A lentivirus containing an empty vector with no target gene was used as a negative control (miR-NC) in the following experiments. All lentiviruses were obtained from Hanheng Chem Technology, Shanghai, China.

For stable overexpression and knockdown of miR-675 in hDPCs, 100 μL lentivirus mixed with 10 μg/mL polybrene (Sigma, St Louis, MO, USA) were used to infect hDPCs (8×10^5 cells in 100-mm dishes) for 24 h. The medium was changed after 24 h and the infected cells were screened in the presence of 1 μg/mL puromycin (Sigma, St Louis, MO, USA) for an additional 3 days. Selected cells with overexpression or knockdown of miR-675 or the empty vector were used as stably infected hDPCs in subsequent experiments.

2.3. Alkaline phosphatase (ALP) staining and activity analysis

After culture in OM for 3, 7 and 14 days, the ALP staining was performed according to the instructions provided in the NBT/BCIP staining kit (Cwbiotech, Beijing, China). In brief, cells from each group were rinsed three times with phosphate-buffered saline (PBS) and fixed in ice-cold 95% ethanol for 30 min at room temperature, washed three times with Millipore-filtered water, and stained with NBT/BCIP solution following the manufacturer's instructions.

ALP activity was determined using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's protocol. Briefly, cells from each group were rinsed three times with PBS, solubilized in 1% Triton X-100, followed by high speed centrifugation for 30 min. The supernatants were collected and analyzed at 405 nm on a spectrophotometer (PerkinElmer, Waltham Mass, MA, USA). ALP activity was normalized to the total protein content.

2.4. Alizarin red staining and quantification of calcification

After 21 days of induction, cells from each group were rinsed three times with PBS, fixed in 4% paraformaldehyde solution for 30 min, and washed three times with distilled water. Then the cells were incubated with 2% Alizarin red (Sigma, St Louis, MO, USA) for 20 min, the dye was removed and the cells were washed with deionized water for three to five times. To quantify the degree of calcification, stained samples were eluted with 100 mM cetylpyridinium chloride (Sigma, St Louis, MO, USA) for 1 h and the optical density of the supernatants at 562 nm was analyzed. Alizarin red intensity was normalized to total protein content of the assayed samples.

2.5. Quantitative real-time polymerase chain reaction (qPCR)

Total RNA from each group of cells under OM induction for 7 days was extracted using Trizol reagent (Life Technologies, Carlsbad, CA, USA) according to manufacturer's procedure. Next, 2 μg of RNA was reverse-transcribed into cDNA using the Superscript First-Strand Synthesis System (Life Technologies, Carlsbad, CA, USA). qPCR was performed on an ABI Prism 7500 Real-Time PCR System (Life Technologies, Carlsbad, CA, USA). Relative mRNA expression was analyzed using the $2^{-\Delta\Delta Ct}$ relative expression method. The sequences of each primer are listed in Table 1.

2.6. Western blot analysis

hDPCs induced for 7 days were lysed in RIPA buffer containing protease inhibitors. An equal amount of total protein was loaded onto

Table 1
Primer sequences used in qPCR.

Gene	Sequences (5′ – 3′)
miR-675	Forward: GTGCTGGTGCGGAGAGG
	Reverse: GTGCAGGGTCCGAGGT
DSPP	Forward: GAGCCACAAACAGAAGCAACAC
	Reverse: TTGGACAACAGCGACATCCTCA
DMP-1	Forward: ACCAGGCACTATGCTAGGTGTT
	Reverse: CTTTGTGGGTCCTTCTATACGC
ALP	Forward: ATGGGATGGGTGTCTCCACA
	Reverse: CCACGAAGGGGAACTTGTC
Nes	Forward: GCCCTGACCACTCCAGTTTA
	Reverse: GGAGTCCTGGATTTCCTTCC
DLX5	Forward: CTCGCTCAGCCACCACCCTCAT
	Reverse: AGTTGAGGTCATAGATTTCAAGGCAC
U6	Forward: CTCGCTTCGGCAGCACA
	Reverse: AACGCTTCACGAATTTGCGT
GAPDH	Forward: GGTCACCAGGGCTGCTTTTA
	Reverse: GGATCTCGCTCCTGGAAGATG