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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

12% sodium dodecyl sulfate polyacrylamide gels and then separated by electrophoresis. Subsequently, the separated proteins were transferred to polyvinylidene difluoride membranes. After blocking, the membranes were incubated with primary antibodies against dentin sialophosphoprotein (DSPP), DNMT3B (Santa Cruz Biotechnology, Santa Cruz, CA, USA), dentin matrix acidic phosphoprotein 1 (DMP-1), DLX3 (Abcam, Cambridge, UK), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH, Proteintech, Chicago, IL, USA) overnight at 4 °C, followed by incubation with anti-rabbit or anti-mouse secondary antibodies. The intensities of the immunoreactive bands obtained by western blot were quantified using Fusion-Capt software (Vilber Lourmat, Paris, France). The relative target band intensity was normalized to that of GAPDH.

2.7. Immunofluorescent staining

At the indicated times, cells grown on sterile glass coverslips were rinsed three times with PBS, fixed in 4% paraformaldehyde for 20 min, treated with 0.1% Triton X-100 for 10 min, and then washed three times with PBS. Thereafter, cells were incubated with the appropriate primary antibody diluted in bovine serum albumin at 4 °C overnight and then incubated with the specified second antibody for 1 h at room temperature in the dark. Cell nuclei were stained with 4′, 6′-diamidino-2-phenylindole (DAPI; Sigma, St Louis, MO, USA), and coverslips were mounted onto a glass slide. Images were obtained with an LSM 5 EXCITER confocal imaging system (Carl Zeiss, Jena, Germany).

2.8. Luciferase assay

The miR-675 mimic oligonucleotide (Mimic) and the corresponding negative control (NC) were obtained from GeneCopoeia (GeneCopoeia Inc, Guangzhou, China). Predicted miR-675 target genes and target binding sites were investigated using TargetScan (http://www.targetscan.org) and miRDB (www.mirdb.org). The reporter vector which contained the promoter sequence of the DLX3 gene was also constructed by GeneCopoeia. Luciferase assays were performed according to the manufacturer's instructions. Briefly, HEK-293T cells were cultured in six-well plates at a density of 1.5×10^5/well. At the indicated times, HEK-293T cells were co-transfected with the reporter vector and the miR-675 mimic or NC oligonucleotide using lipofectamine 3000 (Life Technologies, Grand Island, NY, USA). After transfection for 24 h, cell lysates were collected and measured using a Luc-Pair Duo-Luciferase Assay Kit 2.0 (GeneCopoeia Inc, Guangzhou, China). The light intensity from Renilla luciferase was normalized to that of firefly luciferase.

2.9. Quantitative methylation analysis

Quantitative methylation analyses of the DLX3 promoter were performed using the Sequenom MassARRAY platform (CapitalBio, Beijing, China). Genomic DNA was extracted using a Universal Genomic DNA kit (Cwbiotech, Beijing, China) according to the manufacturer's instructions. A 1 μg DNA sample was converted with sodium bisulfite and the modified DNA was amplified by PCR. Target regions were amplified using the primers listed in Table 2. Methylation levels were analyzed using Epityper software version 1.0 (Sequenom) to produce quantitative results for each CpG site or a cluster of multiple CpG sites.

2.10. DNMT3B activity assay

DNMT3B activity was quantified and analyzed using the DNMT3B Activity Assay Core kit (Epigentek, Farmingdale, NY, USA). Briefly, cells were washed with cold PBS and lysed in RIPA buffer containing protease inhibitors. The lysate was disrupted by ultra-sonication and centrifuged at 12,000*g* at 4 °C for 30 min. Immediately following centrifugation, 5 μL of the supernatant was collected and used to measure DNMT3B activity according to the manufacturer's instructions. Assays were performed in triplicate, and DNMT3B activity was calculated following the manufacturer's instructions.

2.11. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using a Chromatin Immunoprecipitation Assay Kit (Millipore, Billerica, MA, USA) following the manufacturer's instructions. Briefly, DNA-protein complexes were crosslinked in 1% formaldehyde at room temperature for 10 min and then stopped by adding glycine. Genomic DNA-protein complexes were immunoprecipitated using ChIP-grade anti-DNMT3B antibodies (or normal mouse IgG as a negative control). The levels of precipitated DNA were determined by qPCR (primer sequences were shown in Table 3). Relative enrichment of the indicated DNA regions was calculated according to the manufacturer's instructions.

2.12. Statistical analysis

Data were analyzed by one-way analysis of variance using the SPSS version 13.0 software package (SPSS, Chicago, IL, USA). Results are expressed as the mean ± standard deviation from at least three independent trials and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Expression profile of miR-675 during the odontogenic differentiation of hDPCs

To investigate the function of miR-675 in the odontogenic differentiation of hDPCs, we examined the dynamic expression profile of miR-675 in hDPCs after induction. qPCR indicated that miR-675 expression was significantly up-regulated during odontogenic differentiation (Fig. 1A). Meanwhile, odontogenic markers DSPP and DMP-1 were also significantly up-regulated during odontogenic differentiation (Fig. 1 B, C). Western blot analyses further confirmed the expression levels of DSPP and DMP-1 (Fig. 1D). These results suggest that the expression patterns of miR-675 and odontogenic markers are identical and indicate that miR-675 plays an important role in the odontogenic differentiation of hDPCs.

3.2. miR-675 promotes ALP activity and the mineralization ability of hDPCs

A lentivirus was used to overexpress or knockdown of miR-675 in hDPCs, and stably expressing cells were named miR-NC, miR-675, or sh-miR675 groups, respectively. The efficiency of lentivirus infection was more than 90% (Supplementary S1A), and qPCR analyses showed more than five-fold expression in the group overexpressing miR-675 and a ~ 60% decrease in the miR-675 knockdown group compared to

Table 2
Primer sequences used in quantitative methylation sequencing.

Gene	Sequences (5′ – 3′)
DLX3	Left: GTATGGTAGTTAAGGGAGTTGGAGA Right: AAAAAACAAAACTTACAACCAATCAA

Table 3
Primer sequences used in CHIP.

Gene	Sequences (5′ – 3′)
DLX3	Forward: ACGGTGAACCCCTACACCTA Reverse: ATTCCGACTTGGGCGAGTAA

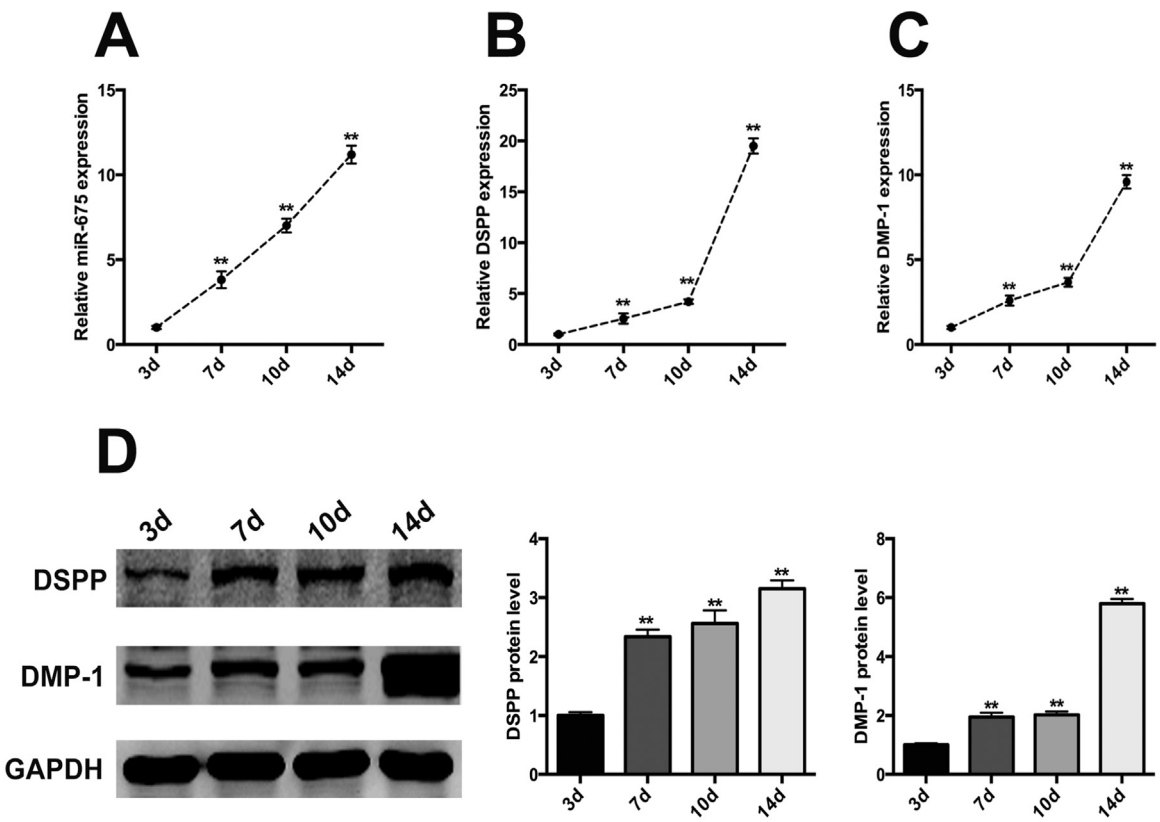

Fig. 1. Dynamic expression profile of miR-675 and odontogenesis-related genes during odontogenic differentiation of hDPCs. **A. B. C.** mRNA expression levels of miR-675, DSPP and DMP-1 on days 3, 7, 10 and 14. **D.** Protein expression levels of DSPP and DMP-1 on days 3, 7, 10 and 14. GAPDH was used as an internal control. Significant differences versus the miR-NC group, *p < 0.05, ** p < 0.01.

the miR-NC group (Supplementary S1B). Along with elevated miR-675 levels, the expression and activity of ALP were significantly up-regulated, while knockdown of miR-675 significantly decreased ALP expression and activity (Fig. 2A, B). The formation of calcified nodules was assessed by Alizarin red staining. Obvious calcified nodules were observed in the miR-675 group, while knockdown of miR-675 dramatically weakened the formation of calcified nodules (Fig. 2C). Quantification analyses of mineralized nodules were in accordance with the staining results (Fig. 2D).

3.3. miR-675 induces odontogenic differentiation of hDPCs

To identify the functional role of miR-675 in the odontogenic differentiation of hDPCs, the expression of odontogenesis-related genes was assessed. qPCR results showed that miR-675 significantly induced the expression of odontogenesis-related genes, including DSPP, DMP-1, ALP, Nestin (Nes), and DLX5, while knockdown of miR-675 robustly down-regulated the expression of those odontogenic genes (Fig. 3A). Western blot analyses and immunofluorescence staining further revealed that miR-675 dramatically increased the expression of the odontogenesis-related proteins DSPP and DMP-1 (Fig. 3B, C). These data clearly illustrate that miR-675 promotes the odontogenic differentiation of hDPCs.

3.4. miR-675 facilitates the odontogenic differentiation of hDPCs by epigenetic regulation of DLX3

As bioinformatics analyses predicted that the 3′-untranslated region (3′-UTR) of DLX3 contains several putative miR-675 binding sites (Fig. 4A), we subcloned these target sites into a luciferase reporter vector. Luciferase reporter assays revealed no significant difference between the Mimic and NC groups (Fig. 4B). However, western blot analyses showed that the expression level of DLX3 in the miR-675 group was significantly up-regulated and the opposite effect was observed in the sh-miR675 group (Fig. 4C). These data suggest that miR-675 cannot directly target the 3′-UTR of DLX3, but promotes the expression of DLX3, which indicates that miR-675 promotes the odontogenic differentiation of hDPCs via DLX3.

To further explore the mechanistic role of miR-675 in the regulation of DLX3, and a previous study showed that the precursor of miR-675 alters genome wide DNMT3B-mediated DNA methylation [23]. Therefore, we investigated whether miR-675 promotes DLX3 expression by regulating the methylation of DLX3. To test this possibility, we used quantitative methylation analyses to measure the methylation level of DLX3. These analyses revealed a low level of methylation in the DLX3 promoter in the miR-675 group compared to the shmiR-675 group, which indicates that hypo-methylation of DLX3 in the miR-675 group corresponds to the promoted expression of DLX3 (Fig. 4D). To further address the regulatory mechanism of miR-675 in DLX3 methylation. Western blot analyses, ChIP assays, and DNMT3B activity analyses were performed. Western blot revealed no changes at the total protein level of DNMT3B (Fig. 4E), DNMT3B ChIP analyses also showed no changes in DNMT3B occupancy at the promoter region of DLX3 (Fig. 4F). However, DNMT3B activity was significantly down-regulated in the miR-675 group and markedly up-regulated in the sh-miR675 group (Fig. 4G). Thus, the miR-675-induced decreased methylation in the DLX3 promoter region was due to the decrease in DNMT3B activity rather than the total expression of DNMT3B or occupancy of DNMT3B at the promoter region of DLX3. These results strongly suggest that miR-675 regulates the expression of DLX3 by changing the methylation level at the DLX3 promoter region rather than by direct interaction between miR-675 and the 3′-UTR of DLX3. Taken together, these data indicate that miR-675 promotes the odontogenic differentiation of hDPCs by epigenetic regulation of DLX3.

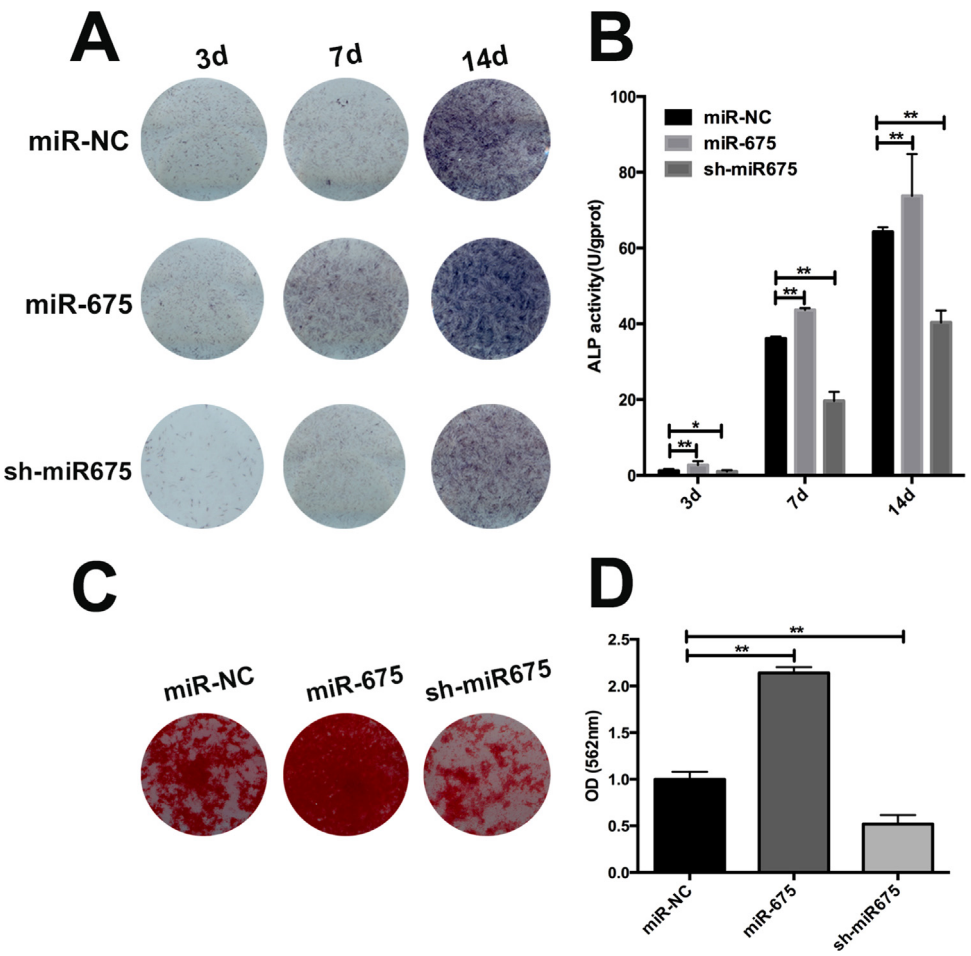

Fig. 2. Effects of miR-675 on ALP expression, activity and the mineralization ability of hDPCs. **A. B.** ALP staining and activity in the miR-NC, miR-675 and sh-miR675 groups on days 3, 7 and 14. **C. D.** Calcified nodule formation of hDPCs was analyzed by Alizarin red staining on day 21, and quantification analyses of Alizarin red were performed. Significant differences versus the miR-NC group, *p < 0.05, ** p < 0.01.

4. Discussion

TDO syndrome is a rare autosomal-dominant disorder with a dominant inheritance mode. The most common manifestations of TDO are related to hair, bone and teeth dysplasia including kinky hair at birth, thickened bones, enamel hypoplasia, and dentin hypoplasia [6]. Mutations in the DLX3 gene are responsible for TDO syndrome. As a member of the Dlx family, DLX3 is a key regulator in dentin development and mineralization. Functional studies have revealed that DLX3 promotes the odontogenic differentiation of hDPCs [24]. Further, in vivo experiments have shown that neural crest deletion of DLX3 in Wnt1-cre mice causes dentin defects, owing to a direct inhibition of interactions between DLX3 and the odontogenic essential marker DSPP [25]. These results suggest that DLX3 is involved in dentinogenesis by regulating target gene expression.

Dentinogenesis is a sophisticated process that involves numerous factors [26,27]. Multiple miRNAs are related to tooth development. Thus, an interesting concept emerges: miRNAs perform their role in tooth development by coordinating with complex signaling networks. Sharp et al. found that miR-200a specifies the fate of dental epithelial cells by repressing β-catenin and identified *Zeb* as a target gene of miR-200a in tooth morphology [28]. Sun et al. reported that crosstalk between miR-34a and the Notch signaling pathway promotes the odontogenic and osteogenic differentiation of stem cells from the apical papilla [29]. Further, Liu et al. [19] demonstrated that miR-145 and miR-143 negatively regulate odontogenic differentiation and dentin formation by interacting with the KLF4 and OSX signaling pathways. Given the clinical features of dentin hypoplasia manifested in TDO patients and the potential role for miRNAs in odontogenic differentiation, we explored the role of miR-675, which was significantly downregulated in this novel mutant DLX3 group, in the odontogenic differentiation process of hDPCs.

Differentiation from hDPCs to functional odontoblasts is a complex process involving numerous genes and transcriptional factors. To explore the role of miR-675 in the odontogenic differentiation of hDPCs, we selected DSPP, DMP-1, ALP, Nes, and DLX5 as odontogenic differentiation markers and examined the formation of calcified nodules. DSPP is a highly phosphorylated protein that modulates dentin mineralization; DMP-1 is a non-collagenous protein that functions as a regulatory molecule in the mineralization process [30]. The role of DMP-1 in dentinogenesis is achieved by its interaction with DSPP; therefore, both DSPP and DMP-1 are essential for dentin formation [31]. ALP plays a crucial role in the early stages of odontogenic differentiation and mineralization [32]. Nes is only expressed in functional odontoblasts and may play a role in odontogenic differentiation during dentinogenesis [33]. The transcription factor DLX5 plays a critical role in odontogenic differentiation, and calcified nodules are a marker of late stages for odontogenic differentiation [9]. In this study, we revealed that miR-675 significantly up-regulates the expression levels of odontogenic-related genes and increases the formation of calcified nodules, which suggests that miR-675 has a positive effect on the odontogenic differentiation of hDPCs. Further, we verified that miR-675 promotes the odontogenic differentiation of hDPCs via DLX3. Previous studies have proven that DLX3 is an essential factor in dentinogenesis

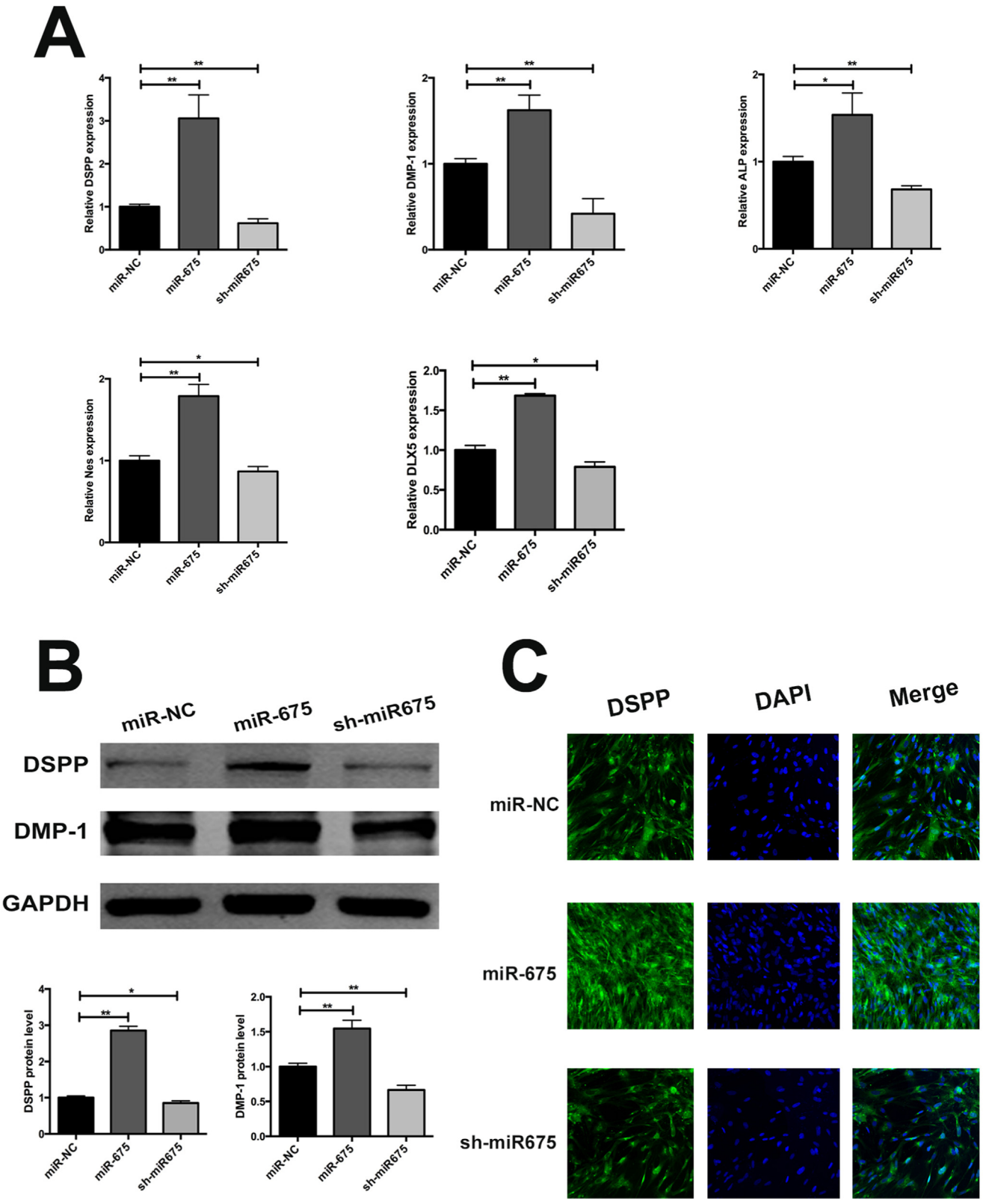

Fig. 3. Role of miR-675 in the odontogenic differentiation of hDPCs. **A. B.** mRNA levels of DSPP, DMP-1, ALP, Nes, and DLX5 were detected in miR-NC, miR-675 and sh-miR675 groups. DSPP and DMP-1 protein levels were measured using western blot. **C.** DSPP protein expression in the miR-NC, miR-675 and sh-miR675 groups after induction to odontogenic lineage was analyzed by immunofluorescent staining. GAPDH was used as an internal control. Significant differences versus the miR-NC group, *$p < 0.05$, ** $p < 0.01$.

and, in this study, we revealed that miR-675 regulates DLX3 expression by altering the DLX3 promoter methylation level. Therefore, we are the first to report that miR-675 positively regulates the odonotgenic differentiation of hDPCs by targeting DLX3.

To the best of our knowledge, this is the first report to demonstrate that miR-675 promotes the odontogenic differentiation of hDPCs via

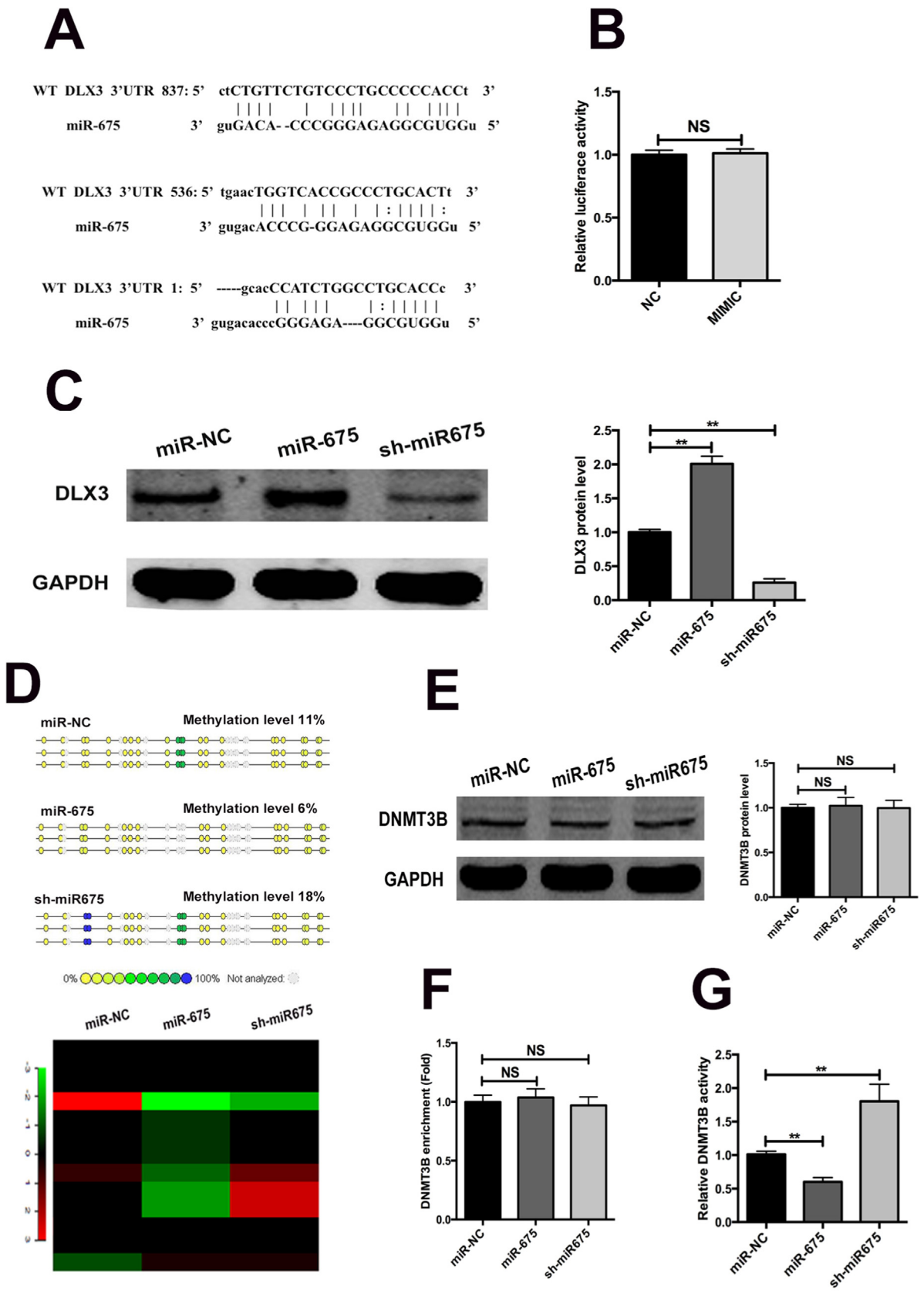

Fig. 4. miR-675 promotes the odontogenic differentiation of hDPCs by the epigenetic regulation of DLX3. **A.** Putative binding sites between miR-675 and 3′-UTR of DLX3. **B.** Relative luciferase activity was measured using the luciferase reporter assay. **C.E.** DLX3 and DNMT3B protein expression in the miR-NC, miR-675, and sh-miR675 groups was determined by western blot. **D.** Methylation levels and heat-map of the DLX3 promoter region in the miR-NC, miR-675, and sh-miR675 groups. **F.** DNMT3B ChIP assays at the promoter region of DLX3. **G.** DNMT3B activities in different groups. NS indicates not significant. Significant differences versus the miR-NC group, *$p < 0.05$, ** $p < 0.01$.

the epigenetic regulation of DLX3, which extends our knowledge on dentin hypoplasia in TDO syndrome.

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Conflict of interest

The authors declare that they have no conflict of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.yexcr.2018.03.035.

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