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DLX3 epigenetically regulates odontoblastic differentiation of hDPCs through H19/miR-675 axis

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ABSTRACT

Objectives: A novel mutation (c.533 A > G; Q178R) in DLX3 gene is responsible for Tricho-Dento-Osseous (TDO) syndrome. As one of features of TDO syndrome is dentin hypoplasia, we explored the mechanism regarding dentin defects in TDO syndrome.

Design: hDPCs were obtained from the healthy premolars, stably expressing hDPCs were generated using recombinant lentiviruses. Quantitative methylation analysis, DNMT3B activity, CHIP, and evaluation of odonto-differentiation ability of hDPCs assays were performed.

Results: Novel mutant DLX3 (MU-DLX3) significantly inhibited the expression of long non-coding RNA H19 and resulted in hyper-methylation of H19 in MU group, rescue studies showed that up-regulation the expression of H19 and demethylation of H19 in MU group were able to rescue the effect of MU-DLX3. Subsequently, miR-675, encoded by H19, was also able to rescue the above effects of MU-DLX3. Thus, we proposed that MU-DLX3 regulated odontoblastic differentiation of hDPCs through H19/miR-675 axis. Through CHIP and DNMT3B activity assays disclosed the underlying mechanism by which MU-DLX3 altered H19 expression and methylation status in MU group by increasing H3K9me3 enrichment and DNMT3B activity.

Conclusions: Our new findings, for the first time, suggest that MU-DLX3 significantly inhibits hDPCs differentiation via H19/miR-675 axis and provides a new mechanism insight into how MU-DLX3 epigenetically alters H19 methylation status and expression contributes to dentin hypoplasia in TDO syndrome.

1. Introduction

Distal-less (Dlx) family belongs to the homeodomain protein family and has a vital role in vertebrate development (Duverger & Morasso, 2008; Hassan et al., 2004). Distal-less homeobox 3 (DLX3), a member of Dlx family, plays a crucial role in epithelium, hair, bone, tooth and placental development (Merlo et al., 2000; Morasso, Markova, & Sargent, 1996; Morasso, Grinberg, Robinson, Sargent, & Mahon, 1999). Previous studies have shown that DLX3 has pivotal functions in mineral matrix deposition and biomineralization which is closely associated with bone and tooth development (Choi et al., 2008; Duverger et al., 2012; Hwang, Mehrani, Millar, & Morasso, 2008). DLX3 mutations cause a rare autosomal-dominant disease named Tricho-Dento-Osseous syndrome (TDO; OMIM190320) (Lee et al., 2008; Wright et al., 2008). The main clinical features of TDO are curly hair, an increased thickness and density of bone, enamel hypoplasia and hypomaturation, dentin hypoplasia and taurodontism (Li et al., 2015; Nieminen et al., 2011; Price, Wright, Kula, Bowden, & Hart, 1998). However, the underlying mechanism is not fully elucidated. Interestingly, our research group lately reported a novel *de novo* missense mutation (c.533 A > G; Q178R) in the homeodomain of DLX3 (Li et al., 2015). As this TDO patient (c.533 A > G; Q178R) has dentin hypoplasia symptom, and our group has identified that this novel mutant DLX3 (MU-DLX3) significantly inhibited odontoblastic differentiation of hDPCs (Zeng et al., 2017). The results reconfirm that DLX3 is required for normal human dental pulp cells (hDPCs) differentiation and dentin deposition. However, its mechanism regarding the novel DLX3 mutation in odontoblastic differentiation of hDPCs was still unknown.

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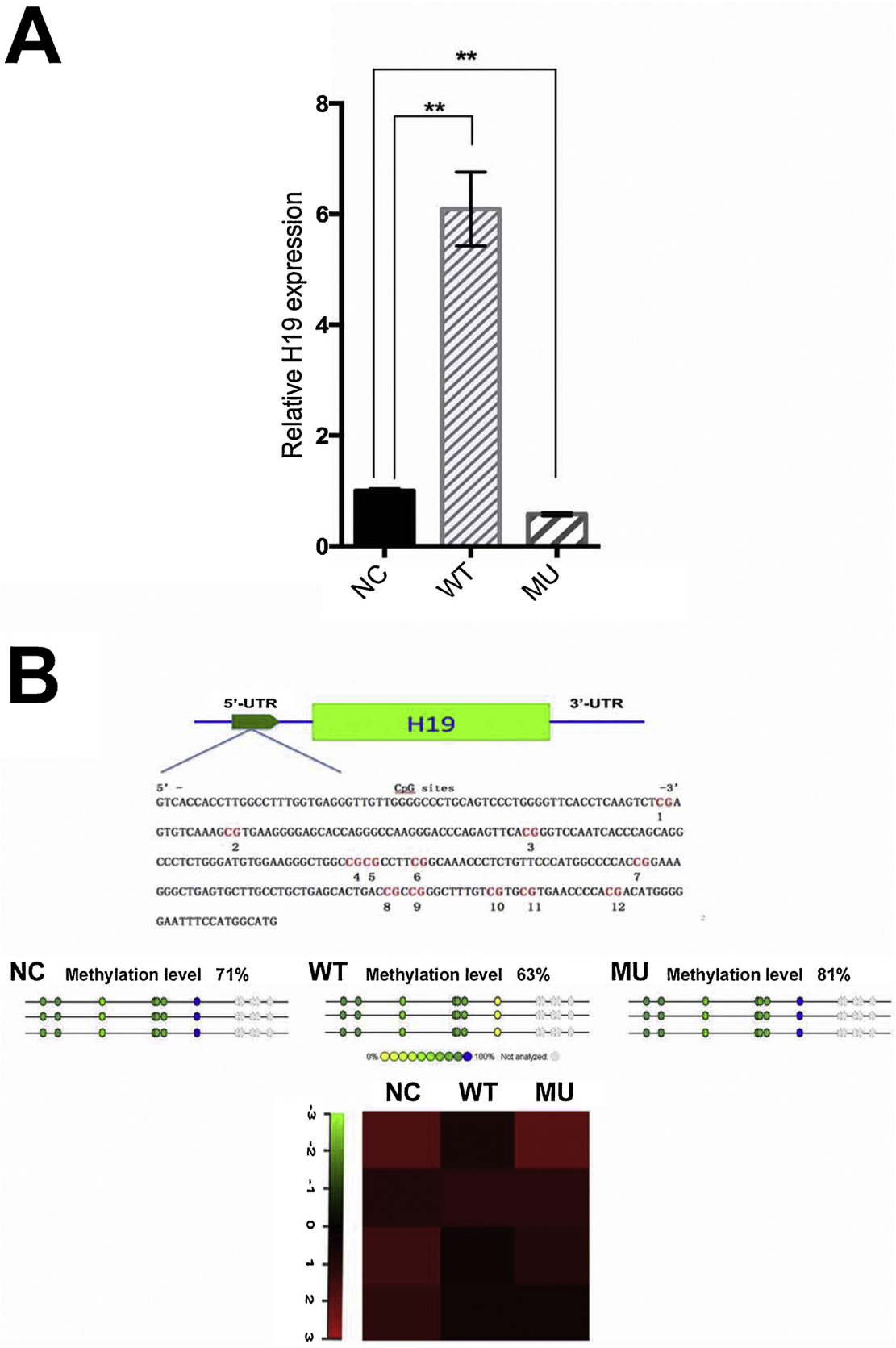

Fig. 1. LncRNA H19 is hypo-expressed and hyper-methylated in MU-hDPCs. (A) Expression level of H19 gene in NC, WT and MU groups. (B) Quantitative methylation level of H19 gene promoter region. The highlights of CpG islands, methylation level and heatmap were shown in H19 gene promoter region.

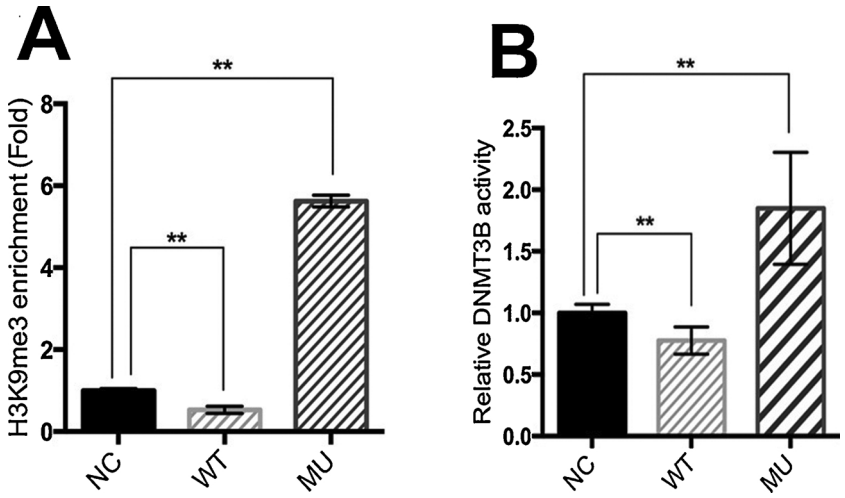

Fig. 2. MU-DLX3 alters H19 expression by increasing the enrichment of H3K9me3 and DNMT3B activity. (A) H3K9me3 CHIP assays at H19 gene promoter region in NC, WT and MU groups. (B) DNMT3B activity was measured in NC, WT and MU groups.

Increasing evidence suggests that long non-coding RNA (lncRNA) H19 plays a crucial role in embryo development, cell differentiation and is closely associated with development-related diseases (Gabory, Jammes, & Dandolo, 2010; Huang, Zheng, Jia, & Li, 2015). H19 is a highly conserved imprinted gene and encodes a ~2.6 kb polyadenylated lncRNA and exerts a variety of functional activities both in the nucleus and in the cytoplasm (Gabory et al., 2010). H19 transgenic mice showed that H19 is involved in the control of growth and survival of embryos (Gabory et al., 2009). H19 also encodes miR-675 that regulates osteoblastic differentiation of mesenchymal stem cells, promotes skeletal muscle regeneration, and enhances carcinogenesis and metastasis (Dey, Pfeifer, & Dutta, 2014; Huang et al., 2015; Li et al., 2014). In addition, altered methylation of H19 are associated with calcific aortic valve disease by silencing Notch1 pathway (Hadji et al., 2016). Further, H19 gene contains deferentially methylated regions (DMRs) that are associated with epigenetic remodeling, and epigenetic demethylation of DNA by 5-Aza-2′ deoxycytidine (5-Aza-CdR) can regulate the expression of H19 (Ishihara, Oshimura, & Nakao, 2006; Ito, Nativio, & Murrell, 2013). Thus, considering the complex function of H19 and the epigenetic regulation of H19 in different disorders, we wondered whether this kind of epigenetic regulation of H19 was involved in TDO syndrome.

Gene methylation is mediated by three DNA methyltransferases (DNMT1, DNMT3 A and DNMT3B), and altered gene methylation causes developmental abnormalities and diseases (Rahmani et al., 2017; Wang, Abu-Amer, O'Keefe, & Shen, 2018; Wu & Santi, 1985). Gene expression is regulated by DNMTs activity and histone modifications cooperatively. Therefore, epigenetics is not only determined by DNMTs activity-mediated gene methylation, but also by histone modifications (Jin et al., 2012). Lysine residues in histone H3, one of histone modifications, is commonly modified by methylation. Tri-methylation of lysines 9 (H3K9me3) and 27 (H3K27me3) of histone H3 are thought to restrict gene expression, while tri-methylation of lysines 4 (H3K4me3), 36 (H3K36me3) and 79 (H3K79me3) of histone H3 activates gene transcription (Lu et al., 2009). In addition, previous studies showed that human lung cancers related gene Mdig could enhance H19 expression by reducing the enrichment of H3K9me3 in the promoter of H19 (Chen et al., 2013), which indicated that H19 expression is under the control of H3K9me3 status. Therefore, we deduced that epigenetic histone modifications would be involved in TDO syndrome.

In this study, we explored the underlying regulatory role of MU-DLX3 in odontoblastic differentiation, and our new findings, for the first time, provide an explanation for dentin hypoplasia phenotype in TDO syndrome from a new insight.

2. Materials and methods

2.1. Cell culture and treatment

This study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (PKUSSIRB-201736082) and followed the Helsinki guidelines. The informed consent was obtained from all participants. Dental pulp tissues were collected from impacted premolars and isolated hDPCs were maintained in a humidified atmosphere with 95% air and 5% CO_2 at 37°C in growth medium (GM) composed of α-MEM supplemented with 10% fetal bovine serum (Gibco, Paisley, UK). For odontoblastic differentiation, cells were cultured in odontoblastic medium (OM), which contains 50 mg/mL ascorbic acid, 100 nmol/L dexamethasone, and 10 mmol/L β-glycerolphosphate (Sigma, St Louis, MO, USA) in GM medium. For DNA demethylation, hDPCs were treated with 10 μmol/L 5-Aza-CdR (Sigma) for 72 h before odontoblastic differentiation.

2.2. Lentivirus infection and establishment of stably infected hDPCs

Recombinant lentiviruses carrying wild-type DLX3 (WT), novel MU-DLX3 (MU) and the control (NC), recombinant lentiviruses containing H19 (H19) and miR-675 (miR-675) expression cassette were obtained from GeneChem company (GeneChem, Co.,Ltd. Shanghai, China). The lentiviruses were used to infect hDPCs and establish stably expressing hDPCs. Infection of the hDPCs was performed by exposing hDPCs to lentiviruses mixed with polybrene (5 μg/mL) for 12 h. The medium was changed at 12 h after infection, and the infected cells were cultured in the presence of 1 μg/mL puromycin to establish stable infected hDPCs. After idenfication of the targeted gene expression, the screened cells were used as the stably infected hDPCs in subsequent experiments. Hereafter, wild type and mutant DLX3 stably infected hDPCs are named WT-hDPCs and MU-hDPCs, respectively.

2.3. Alkaline phosphatase (ALP) staining and quantification of ALP activity

hDPCs were cultured in 12-well plates and induced by OM. At the indicated time points, ALP staining was performed based on the protocol provided in the NBT/BCIP staining kit (Cwbiotech, Beijing, China). ALP activity was measured using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction.

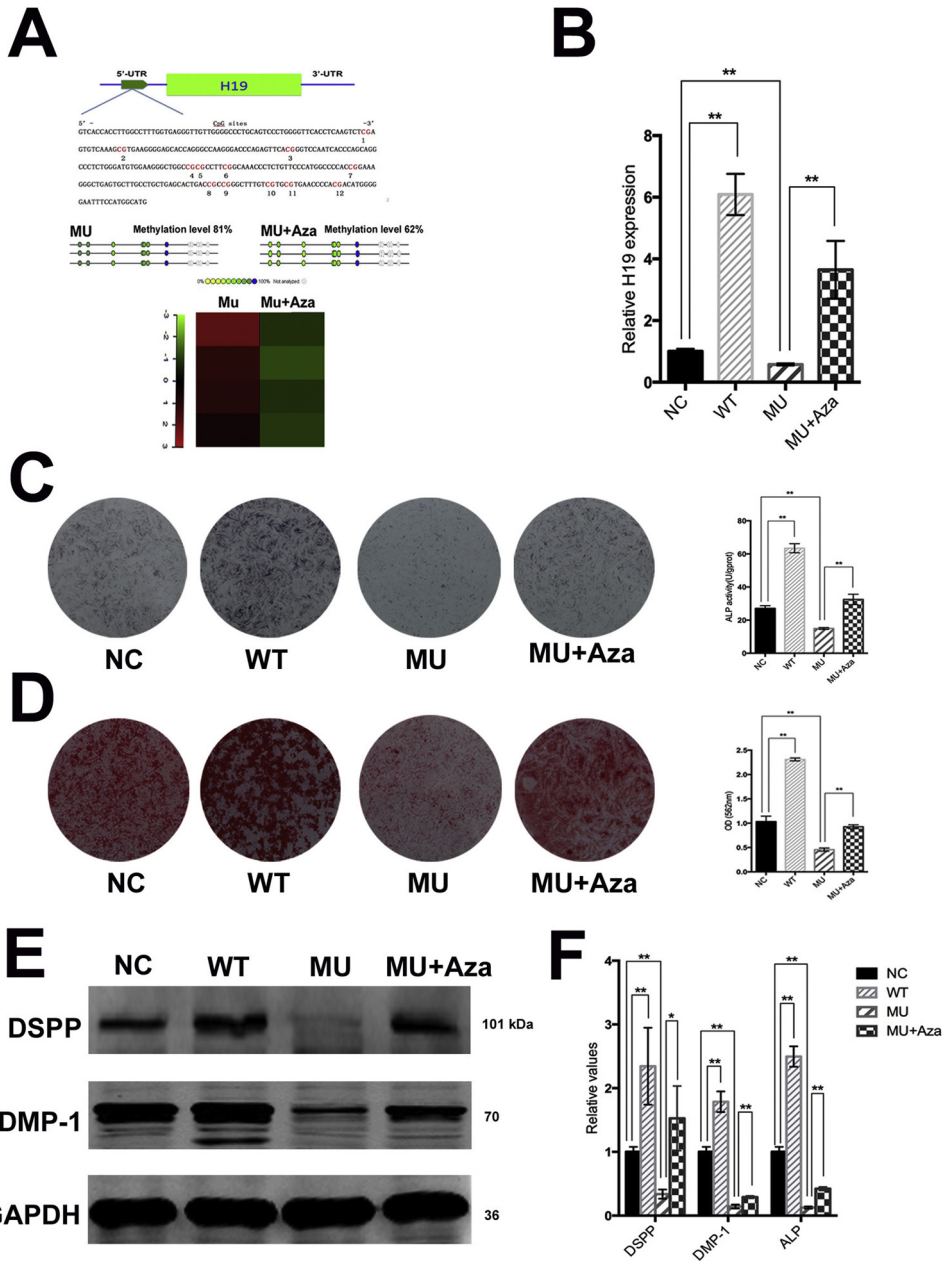

Fig. 3. H19 methylation is involved in odontoblastic differentiation of MU-hDPCs. 5-Aza-CdR was used to decrease the methylation level of genes. (A) Methylation level of H19 in MU-hDPCs after 5-Aza-CdR treatment. (B) The expression level of H19 in MU-hDPCs after 5-Aza-CdR treatment. (C, D) The ALP expression, activity, and calcified deposition in MU-hDPCs after 5-Aza-CdR treatment. (E, F) Protein levels of DSPP and DMP-1 detected by western blot, as well as odontogenesis-related genes expression levels detected by qPCR in MU-hDPCs after 5-Aza-CdR treatment.

2.4. Alizarin red staining

At the indicated time, Alizarin red staining and quantification were performed according to the manufacturer's instruction. Briefly, cells were rinsed with cold PBS for 3 times and fixed in 95% ethanol for 20 min, and washed by distilled water for 3 times. The cells were incubated with 2% Alizarin red pH 4.2 (Sigma) for 10 min at room temperature, then the stained samples were eluted with 100 mM cetylpyridinium chloride (Sigma) for 1 h, and the released Alizarin red was analyzed using a spectrophotometric instrument at 562 nm.

2.5. Quantitative polymerase chain reaction (qPCR)

Total RNA was isolated with TRIzol reagent (Life Technologies, Carlsbad, CA, USA) and 2 μg of RNA was reverse-transcribed into cDNA. qPCR reactions were conducted with the ABI Prism 7500 Real-time PCR System (Life Technologies) by using SYBR green master mix (Roche Diagnostics, Indianapolis, IN, USA). The relative mRNA expression was normalized to glyceraldehyde phosphate dehydrogenase (GAPDH), and calculated using the $2^{-\triangle\triangle Ct}$ method. The sequences of each primer are listed in Supplementary Table 1.

2.6. Western blot assay

At the indicated time, cells were lysed in RIPA buffer with protease inhibitors. Forty micrograms of total protein were loaded into a 12% sodium dodecyl sulfate polyacrylamide gel for electrophoresis, and then transferred to polyvinylidene difluoride membranes. The membranes were blocked in 5% skim milk for 1 h and incubated with antibodies against DMP-1, GAPDH (Abcam, Cambridge, UK), DSPP (Santa Cruz

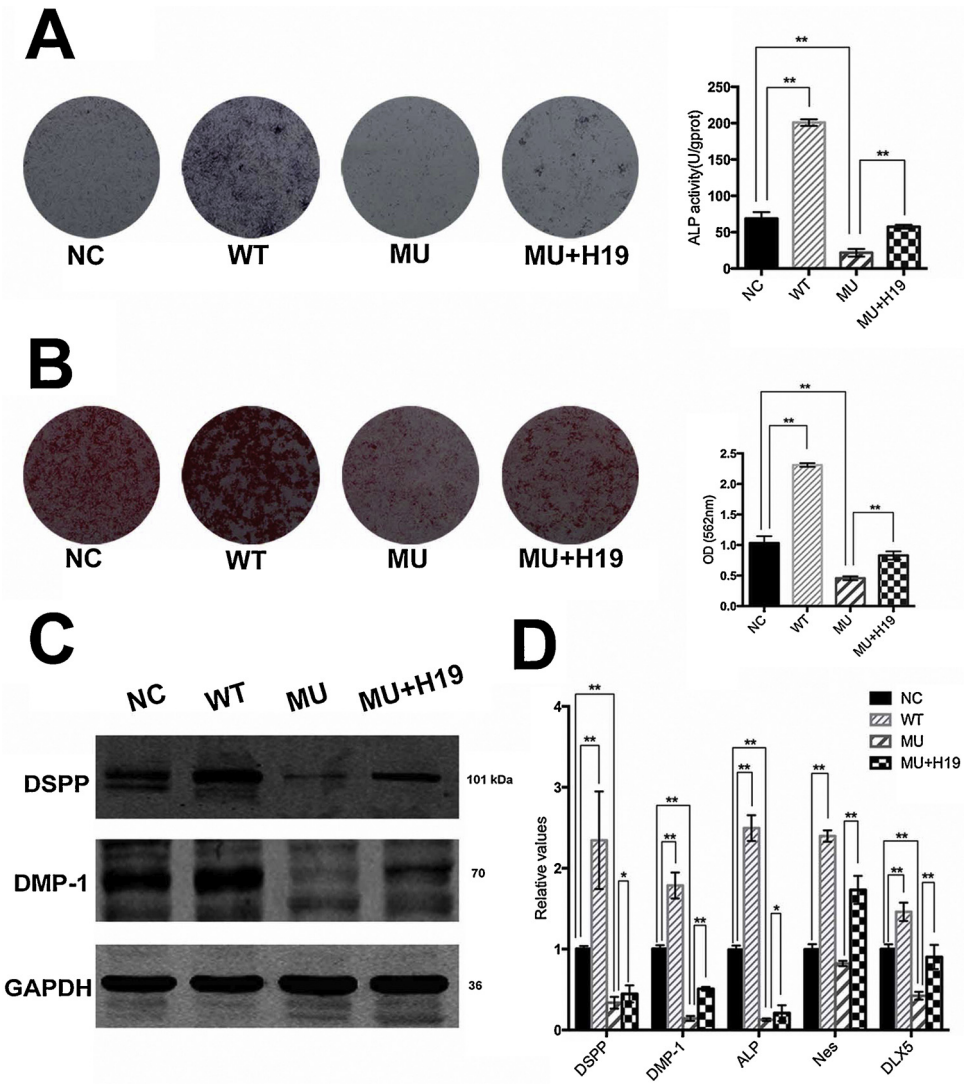

Fig. 4. H19 regulates the odontoblastic differentiation of MU-hDPCs. Lentivirus-H19 was used to overexpress H19 in MU-hDPCs. (A, B) Images of ALP staining and ALP activity at day 7, Alizarin red staining assays and quantification at day 21 post-odontoinduction. (C, D) Western blots of DSPP, DMP-1, GAPDH and odontogenesis-related genes expression after odontoblastic induction. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Biotechnology Inc., Santa Cruz, CA, USA) separately at 4°C overnight, then followed by peroxidase-linked secondary antibodies (1:10,000) for 1 h. The immunoreactive bands were visualized after incubation of Pierce ECL Western Blotting Substrate.

2.7. Immunofluorescent staining

At the indicated time, cells grown on sterile glass coverslips were fixed with 4% formaldehyde for 30 min at room temperature, permeabilized with 0.1% Triton X-100 for 10 min, and followed by the incubation in blocking buffer for 30 min. After that, cells were incubated with primary antibody diluted in bovine serum albumin at 4 °C overnight, and then incubated in the specified second antibody for 1 h at room temperature in the dark. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) and the coverslips were mounted on a glass slide. Images were obtained under a Leica TCS SP8 X confocal imaging system (Leica, Wetzlar, Germany).

2.8. Quantitative methylation analysis

Quantitative methylation analysis of the H19 gene promoter was performed using the Sequenom MassARRAY platform (CapitalBio, Beijing, China). Genomic DNA was extracted using a Universal Genomic DNA kit (Cwbiotech). DNA sample was converted with sodium bisulfite and the modified DNA was amplified by PCR. The target regions were amplified using the primers listed in Supplementary Table 2. The methylation level of gene was analyzed using Epityper software version 1.0 (Sequenom).

2.9. DNMT3B activity assay

Quantification of DNMT3B activity was performed using DNMT3B Activity Assay Core kit (Epigentek, Farmingdale, NY, USA). Specifically, cells were washed with cold PBS and lysed in RIPA buffer with protease inhibitors. The lysate was ultra-sounded and centrifuged at 12,000 g at 4 °C for 30 min, 5 μL of the supernatant was collected and used for DNMT3B activity measurement according to the manufacture's instructions. Spectrophotometric device (PerkinElmer) was used to measure the absorbance of the samples.

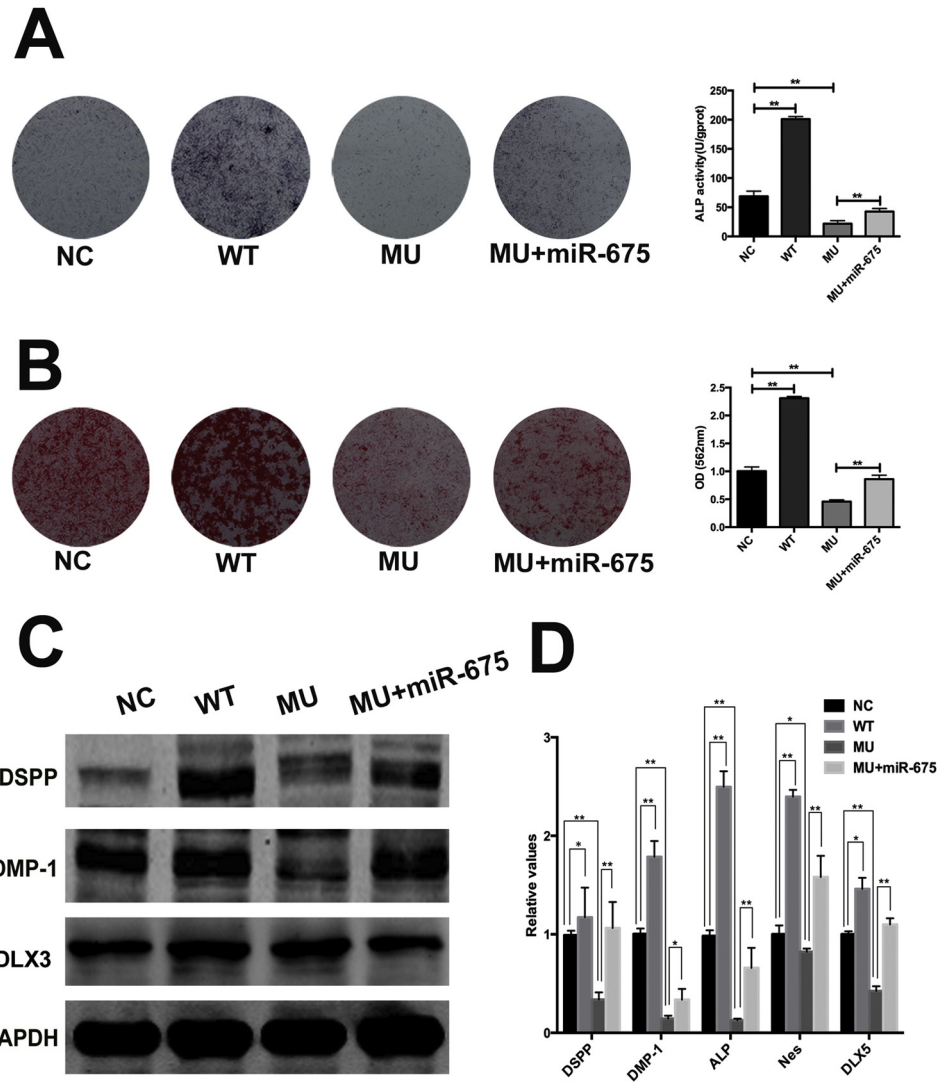

Fig. 5. miR-675 rescues the odontoblastic differentiation of MU-hDPCs. (A, B) ALP staining and activity assays at day 7 after odontoblastic induction, Alizarin red staining assays and quantification of alizarin red staining at day 21 post-odontoinduction. (C, D) Western blots of DSPP, DMP-1, DLX3、GAPDH and odontogenesis-related genes expression after odontoblastic induction (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

2.10. Chromatin immunoprecipitation (ChIP)

ChIP assays were analyzed using a Chromatin Immunoprecipitation Assay Kit (Millipore, Billerica, MA, USA), following the manufacturer's instruction. Specifically, 1% formaldehyde was used to perform cross-linking, the reaction was stopped by glycine after 10 min. Genomic DNA-protein complexes were immunoprecipitated using CHIP-grade anti-H3K9me3 antibody. Normal mouse IgG was as a negative control. Relative levels of precipitated DNA were measured by qPCR (primer sequences were shown in Supplementary Table 3). The relative enrichment of the indicated DNA regions were calculated according to the manufacturer's instructions.

2.11. Statistical analysis

Statistical analyses were performed by SPSS version 13.0 software package (SPSS Inc., Chicago, IL, USA). Statistical significance was determined by one-way analysis of variance and $p < 0.05$ was considered statistically significant.

3. Results

3.1. H19 is significantly down-regulated and hyper-methylated in MU-hDPCs

To explore the role of lncRNAs in TDO syndrome, we performed high throughput analysis. As highthroughput analysis revealed that long noncoding RNA H19 is the strongest down-regulated lncRNA in MU-hDPCs compared with gender- and age-matched WT-hDPCs. Meanwhile, the specified sequences of H19 promoter are statistically hyper-methylated. Therefore, we used qPCR and quantitative methylation analysis to validate the expression and methylation level of H19 in MU-hDPCs and WT-hDPCs. qPCR results showed that H19 is markedly down-regulated in MU-hDPCs, whereas the opposite effect is observed in WT-hDPCs (Fig. 1A). Quantitative methylation analysis also revealed that H19 promoter is highly methylated in MU-hDPCs compared with WT-hDPCs (Fig. 1B). Therefore, the high throughput analysis is believable.

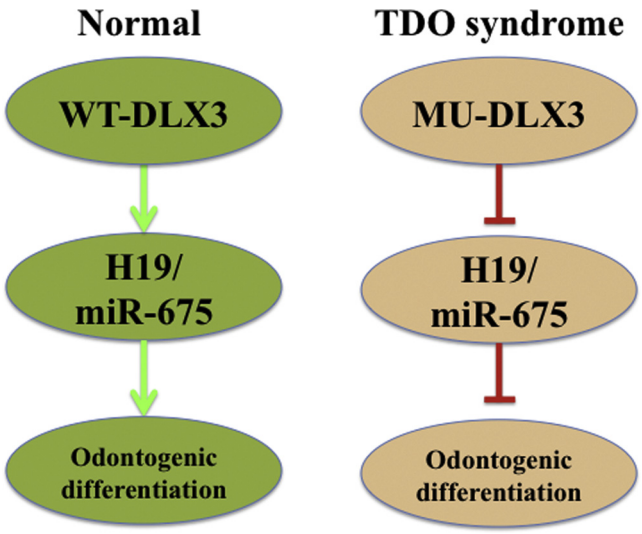

Fig. 6. Schematic of MU-DLX3 epigenetically odontoblastic differentiation of hDPCs through H19/miR675 axis. Green arrow means promoting effect, while red T means inhibiting role (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

3.2. MU-DLX3 alters H19 expression and methylation by increasing H3K9me3 enrichment and DNMT3B activity

The expression and methylation of H19 is regulated by histone modification and DNMTs activity in the promoter region of H19. To test the hypothesis that MU-DLX3 represses H19 transcription by affecting the level of H3K9me3, we determined the abundance of H3K9me3 in the specified promoter region of H19 by ChIP assays in MU-hDPCs and WT-hDPCs. There is no H3K9me3 enrichment in the promoter of H19 gene by using IgG as control. MU-DLX3 increased the enrichment of H3K9me3 in the promoter region of H19 gene in MU-hDPCs. In contrast, WT-DLX3 diminished the enrichment of H3K9me3 in the promoter region compared to the MU group (Fig. 2A). As H19 methylation is regulated by DNMTs activity, we screened the activity of DNMTs and found that the DNMT3B activity was up-regulated in MU-hDPCs, whereas the opposite effect was observed in WT-hDPCs (Fig. 2B). No significant difference of DNMT1 and DNMT3A was found among the groups (data not shown). These data clearly indicate that MU-DLX3 is capable of repressing H19 expression by increasing the enrichment of H3K9me3 in the promoter region and the DNMT3B activity.

3.3. H19 methylation is involved in odontoblastic differentiation of MU-hDPCs

As quantitative methylation analysis revealed that H19 promoter is highly methylated in MU-hDPCs, in order to determine whether the methylation level of H19 is involved in differentiation of MU-hDPCs, we treated MU-hDPCs with 5-Aza-CdR for 72 h and measured the odontoblastic abilities of MU-hDPCs. A treatment with 5-Aza-CdR significantly decreased the methylation level of H19 and greatly increased the expression of H19 by more than fivefold in MU-hDPCs (Fig. 3A, B). The odontoblastic differentiation ability of MU-hDPCs were markedly rescued by increased ALP expression and activity, calcified deposition, protein levels of DSPP and DMP-1, and odontogenesis-related genes DSPP, DMP-1, ALP expression levels after 5-Aza-CdR treatment (Fig. 3C–F). Taken together, these data suggest that the hyper-methylation of CpG in the promoter region of H19 in TDO syndrome is associated with the expression of H19 and the odontoblastic differentiation of MU-hDPCs.

3.4. H19/miR-675 mediates the odontoblastic function of MU-DLX3

As H19 gene was significantly down-regulated in MU-hDPCs, therefore, we performed rescue experiments to identify whether the impaired odontoblastic differentiation effect by MU-DLX3 was mediated by H19 gene. We firstly transfected Lentivirus-H19 into the MU-hDPCs to compensate the hypo-expression of H19 in MU-hDPCs. Results showed that exogenous H19 can partially rescue the inhibition of ALP expression and activity, as well as calcified deposition in MU-hDPCs (Fig. 4A, B). Meanwhile, western blot analysis showed that the protein levels of DSPP and DMP-1 were rescued by exogenous H19, and qPCR results also revealed that the odontogenesis-related genes DSPP, DMP-1, ALP, Nes and DLX5 mRNA expression levels were rescued by H19 (Fig. 4C, D). As the exon1 of H19 can encode miR-675, we further compensated miR-675 into the MU-hDPCs. Results indicated that exogenous miR-675 partially rescued the odontoblastic differentiation of MU-hDPCs (Fig. 5A–D). Those results demonstrate that MU-DLX3 regulates odontoblastic differentiation of hDPCs via H19/miR-675 axis.

Taken together, MU-DLX3 results in hyper-methylation and hypo-expression of H19 in MU-hDPCs by increasing the activity of DNMT3B and the enrichment of H3K9me3. However, improving the H19/miR-675 expression in MU-hDPCs by 5-Aza-CdR treatment or exogenous H19/miR-675 can rescue the odontoblastic differentiation of MU-hDPCs, suggesting that MU-DLX3 weakens the odontoblastic differentiation of MU-hDPCs through repression H19/miR-675 (Fig. 6).

4. Discussion

Herein this is the first work to report on the epigenetic dysregulation of H19 in TDO syndrome. Our studies provide a reasonable explanation for abnormal dentin development in TDO patients with DLX3 mutation (Q178R). Owing to the new findings that MU-DLX3 results in hyper-methylation and hypo-expression of H19 in hDPCs, rescue studies revealed that the phenotype of dentin hypoplasia is attributed to dysregulation of H19/miR-675 axis caused by DLX3 mutation. Therefore, the in-depth functional investigations extend our knowledge of DLX3 function in odontoblastic differentiation, and functional alterations caused by DLX3 mutation.

Works performed in the last several years have demonstrated that DLX3 is a key regulator in dentinogenesis, and mineralization of the dentin is a critical event associated with dentin hypoplasia of TDO syndrome (Dong et al., 2005; Duverger et al., 2012; Nieminen et al., 2011). In vitro function studies have revealed that DLX3 has the potential to promote odontoblastic differentiation. In other words, overexpression of DLX3 in oodontoprogenitors promotes odontoblastic differentiation (Li, Yang, & Fan, 2012). These results are confirmed with the in vivo results (Duverger et al., 2012). Neural crest deletion of DLX3 in Wnt1-cre mice presents major dentin defects throughout the lifetime due to directly weaken odontoblastic differentiation and down-regulation of odontoblastic essential marker DSPP (Duverger et al., 2012). Undoubtedly, these results show that DLX3 is a positive regulator in dentinogenesis. Meanwhile, transgenic mice expressing mutant DLX3 (c.571_574delGGGG) driven by a mouse 2.3 Col1A1 promoter model present dentin hypoplasia and taurodontism owing to mutant DLX3 disrupts odontoblast polarization and differentiation (Choi et al., 2010). The results are consistent with our findings in this study. We find that the novel MU-DLX3 (c. 533 A > G; Q178R) significantly weakens the odontoblastic potential of hDPCs by down-regulation of odontogenesis-related genes and mineralization ability.

Given the fact that dentin formation is an intricate process directed by many factors, an interesting concept emerges is that lncRNAs perform a key role in mineralization and human disorders by coordinating with other signal pathways (Chen et al., 2016; Hadji et al., 2016; Huang et al., 2015). Therefore, we performed high-throughout analysis and found that lncRNA H19 is significantly hypo-expressed in TDO syndrome. Then we detected the expression of H19 in MU-hDPCs and

found that H19 was also hypo-expressed in MU-hDPCs. Hence, H19 is deduced to play a vital role in differentiation and mineralization of hDPCs. As quantitative methylation analysis revealed that hypo-expressed of H19 in MU-hDPCs is related to the hyper-methylation in H19 promoter region. Therefore, we treated MU-hDPCs with 5-Aza-CdR, and found that methylation level of H19 in MU-hDPCs was significantly decreased and H19 expression level was increased dramatically. Meanwhile, the differentiation and mineralization of MU-hDPCs were improved after 5-Aza-CdR treatment. Further, we found that H19/miR-675 could partially rescue the inhibition of differentiation and mineralization in MU-hDPCs. Taken together, the above results demonstrates that MU-DLX3 regulates the differentiation and mineralization of hDPCs through H19/miR-675 axis through epigenetic regulation. In addition, our results firstly shows that lncRNA H19 is involved in the process of odonto-differentiation of hDPCs.

Epigenetic regulation is also closely associated with histone modifications and DNMTs-mediated DNA methylation. However, details of H19 methylation changing in TDO syndrome are still poorly understood. H3K9me3 histone has emerged as a key regulator in repressing gene expression by interacting with heterochromatin protein 1 (Bannister et al., 2001; Lachner, O'Carroll, Rea, Mechtler, & Jenuwein, 2001). Enrichment of H3K9me3 on specific gene promoter can suppress the gene transcription (Chen et al., 2013). Meanwhile, dysregulation of H3K9me3 has been observed in different human disorders (Chen et al., 2013; Chi, Allis, & Wang, 2010; Li et al., 2017). Thus, we wondered that whether aberrant expression of H19 in TDO syndrome is regulated by H3K9me3. Of note, our study showed that H3K9me3 is highly enriched in the promoter region of H19 in MU group, while the opposite effect was observed in WT group, indicating that highly enrichment of H3K9me3 in H19 promoter inhibits the transcription of H19. Being a dynamic process, gene methylation is regulated by DNMTs activity. DNMTs activity plays an essential role in DNA methylation (Samudio-Ruiz & Hudson, 2012). Therefore, we detect whether aberrant methylation of H19 in TDO syndrome is associated with DNMTs activity too. Our results demonstrated that DNMT3B activity changed dramatically. As shown in Fig. 2, DNMT3B activity was dramatically up-regulated in MU-hDPCs. These results support the notion that MU-DLX3 increases methylation of H19 promoter in hDPCs was due to the changes in DNMT3B activity. Further, hyper-methylation of H19 can also inhibit the expression of H19. Therefore, the decreased expression of H19 in MU-hDPCs is caused by the enrichment of H3K9me3 and hyper-methylation in the promoter region of H19.

Taken together, our data, for the first time, provide a new mechanism for TDO syndrome that novel MU-DLX3 weakens the odontoblastic differentiation of hDPCs by repressing the expression of H19/miR-675 through epigenetic regulation. Our new findings indicate that the repressed expression of H19/miR-675 is due to the enrichment of H3K9me3 in the promoter region of H19 and the hyper-methylation of H19/miR-675 via DNMT3B activity.

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Conflict of interest

None declared.

Ethical approval

This study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology (PKUSS-IRB-201736082).

Author contributions

L.Z wrote the draft, L.Z and S.S performed the experiments. Y.L, H.L and D.H assisted the experimental skills. Z.M, W.Y and H.F designed the project, revised the manuscript and their grants supported the project.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.archoralbio.2019.04.009.

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