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DLX3 mutation negatively regulates odontogenic differentiation of human dental pulp cells

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ABSTRACT

Objectives: The purpose of this study was to investigate the role of a novel mutant DLX3 on the odontogenic differentiation of human dental pulp cells (hDPCs) in tricho-dento-osseous (TDO) syndrome.
Design: hDPCs were obtained from the healthy premolars, stably-expressing wild-type DLX3 (WT), novel mutant DLX3 (Mu) and control vector (NC) cells were generated using recombinant lentiviruses. The proliferation rates of WT-hDPCs and Mu-hDPCs were measured by CCK8 assay. Odonto-differentiation of hDPCs was assessed by alkaline phosphatase (ALP) activity assay, and mineralization ability was assessed by Alizarin red staining. Odontogenic markers, including DMP-1, DSPP, Nes, ALP, and DLX5, were analyzed using real-time polymerase chain reaction (qPCR). DMP-1 and DSPP expressions were further confirmed by Western blotting.
Results: CCK8 results showed that the novel mutant DLX3 decreased the proliferation rate of hDPCs compared with wild-type DLX3. qPCR showed that the novel mutant DLX3 weakened odontogenic differentiation by downregulating the expression of odontogenic genes. These results were further confirmed by Western blotting and ALP activity assay. Additionally, Alizarin red staining showed that the novel mutant DLX3 decreased the mineralization of hDPCs compared with wild-type DLX3.
Conclusions: Novel *de novo* mutation of DLX3 significantly decreases the proliferation rate and inhibits the odontogenic differentiation and mineralization of hDPCs, suggesting that this novel mutation of DLX3 can influence the dentinogenesis in TDO syndrome.

1. Introduction

Distal-less (Dlx) was originally identified in the homeodomain, which is important in the patterning of many tissues during vertebrate development (Merlo et al., 2000; Morasso, Markova, & Sargent, 1996). The Dlx family is composed of six individual members. DLX3 is mapped on chromosome 17q21 and plays essential roles in the development of the placental, epidermis and ectodermal appendages (Chui et al., 2010; Hassan et al., 2004; Morasso, Markova et al., 1996). Deletion of the DLX3 gene in mice results in placental death between embryonic days 9.5 and 10 (Morasso, Grinberg, Robinson, Sargent, & Mahon, 1999). Importantly, DLX3 is required for the entire cycle of hair follicle differentiation, and for skeletal formation and development, and for tooth morphogenesis (Hwang, Mehrani, Millar, & Morasso, 2008; Isaac et al., 2014; Zhao, Stock, & Buchanan, 2000).

DLX3 mutation is responsible for tricho-dento-osseous (TDO) syndrome, a rare autosomal dominant disorder with a dominant inheritance mode. (Li et al., 2015; Price, Bowden, Wright, Pettenati, & Hart, 1998). The primary clinical features of TDO are mainly manifested in the hair, teeth, and bones, including kinky hair at birth, enamel hypoplasia, dentin hypoplasia, taurodontism, and thickened bones (Li et al., 2015; Wright, Kula, Hall, Simmons, & Hart, 1997). This indicates that the DLX3 mutation exerts an abnormal effect on the development of hair, bone, and teeth. Other minor abnormalities associated with TDO are nail splitting, skin lesions and dental abscesses (Quattromani et al., 1983; Mayer, Baal, Litschauer-Poursadrollah, Hemmer, & Jarisch, 2010; Wright et al., 1997), although these abnormalities are not observed in all affected individuals. Recently, our research group reported a novel mutation of DLX3 in a Chinese TDO patient who is receiving treatment at our hospital (Li et al., 2015). Unfortunately, the teeth became residual crowns or roots in this patient. Therefore, it remains difficult to determine whether this patient has

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taurodontism or whether the dentinogenesis process is impeded. As previous studies demonstrated irregularities in dentinal tubules, canal-like defects in dentin, and enlarged dental pulp in TDO patients (Nieminen et al., 2011), we deduced that the novel mutant DLX3 can affect the process of dentinogenesis.

Human dental pulp cells (hDPCs) are the only source of cells for dentinogenesis. hDPCs can differentiate into odontoblasts, and the normal differentiation of hDPCs is essential for dentin development and formation (Iida et al., 2010). In TDO patients, affected individuals have enlarged pulp chambers or dentin defect, indicating that DLX3 mutation is associated with imperfect dentinogenesis and may play a differential role in disrupting the development of this mineralized tissue (Nieminen et al., 2011; Price et al., 1999). Given the effects of DLX3 on dentinogenesis, and little is known about this novel mutation of DLX3 in hDPCs proliferation and odontogenic differentiation. We examined the direct influence of this novel mutant DLX3 on the proliferation and odontogenic differentiation of hDPCs.

To characterize the effect of the novel mutant DLX3 on the proliferation and odontogenic differentiation of hDPCs, a stably expressing novel mutant DLX3 and wild-type DLX3 in hDPCs were established and used to examine the effects on proliferation and odontogenic differentiation exerted by the mutation of DLX3. The effect of the mutant DLX3 on the proliferation and odontogenic differentiation of hDPCs was assessed using Cell Counting Kit-8 (CCK8), quantitative real-time polymerase chain reaction (qPCR), Western blot analysis, ALP activity, and the formation of calcified nodules, which helps to clarify the regulatory role of this novel mutant DLX3 in hDPCs.

2. Materials and methods

2.1. Cell culture

This study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology. Three individuals participated in this study with informed consent. Dental pulp tissues were obtained from premolars extracted due to orthodontic treatment. Pulp tissues from the three donors were cut into about 1-mm^3 pieces, and subjected to isolate hDPCs. Cell isolation was performed as described previously (Guzmán-Uribe, Estrada, Guillén Ade, Pérez, & Ibáñez, 2012). Cells were cultured in a humidified atmosphere with 5% CO_2 and 95% air at 37 °C in alpha essential medium (Gibco, Paisley, UK) supplemented with 10% fetal bovine serum (Gibco), and 1% penicillin-streptomycin (Gibco). All cells used in this study were between the second and sixth passages. For odontogenic differentiation, cells were cultured in odontogenic medium (OM), which contains 50 μg/ml ascorbic acid, 100 nmol/L dexamethasone, and 10 mmol/L β-glycerolphosphate (Sigma, St Louis, MO).

2.2. Lentivirus construction and establishment of stably infected hDPCs

The full-length of human wild-type DLX3 cDNA and novel mutant DLX3 (c.533 A > G) cDNA were sub-cloned with EcoR I and BamHI into pHBLV-CMVIE-ZsGreen-T2A-puro vector to generate lentiviruses overexpressing wild-type DLX3 and the novel mutant DLX3, respectively; lentivirus containing a green fluorescent protein (GFP) tag with no target gene was used as a negative control (NC). The recombinant vector and control vectors were transfected, along with the packaging vectors psPAX2 and pMD2.G into 293T cells to produce the lentivirus. The supernatant was harvested, filtered, and concentrated at 2 days post-transfection. To establish stable overexpression of human wild-type DLX3 or mutant DLX3 in hDPCs, 200 μL of lentivirus mixed with 10 μg/ml polybrene (Sigma) were used to infect the second passage of hDPCs (5×10^5 cells in 100 mm dishes) for 12 h. The medium was changed 12 h post-infection and incubated for another 48 h, then cells were cultured in the presence of 2 μg/ml puromycin (Sigma). Cells were observed under a fluorescent microscope to identify their infection efficiency. qPCR and Western blotting were used to detect the expression of DLX3 in surviving cells after puromycin selection. Cells stably expressing wild-type DLX3 or mutant DLX3 were named WT-hDPCs and Mu-hDPCs, respectively.

2.3. Cell proliferation assay

Cell proliferation was performed using Cell Counting Kit-8 (CCK8) (Dojindo, Kumamoto, Japan) assay following the manufacturer's protocol. Stably infected hDPCs were seeded in 96-well plates (1×10^3/well) in triplicate and cultured in complete medium for 9 days. The medium was changed at 2-day intervals. The supernatant was removed and the hDPCs were incubated in complete medium containing 10% CCK8 reagent for another 2 h. The optical density at 450 nm was measured using a microplate reader (BioTek, Winooski, VT).

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

hDPCs were cultured in 12-well plates and under the OM induction for 3,7, and 14 days. At the indicated time, ALP staining was performed based on the protocol included in the NBT/BCIP staining kit (Cwbiotech, Beijing, China). Briefly, the cultured cells were rinsed 3 times with phosphate buffer saline (PBS) and fixed in 95% ethanol for 30 min, washed 3 times with millipore-filtered water, and incubated in NBT/BCIP solution for another 30 min. ALP activity was measured using an ALP assay kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacturer's protocol, the cultured cells were rinsed with PBS for 3 times, lysed by 1% Triton X-100 and then collected into tubes, followed by three times of freezing and thawing. ALP activity was analyzed at 405 nm using a spectrophotometric instrument (PerkinElmer, Waltham Mass, MA). ALP activity relative to the NC group was calculated after normalization to total protein concentration.

2.5. Alizarin red staining and quantification of mineralization

Cells were cultured in 12-well plates and cultured in OM for 21 days, and mineralization was analyzed using an Alizarin red staining assay. At the indicated time, cells were rinsed with PBS for 3 times and fixed in 95% ethanol for 30 min, then washed the cells with distilled water for 3 times. The cell layer was stained with 2% Alizarin red pH 4.2 (Sigma) for 20 min at room temperature. To quantify the degree of mineralization, 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. The Alizarin red intensity was calculated relative to the that of NC group after normalization to total protein concentration.

2.6. Real-time quantitative polymerase chain reaction (qPCR)

Total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, CA) and 2 μg of RNA was reverse-transcribed into cDNA. qPCR was conducted with the ABI Prism 7500 Real-Time PCR System (Life Technologies) using SYBR green master mix (Roche Diagnostics, Indianapolis, IN). The relative mRNA expression was normalized to glyceraldehyde phosphate dehydrogenase (GAPDH), and calculated using the $2^{-\Delta\Delta Ct}$ method. The primers for DLX3, DLX5, dentin sialophosphoprotein (DSPP), dentin matrix acidic

Table 1
Primer sequences used in qPCR.

Gene	Sequences (5′-3′)
DLX3	Forward: CCTATGGCCAGACGGTGAAC Reverse: CCTTCTTGGGCTTCCCATTC
DLX5	Forward: CTCGCTCAGCCACCACCCTCAT Reverse: AGTTGAGGTCATAGATTTCAAGGCAC
DSPP	Forward: GAGCCACAAACAGAAGCAACAC Reverse: TTGGACAACAGCGACATCCTCA
DMP-1	Forward: ACCAGGCACTATGCTAGGTGTT Reverse: CTTTGTGGGTCCTTCTATACGC
ALP	Forward: ATGGGATGGGTGTCTCCACA Reverse: CCACGAAGGGGAACTTGTC
Nes	Forward: GCCCTGACCACTCCAGTTTA Reverse: GGAGTCCTGGATTTCCTTCC
GAPDH	Forward: GGTCACCAGGGCTGCTTTTA Reverse: GGATCTCGCTCCTGGAAGATG

phosphoprotein-1 (DMP-1), ALP, nestin (Nes), GAPDH are listed in Table 1.

2.7. Western blotting

Cells were collected and lysed in RIPA buffer with protease inhibitors. Forty micrograms of total protein were loaded into a sodium dodecyl sulfate polyacrylamide gel for electrophoresis and subsequently transferred to a polyvinylidene difluoride membrane and blocked in 8% skim milk for 1 h. The membranes were then incubated with anti-DLX3 (Abcam, Cambridge, UK), anti-DMP-1 (Abcam), anti-DSPP (Santa Cruz Biotechnology, Inc, Santa Cruz, CA), and anti-GAPDH (Proteintech, Chicago, IL) antibodies overnight at 4 °C, followed by peroxidase-linked secondary antibodies (1:10,000) for 1 h. The immunoreactive bands were detected on an Odyssey infrared imaging system (Odyssey LI-COR Biosciences, Lincoln, NE). GAPDH served as an internal control.

2.8. Statistical analysis

Three independent experiments were performed. All data are expressed as means and standard deviation. Statistical analyses were performed by SPSS version 13.0 software package (SPSS Inc, Chicago, IL). The data was analyzed by one-way analysis of variance and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Identification of stable expression of WT-DLX3 and Mu-DLX3 in hDPCs

Stable expressions of wild-type DLX3 and mutant DLX3 in hDPCs were generated as stated above. Because the infected lentiviruses carried the GFP tag, we used fluorescent detection to monitor infection efficiency. GFP expression was observed in more than 90% stably infected hDPCs under fluorescent microscopy (Fig. 1A–C). Additionally, the DLX3 mRNA and protein levels were significantly increased in WT-hDPCs and Mu-hDPCs compared with NC group (Fig. 1D–F, $p < 0.05$). These results show that hDPCs stably expressing wild-type and mutant DLX3 were successfully established.

3.2. Mutant DLX3 markedly suppresses the proliferation of hDPCs

To investigate the effect of mutant DLX3 on the proliferation rate of hDPCs, CCK8 assay was performed. As shown in Fig. 2, mutant DLX3 significantly inhibited the proliferation rate of hDPCs from day 1 to day 9 ($p < 0.05$). Although wild-type DLX3 can also decrease the cell proliferation rate, the inhibition efficiency was lower than that with novel mutant DLX3, and there was no statistical difference between the WT and NC groups at days 3, 5, and 9 ($p > 0.05$).

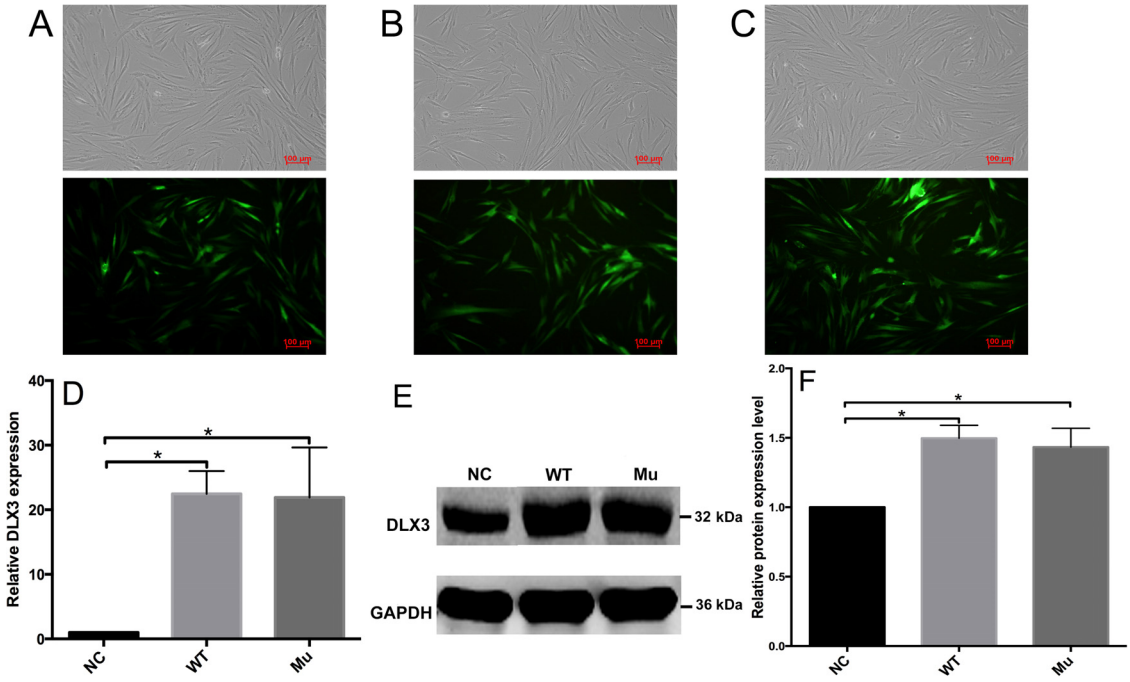

Fig. 1. Identification of stably expressed DLX3 in hDPCs. GFP expression was observed in stably infected hDPCs in the NC (A), WT (B), and Mu (C) groups. DLX3 mRNA levels were significantly increased in the WT and Mu groups (D), and DLX3 protein levels were markedly higher in the WT and Mu groups (E and F). GAPDH was used as an internal control. *Indicates the statistically significant differences, $p < 0.05$.

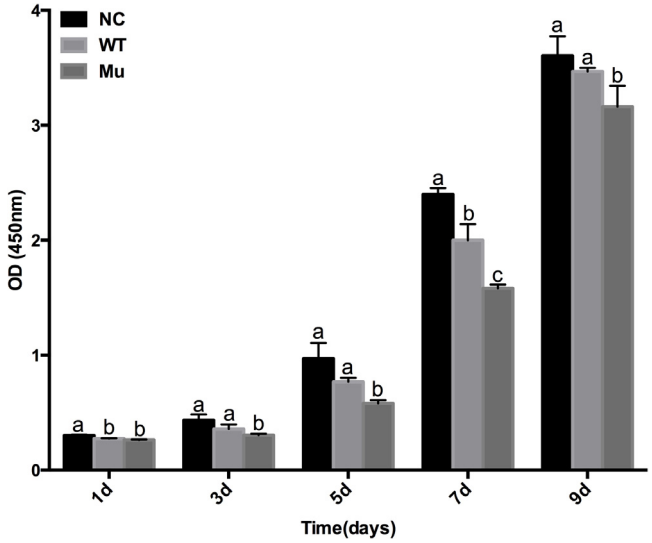

Fig. 2. Effects of novel mutant DLX3 on the proliferation rate of hDPCs. Cell growth in each group was measured by CCK-8 assay. The Mu group exhibited the lowest proliferation activity, followed by the WT and NC groups. Different letters for the same day indicate statistically significant differences, $p < 0.05$.

3.3. Mutant DLX3 weakens the ALP activity and mineralization ability of hDPCs

To examine the effect of novel mutant DLX3 on the odontogenic differentiation and mineralization potential of hDPCs, ALP activity assay and Alizarin red staining were performed. Results showed that the Mu group had the lowest ALP expression (Fig. 3A) and the lowest level of ALP activity (Fig. 3B), whereas wild-type DLX3 significantly increased the ALP expression and activity at days 3,7, and 14 ($p < 0.05$). Alizarin red staining results indicated that the WT group had the highest formation of mineralized nodules ($p < 0.05$), whereas the novel mutant DLX3 obviously decreased the formation of calcified nodules (Fig. 4A–C). Quantification analysis was in agreement with the staining results (Fig. 4D).

3.4. Mutant DLX3 inhibited odontogenic differentiation of hDPCs

To further determine the effects of mutant DLX3 on the regulation of the odontogenic differentiation potential in hDPCs, the expressions of odontogenesis-related genes were examined. Mutant DLX3 significantly down-regulated the mRNA expression levels of DMP-1, DSPP, Nes, ALP, and DLX5 ($p < 0.05$), whereas wild-type DLX3 greatly up-regulated these mineralization genes in hDPCs (Fig. 5A, B). Western blot analysis demonstrated that the novel mutant DLX3 evidently decreased, whereas wild-type DLX3 greatly increased the expressions of the odontogenesis-related proteins DMP-1 (Fig. 5C) and DSPP (Fig. 5D).

4. Discussion

The TDO patient with the novel *de novo* mutation of DLX3 showed distinct clinical features of TDO in bones, hair and teeth. DLX3 is an essential transcriptional factor in the proliferation and differentiation of odontoblasts (Li, Yang, & Fang, 2012). Given the clinical characteristics of TDO syndrome and the function of DLX3 in tooth development, we wondered whether this novel mutant DLX3 disrupts the proliferation and odontogenic differentiation of hDPCs. Therefore, we focused on the effects of this novel mutant DLX3 on proliferation and odontogenic differentiation of hDPCs.

Dentinogenesis is a multi-step process which dentin is formed by a progressive cytodifferentiation of hDPCs to mature odontoblasts. Multiple genes are involved in this process (Huang & Chai, 2012). hDPCs are a neural crest-derived mesenchymal progenitors endowed with plasticity and multi-potency and capable of differentiation into odontoblasts. hDPCs play a critical role in reparative regeneration in response to dental pulp infection, dental injury, and irreversible caries damage (Lv et al., 2016; Tecles et al., 2005). Therefore, the cell proliferation rate has important implications for dental development and repair. To examine the effects of the novel mutant DLX3 on the proliferation of hDPCs, cell growth rates were measured using CCK8. The results show that the mutant DLX3 markedly suppressed the proliferation rate of hDPCs, and this effect was much stronger than that of wild-type DLX3. Li et al. (2012) results showed that wild-type DLX3 can inhibit the proliferation rate of hDPCs. Our results are consistent with their study and further show that this novel mutation can influence the proliferation of hDPCs.

Differentiation from multipotent stem cells to functional odontoblasts is a continuous process involving a number of genes and transcriptional factors. DMP-1 and DSPP belong to a family of proteins known as SIB-LINGs. DSPP is a major non-collagenous protein in tooth dentin and is essential for dentin mineralization; DMP-1 is another non-collagenous extracellular matrix protein, which promotes the differentiation of hDPCs and has a mineralization regulatory function (Alvares, Kanwar, & Veis, 2006; Prescott et al., 2008). Nestin (Nes) and ALP play roles in odontogenic differentiation (Woltgens, Lyaruu, Bronckers, Bervoets, & Van Duin, 1995; About, Laurent-Maquin, Lendahl, & Mitsiadis, 2000). DLX5, another Dlx family member, is a transcriptional factor that plays an important role in odontogenic differentiation (Li et al., 2008). To identify the effects of novel mutant DLX3 on the odontogenic differentiation of hDPCs, DMP-1, DSPP, Nes, ALP, and DLX5 were selected as makers of odontogenic differentiation and

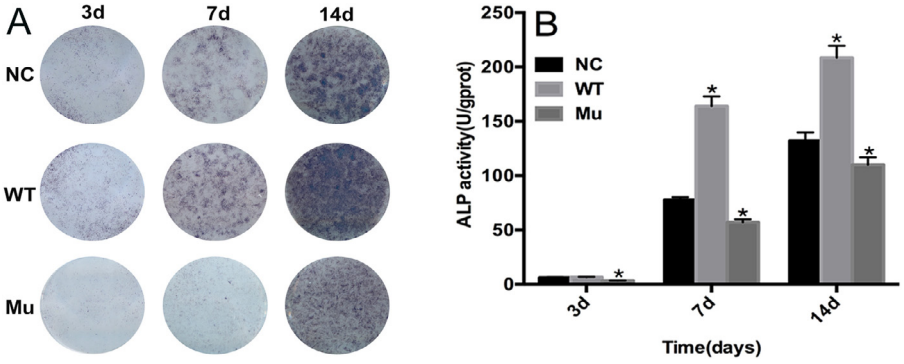

Fig. 3. Effects of novel mutant DLX3 on ALP expression and activity in hDPCs. ALP staining in the NC, WT and Mu groups on day 3,7 and 14 (A). ALP activity of the NC, WT and Mu groups on day 3,7 and 14 (B). *Indicates the statistically significant differences versus the NC group, $p < 0.05$.

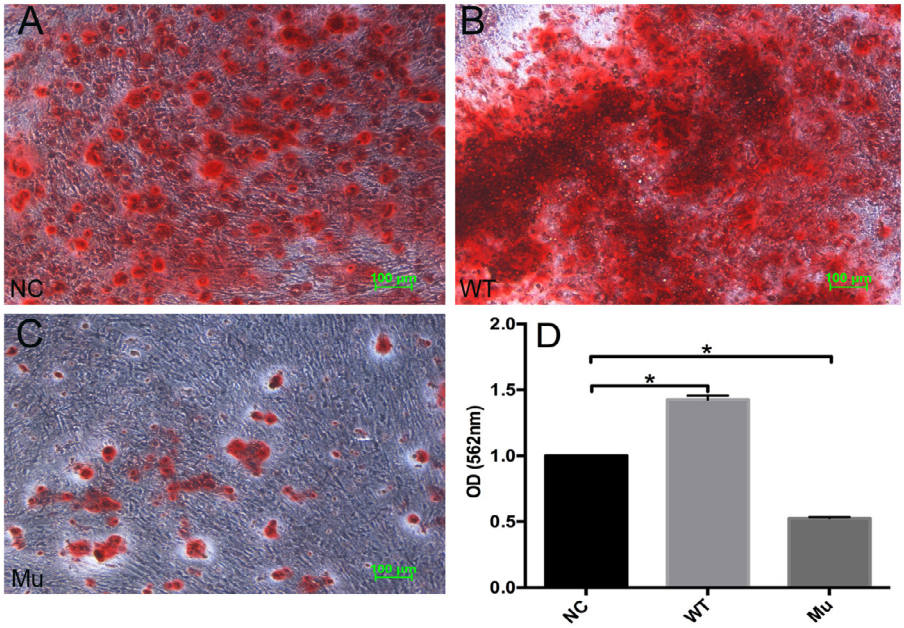

Fig. 4. The effects novel mutant DLX3 on the mineralization ability of hDPCs. Calcified nodule formation in hDPCs was analyzed by Alizarin red staining on day 21 (A–C) and quantification of Alizarin red (D). *Indicates the statistically significant differences versus the NC group, $p < 0.05$.

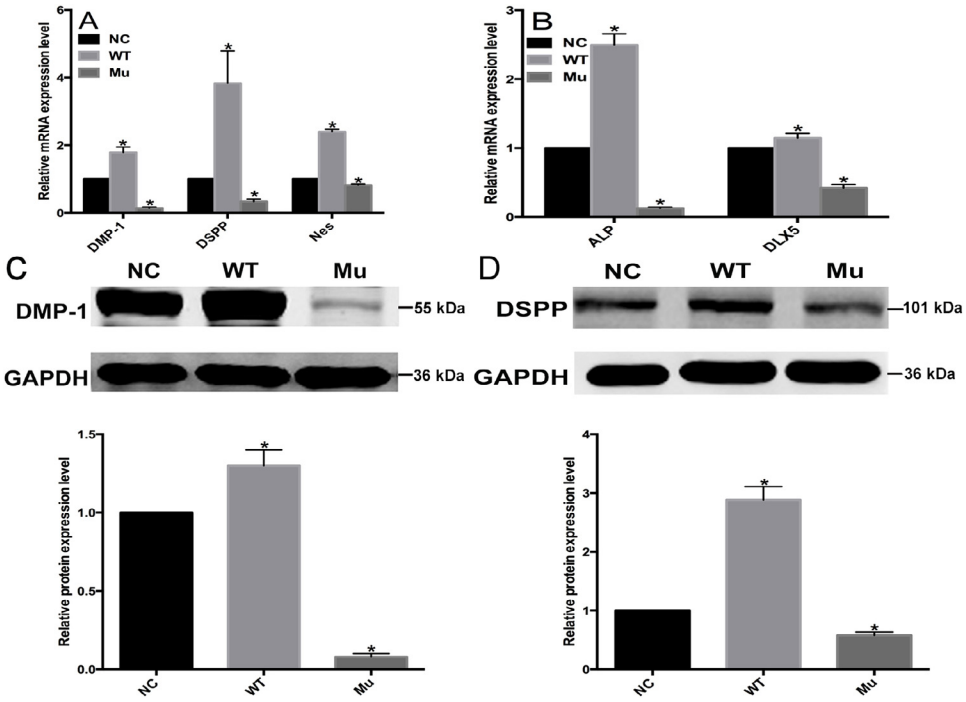

Fig. 5. Effects of mutant DLX3 on the odontogenic differentiation of hDPCs. mRNA expression levels of DMP-1, DSPP, Nes, ALP, and DLX5 were measured by qPCR on day 7 (A, B). Protein levels of DMP-1 (C) and DSPP (D) were detected by Western blotting on day 7. GAPDH was used as an internal control. *Indicate the statistically significant differences versus the NC group, $p < 0.05$.

mineralization in hDPCs. Our results indicate that mutant DLX3 significantly inhibits the expression levels of DMP-1, DSPP, Nes, ALP and DLX5. These data illustrate that the novel mutant DLX3 has a negative effect on the odontogenic differentiation of hDPCs, which is consistent with the results that the mutant DLX3 inhibited the transcriptional activation of enamel matrix protein (EMP) genes during amelogenesis (Zhang et al., 2015).

ALP plays a critical role in the early stages of mineralization, and calcified nodules are considered as an indicator of late-stage mineralization. To further confirm the effect of novel mutant DLX3 on odontogenic differentiation, we performed ALP activity and Alizarin red staining assays to determine the mineralization of hDPCs. The results showed that ALP expression and calcium deposition were lower in the Mu group, whereas the opposite

effects were observed in the WT group, which is agreement with Pekka's clinical results (Nieminen et al., 2011).

Our results demonstrate that this novel mutant DLX3 can markedly suppress the proliferation and odontogenic differentiation of hDPCs by decreasing the expression of mineralization-related genes and calcium nodule formation. These results help to elucidate the negative regulatory effects of novel mutant DLX3 on the proliferation and odontogenic differentiation of hDPCs.

Conflict of interest

None.

Funding

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Ethical approval

This study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology. ALL performances were conducted in compliance with established guidelines.

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