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# DLX3 regulates osteogenic differentiation of bone marrow mesenchymal stem cells via Wnt/β-catenin pathway mediated histone methylation of DKK4

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ABSTRACT

*Objective:* Distal-less homeobox 3 (DLX3) is an important transcription factor involved in the osteogenic differentiation of bone marrow mesenchymal stem cells (BMSCs). However, the underlying mechanism is not clear. This study investigated the underlying mechanism of DLX3 in osteogenic differentiation.
*Methods:* DLX3 overexpression and knockdown in cells were achieved using lentiviruses. The osteogenic differentiation of BMSCs was detected using alkaline phosphatase expression, alizarin red staining, real-time quantitative polymerase chain reaction (RT-qPCR), Western blotting, and chromatin immunoprecipitation (ChIP) assays.
*Results:* DLX3 overexpression promoted the osteogenic differentiation of BMSCs, whereas DLX3 knockdown reduced the osteogenic differentiation of BMSCs. RT-qPCR and Western blotting assays showed that DLX3 modulated osteogenic differentiation via the Wnt/β-catenin pathway. ChIP-qPCR showed that DLX3 knockdown promoted DKK4 expression by decreasing the enrichment of histone H3 lysine 27 trimethylation (H3K27me3) in the promotor region of DKK4.
*Conclusion:* Our data implied that DLX3 regulated Wnt/β-catenin pathway through histone modification of DKK4 during the osteogenic differentiation of BMSCs.


## 1. Introduction

Distal-less homeobox 3 (DLX3) is a member of the DLX family transcription factors, which has six members in humans: DLX1–6 [1,2]. DLX3 plays a crucial role in embryogenesis and development of organs, including the epidermis and ectodermal appendages [3,4]. Dlx3-null mice die as embryos due to placental dysplasia caused by reductions in the placental vasculature and levels of placental growth factors [5]. DLX3 mutations are responsible of the rare tricho-dento-osseous syndrome, which involves hypoplasia of hair, enamel, and dentin as well as a high bone mineral density [6]. A few DLX3 mutations have been identified. *In vitro*, functional study showed that a 4-base-pair deletion mutation in DLX3 enhanced osteogenic differentiation of bone marrow stromal cells [7]. Previously, we discovered a DLX3 missense mutation (c.533A > G, Q187R) that caused a decrease in the osteogenic differentiation potential of bone marrow mesenchymal stem cells (BMSCs) [8]. These indicate that DLX3 is essential for bone formation, however, the mechanism of DLX3 in bone formation is still unclear.

The Wnt/β-catenin pathway is an important biochemical pathway that is involved in the development of multiple tissues; it also plays a vital role in the regulation of bone formation [9]. The Wnt/β-catenin pathway regulates the proliferation, differentiation, and apoptosis of osteoblasts [10]. It also mediates the negative effects on osteoclastogenesis via acting either directly on osteoclast differentiation or indirectly production of osteoprotegerin in osteoblasts [9,11]. Wnt ligands activate the Wnt/β-catenin pathway by binding to receptor Frizzled (FZD) or co-receptor lipoprotein receptor-related protein 5/6 (LRP5/6). Activation of these receptors inhibits the degradation of β-catenin, which leads to the accumulation of β-catenin in the cytoplasm, and then translocates to the nucleus and

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subsequently activates transcription [12]. Dickkopf (DKK) family members, including DKK1–4, regulates the activity of the Wnt/β-catenin pathway, which are antagonists of the Wnt/β-catenin pathway by blocking the interaction between Wnt ligands and LRP5/6 [9]. Of the four members, DKK1 has been well studied. Inhibition of DKK1 promotes osteogenesis *in vitro* and *in vivo* [13,14]. As a newly discovered member, DKK4 has demonstrated the function in vertebrate development, such as bone formation [12]. Interestingly, Dkk4-knockdown MC3T3-E1 cells showed higher alkaline phosphatase activity than Dkk1-knockdown cells [12]. It indicates that DKK4 acts as an important role in osteogenesis. Studies reveal that epigenetic modifications are involved in regulating Wnt/β-catenin pathway [15,16]. Among of them, histone H3 lysine 27 trimethylation (H3K27me3), as a marker of gene repression, is an important modification involving in Wnt/β-catenin pathway [17,18]. H3K27me3 acts as a negative regulator of Wnt/β-catenin pathway through regulating WNT ligands or DKKs expression [19,20].

Here, we explore the underlying molecular mechanism by which DLX3 regulates the osteogenic differentiation of BMSCs. We found that DLX3 promoted the osteogenic differentiation of BMSCs by activating the Wnt/β-catenin pathway.

## 2. Materials and methods

### 2.1. Cell isolation, culture and differentiation

This study was approved by the Ethics Committee of Peking University School and Hospital of Stomatology. Informed consent was obtained from all participants. Primary BMSCs were isolated from alveolar bone of healthy volunteers as described previously [8]. The primary BMSCs were cultured in alpha minimal essential medium (α-MEM) containing 10% fetal bovine serum and 1% penicillin–streptomycin and 1% L-glutamine (Thermo Fisher Scientific, Carlsbad, CA, USA). The osteogenic differentiation of BMSCs was performed in osteogenic differentiation medium (ODM), which contains $10^{-7}$ M dexamethasone, 50 μg/mL ascorbic acid 2-phosphate, and 10 mM glycerol 2-phosphate in α-MEM (Sigma-Aldrich, St. Louis, MO, USA). The differentiation medium was replaced with fresh medium every 3 days.

### 2.2. Lentiviral infection

To achieve stable overexpression of DLX3 and knockdown of DLX3 (ShDLX3), passage 3 BMSCs were infected with DLX3, ShDLX3, or negative control lentiviruses (DLX3-NC and ShDLX3-NC) (Hanheng Chem Technology, Shanghai, China).

### 2.3. Alkaline phosphatase (ALP) staining and ALP activity

BMSCs infected with lentiviruses were cultured in six-well plates in ODM for 7 days to induce differentiation. ALP staining was performed using the NBT/BCIP staining kit based on the manufacturer's protocol (Cwbiotech, Beijing, China). ALP activity was measured using an ALP assay kit (Nanjing Jiancheng, Nanjing, China), according to the manufacturer's protocol.

### 2.4. Alizarin red staining (ARS)

Cells infected with lentiviruses were induced by ODM in six-well plates for 3 weeks. ARS and quantification of the staining intensity (Sigma-Aldrich) were performed according to the manufacturer's protocol. The ARS intensity was quantified spectroscopically at 562 nm.

### 2.5. Real time-quantitative polymerase chain reaction (RT-qPCR)

Total RNA was isolated from each sample using TRIzol reagent (Thermo Fisher Scientific) and reverse transcribed into cDNA using GoScript™ Reverse Transcriptase (Promega, Madison, WI, USA). RT-qPCR was performed using SYBR green master mix (Roche, Indianapolis, IN, USA) on the ABI QuantStudio 3 system (Thermo Fisher Scientific). Gene expression was calculated using the $2^{-\Delta\Delta CT}$ method and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The primers used are listed in Supplementary Table 1.

### 2.6. Western blotting

Cells infected with lentiviruses were lysed with RIPA buffer supplemented with proteinase inhibitors and PMSF (Solarbio, Beijing, China). The protein concentration was determined using the BCA Protein Assay (Thermo Fisher Scientific). Then, 30 μg of each sample were separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The proteins were transferred to nitrocellulose membranes (Thermo Fisher Scientific) and blocked with 5% bovine serum albumin (Solarbio) for 1 h. The membranes were incubated with primary antibodies against DLX3, ALP, RUNX2, OCN, DKK4, β-catenin, β-catenin (Ser45), and GAPDH (Abcam, Cambridge, UK), separately, with gentle shaking at 4 °C overnight. Bound antibodies were visualized using a horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG (Proteintech Group, Rosemont, IL, USA) and a chemiluminescence kit (Proteintech Group). GAPDH served as the loading control.

### 2.7. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed using a ChIP assay kit (Millipore, Billerica, MA, USA), according to the manufacturer's protocol. Briefly, cells were fixed with 1% formaldehyde for 10 min at room temperature. Chromatin was extracted from the nuclei. The chromatin was then sheared to 200–1000 bp fragments by sonication (Qsonica, Newtown, CT, USA). An antibody against H3K27me3

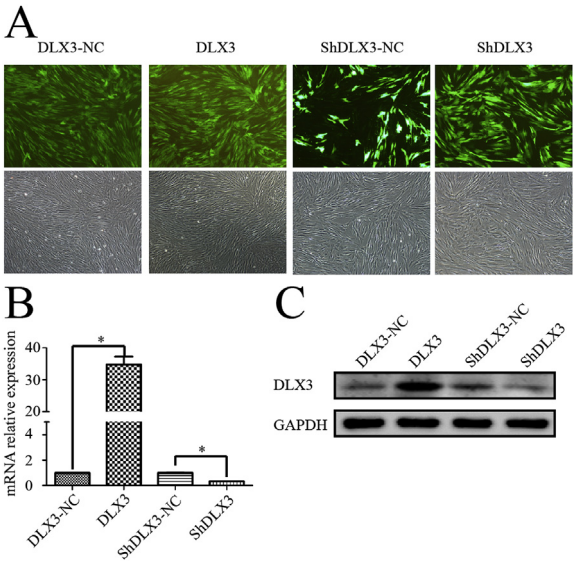


**Fig. 1.** DLX3 expression in BMSCs. (A) GFP expression was observed in BMSCs in the negative control (DLX3-NC and ShDLX3-NC), DLX3-overexpression (DLX3), and DLX3-knockdown (ShDLX3) groups. (B and C) DLX3 mRNA and protein levels were markedly increased in the DLX3 group and decreased in the ShDLX3 group. GAPDH served as the loading control ($^*p < 0.05$, n = 3).

(Abcam) was applied to immunoprecipitate DNA, which was then analyzed by RT-qPCR. The purified DNA was used to analyze the DKK4 promotor using the following primers: (forward) 5′-CGAGCCTTCCTACTACTTGGC-3′ and (reverse) 5′-TGGGGGCTCTTTCCTTTATCC-3'.

### 2.8. Statistical analysis

Statistical analysis was performed using SPSS 20.0 (IBM SPSS, Chicago, IL, USA). Group differences were assessed using one-way analysis of variance followed by Dunnett's multiple comparison post hoc test. $P < 0.05$ was considered statistically significant. All data are expressed as means ± standard deviation.

## 3. Results

### 3.1. Identification of stable overexpression and knockdown of DLX3 in BMSCs

Stable overexpression of DLX3 (DLX3) and knockdown of DLX3 (ShDLX3) in BMSCs were generated as described above. Green fluorescent protein (GFP) as a tag of lentiviruses was observed in >90% of the stably infected BMSCs by fluorescent microscopy (Fig. 1A). RT-qPCR and Western blotting showed that DLX3 mRNA and protein levels were significantly increased in the DLX3-overexpression group and decreased in the DLX3-knockdown group compared with the negative controls (DLX3-NC and ShDLX3-NC) (Fig. 1B and C). This indicated that BMSCs successfully overexpressed or knocked down DLX3.

### 3.2. The effect of DLX3 on osteogenic differentiation of BMSCs

To assess the effect of DLX3 on the osteogenic differentiation of BMSCs, cells were infected with negative control (DLX3-NC and ShDLX3-NC), DLX3-overexpression, or ShDLX3 lentiviruses and then induced to differentiate in ODM. Osteoinduction for 7 days, ALP staining and activity analysis showed that DLX3 overexpression significantly promoted ALP expression and activity, whereas DLX3 knockdown significantly decreased ALP expression and activity (Fig. 2A). Osteoinduction for 21 days, ARS analysis

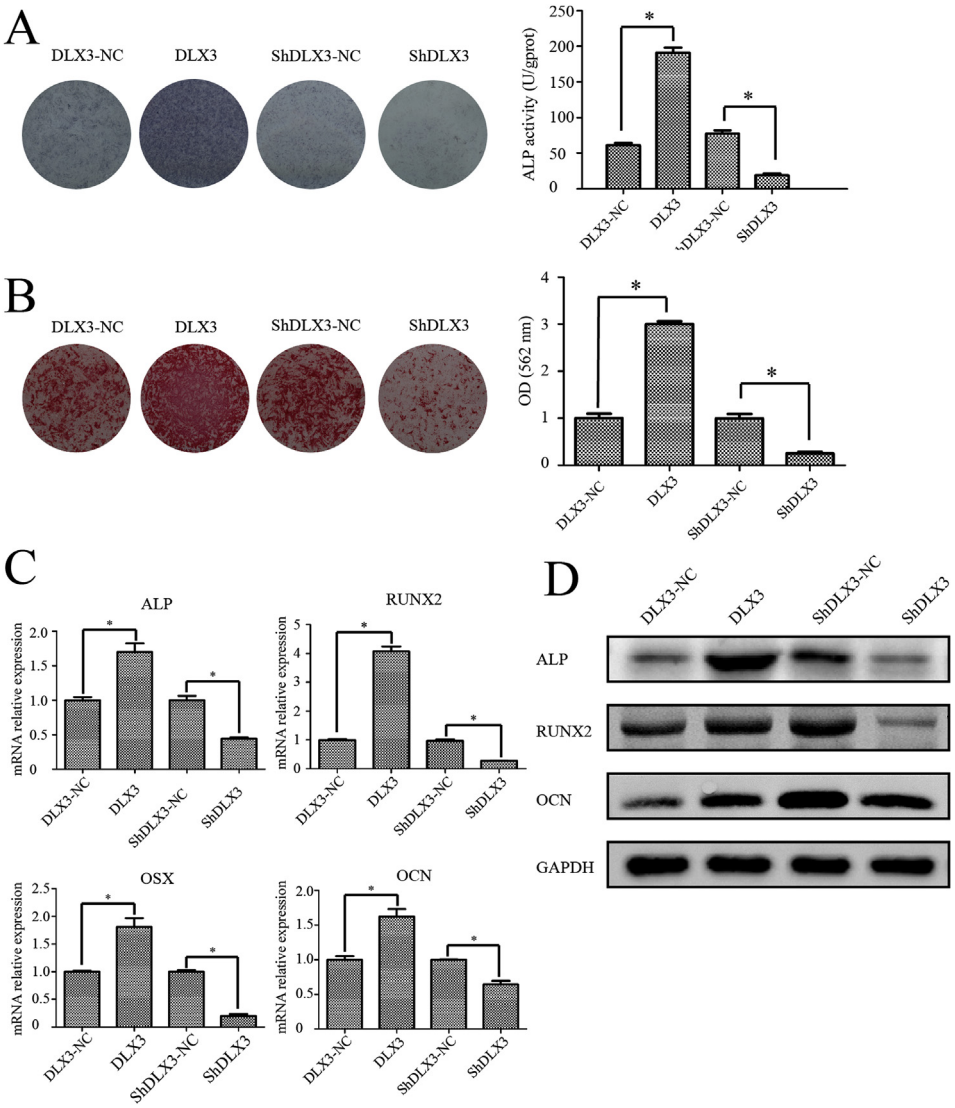


**Fig. 2.** The effects of DLX3 on the osteogenic differentiation of BMSCs. (A) ALP expression and activity after induction of the cells in ODM for 7 days. (B) Calcified matrix and quantification of Alizarin red staining intensity after induction in ODM for 21 days. (C and D) The expression of osteogenesis-related genes was detected by RT-qPCR and Western blotting at 7 days after osteoinduction (*$p < 0.05$, n = 3). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

showed that the DLX3-overexpression cells formed the most calcified matrix, whereas DLX3 knockdown reduced the formation of calcified matrix (Fig. 2B). Overexpression of DLX3 also increased the mRNA expression of osteogenic-related genes, including ALP, RUNX2, OSX, and OCN, whereas DLX3 knockdown had the opposite effects on osteogenic differentiation (Fig. 2C). Furthermore, ALP, RUNX2, and OCN proteins showed a similar tendency as their respective mRNAs (Fig. 2D). Our data supported DLX3 is a positive regulator in osteogenic differentiation of BMSCs.

### 3.3. DLX3 regulation of osteogenesis of BMSCs involving in the Wnt/β-catenin pathway

To explore the molecular mechanism underlying how DLX3 functions in the regulation of BMSC osteogenic differentiation, we evaluated the effect of DLX3 on the Wnt/β-catenin pathway during osteogenesis of BMSCs. DLX3 overexpression significantly promoted β-catenin expression in mRNA and protein levels, whereas knockdown of DLX3 had the opposite effects (Fig. 3A and B). Additionally, activated β-catenin (Ser45) was increased in the DLX3-overexpression group and reduced in the knockdown group (Fig. 3B). To further confirm Wnt/β-catenin pathway involving in this process, the DLX3-knockdown cells were treated with a Wnt agonist (1 μM/mL, S8178, Selleckchem, Houston, TX, USA). ALP staining and ARS analysis showed that the Wnt agonist promoted osteogenesis, significantly (Fig. 3C and D). RT-qPCR and Western blotting analysis showed that the Wnt agonist increased osteogenic-related gene expression (Fig. 3E and F). Our data

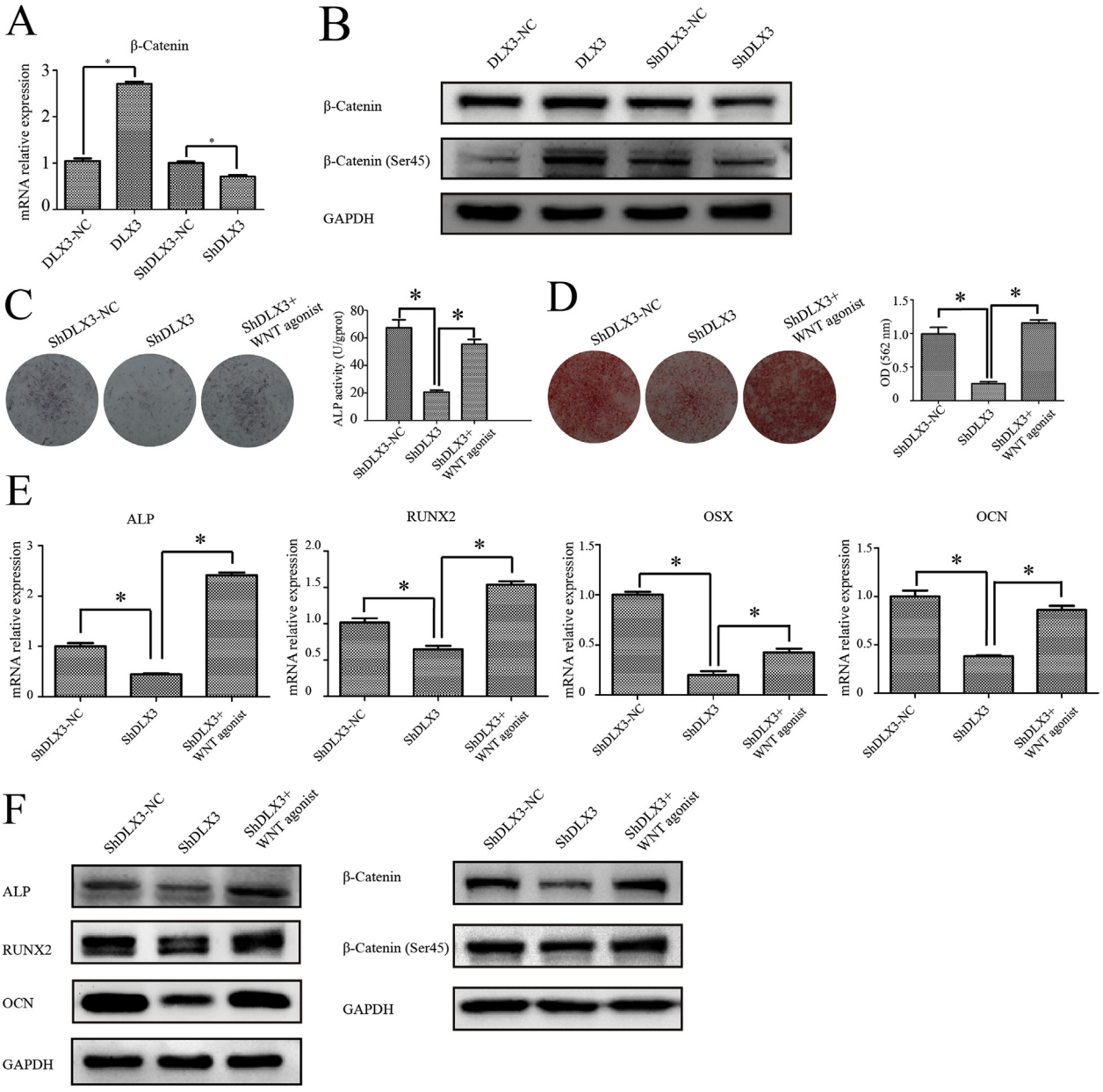


**Fig. 3.** DLX3 regulation of osteogenesis of BMSCs via the Wnt/β-catenin pathway. (A and B) β-catenin mRNA and protein levels during osteogenic differentiation of BMSCs in the DLX3-overexpression and -knockdown groups. β-catenin (Ser45) indicated activated β-catenin at 7 days after osteoinduction. (C and D) ALP expression, and activity and calcified matrix in DLX3-knockdown cells treated with a Wnt agonist at 7 and 21 days after osteoinduction. (E) The expression of osteogenesis-related genes was determined by RT-qPCR in the DLX3-knockdown cells treated with the Wnt agonist at 7 days after osteoinduction. (F) The ALP, RUNX2, OCN, β-catenin, and activated β-catenin (Ser45) protein levels determined by Western blotting in DLX3-knockdown cells treated with the Wnt agonist at 7 days after osteoinduction (*$p < 0.05$, n = 3).

indicated that DLX3 regulated osteogenic differentiation of BMSCs through Wnt/β-catenin pathway.

### 3.4. DLX3 knockdown promotes DKK4 expression by decreasing enrichment of H3K27me3 at the promotor region

To further identify how DLX3 regulates Wnt/β-catenin pathway during osteogenesis of BMSCs, we performed high throughout assay. DKK4, an antagonist of Wnt/β-catenin pathway, underwent the greatest change among Wnt/β-catenin pathway-related gene (data not shown). RT-qPCR and Western blotting assays showed that expression of DKK4 was increased in ShDLX3 group (Fig. 4A and B). Studies reveal H3K27me3 regulate Wnt/β-catenin pathway via DKK1 expression [21,22]. To further test whether DLX3 knockdown promotes DKK4 transcription by reducing H3K27me3 levels at the promotor region, we examined H3K27me3 enrichment at the DKK4 promoter using ChIP assays. There is no H3K27me3 enrichment at the DKK4 promotor in the DLX3-NC and ShDLX3-NC groups using IgG as a control. DLX3 knockdown decreased H3K27me3 enrichment at the DKK4, whereas DLX3 overexpression increased H3K27me3 enrichment at the DKK4 promotor region (Fig. 4C). These data indicated that DLX3 regulate DKK4 expression through H3K27me3 enrichment in the promotor region of DKK4.

## 4. Discussion

DLX3 is an important transcription factor involved in the osteogenic differentiation of mesenchymal stem cells [8]. However, the mechanism by which DLX3 regulates the osteogenic differentiation of mesenchymal stem cells is not clear. Here, our present investigation implied that DLX3 regulated the osteogenic differentiation of BMSCs via the Wnt/β-catenin pathway through histone modification of DKK4.

DLX3 is a crucial factor of bone formation [23]. Our results are consistent with the previous research that DLX3 overexpression promoted osteogenesis of BMSCs [8]. Whereas, how DLX3 regulation of osteogenesis of BMSCs needs further study. The Wnt/β-catenin pathway is important for the regulation of osteogenic differentiation [24]. *In vitro*, the activated Wnt/β-catenin pathway promoted osteogenesis of aortic valve interstitial cells [25]. Inhibition of the Wnt/β-catenin pathway in mice decreased bone formation [26]. In this study, we found DLX3 promoted the expression and activity of β-catenin, whereas DLX3 knockdown decreased both. Treatment with a Wnt agonist rescued the osteogenic potential of DLX3 knockdown cells. Therefore, DLX3 modulates osteogenesis by BMSCs via the Wnt/β-catenin pathway. However, Zhan et al. reported that DLX3 promoted proliferation of dental pulp stem cells by inactivation of Wnt/β-catenin pathway [27]. It may be associated with different types of cells or condition of differentiation.

DKK4 is a newly discovered member of the DKK family and is an antagonist of the Wnt/β-catenin pathway that binds LRP5/6 [28]. Compared with DKK1, DKK4 has not been well studied. Hiramitsu et al. reported that DKK4 has negative effects on the osteogenesis of osteoblasts via inhibition of the Wnt/β-catenin pathway [12]. Recently, Li et al. revealed that a similar result that upregulation of Dkk4 inhibited the Wnt/β-catenin pathway in disuse osteoporosis mice [29]. It indicates that DKK4 is a negative regulator of osteogenesis. In this study, DKK4 expression was increased in the DLX3-knockdown group and decreased in the DLX3-overexpression group. Combined with high throughout data, our results indicate that DLX3 is a negative regulator of DKK4. However, the details of how DLX3 regulates DKK4 are not clear. Histone modifications have been reported involving in regulating Wnt/β-catenin pathway. H3K27me3 is an important histone modification and regulate Wnt/β-catenin pathway via DKK1 [30,31]. Thus, we wondered that whether DLX3 regulates DKK4 expression via H3K27me3 enrichment. Of note, our results demonstrated greater H3K27me3 enrichment in the DKK4 promoter region in the DLX3-overexpression group and lower enrichment in the DLX3-knockdown group. These results indicate that DLX3 regulates the enrichment of HEK27me3 at the DKK4 promoter, affecting DKK4 transcription.

Overall, our explored the intrinsic mechanism by which DLX3 regulates osteogenic differentiation of BMSCs. Our data implied that DLX3 regulated Wnt/β-catenin pathway through histone modification of DKK4 during the osteogenic differentiation of BMSCs.

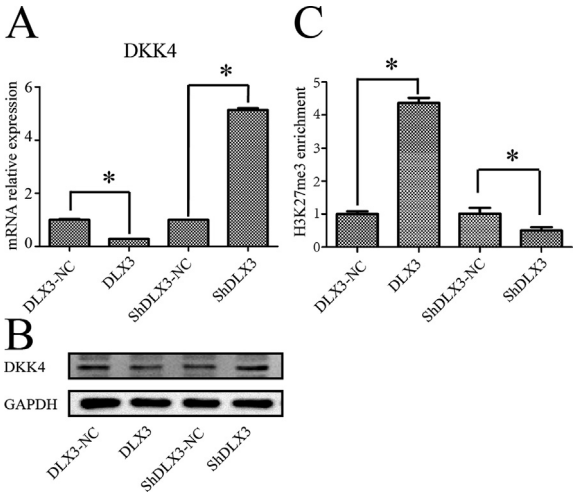


**Fig. 4.** DLX3 regulation of DKK4 via the histone modification. (A and B) DKK4 mRNA and protein levels in the DLX3-NC, DLX3, ShDLX3-NC, and ShDLX3 groups at 7 days after osteoinduction. (C) H3K27me3 levels at the DKK4 gene promoter region detected by ChIP assays in the negative control (DLX3-NC and ShDLX3-NC), DLX3-overexpression (DLX3), and DLX3-knockdown (ShDLX3) groups at 7 days after osteoinduction ($^*p < 0.05$, n = 3).

## Declaration of interest

The authors have declared no conflict of interest.

## Acknowledgments

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.06.029.

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