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Dlx2 overexpression enhanced accumulation of type II collagen and aggrecan by inhibiting MMP13 expression in mice chondrocytes

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ABSTRACT

Genetic studies revealed a crucial role of Distal-homebox (Dlx) genes in skeletal development, and our previous study demonstrated overexpressing Dlx2 in neural crest cells led to abnormal cartilage structure, including ectopic cartilage in the maxillary region and nasal bone in mice. The aim of this study was to investigate how *Dlx2* overexpression affects chondrogenesis in mouse chondroblast cell line TMC23 and the underlying mechanism. We first demonstrated that *Dlx2* expression was upregulated during chondrogenesis in TMC23 cells. Moreover, forced overexpression of *Dlx2* in TMC23 cells led to increased accumulation of aggrecan and type II collagen, markers of early chondrocyte differentiation, but had little effect on mRNA and protein levels of *Aggrecan* and *Col2α1*, type II collagen gene. Importantly, *Dlx2* overexpression decreased mRNA and protein levels of *MMP13*, a major collagenase degrading aggrecan and type II collagen during late stages of chondrogenesis. Luciferase-reporter and Chromatin-immunoprecipitation analysis demonstrated that *MMP13* promoter contained two Dlx2-response elements, and Dlx2 inhibited *MMP13* expression by directly binding to these two elements. Based on these observations, we propose that forced overexpression of *Dlx2* enhances early chondrocyte differentiation by increasing accumulation of type II collagen and aggrecan, but interferes later stages of chondrocyte differentiation through inhibiting *MMP13* expression.

1. Introduction

The distal-less homeobox (Dlx) gene family is one of the crucial homeobox families in mammalian development, and it contains six members (Dlx1-6), three convergent pairs (Dlx1/2, Dlx3/4, and Dlx5/6) structurally and functionally expressing in the first and second branchial arch region during vertebrate development [1]. *Dlx2* is upregulated during days 9.5 and 10.5 of embryo development in the first branchial arch in mice. This upregulation is essential for the development of the primordium, since it results in the development of the maxillofacial skeletal patterns and $Dlx2^{-/-}$ deficient mice exhibits craniofacial malformation and died immediately after birth [2]. Therefore, Dlx2 plays an important role in craniofacial development.

Endochondral ossification involves progressive differentiation of proliferative chondrocytes to hypertrophic cells. During terminal chondrocyte differentiation, cartilage converts into a vascularized tissue, which supports atrix remodeling, further cartilage calcification and recruitment of osteogenic cells [3]. Chondrocyte, the only cell type in the cartilage, secretes type II collagen, type X, aggrecan and other proteins to constitute extracellular matrix that maintains the normal structure of cartilage [4]. Dlx genes are expressed in chondrocytes at various stages of endochondral ossification. Sharing strong sequence similarity with Dlx2, Dlx3 and Dlx5 are expressed in chondroprogenitor cells, pre-hypertrophic chondrocytes and hypertrophic chondrocytes [5,6]. Our previous study demonstrated that overexpressing *Dlx2* in neural crest cells (NCCS) leads to ectopic cartilaginous, abnormal skeletal structure in maxillary and mandibular region and less mineralized craniofacial bones in mice [7]. However, little is known about the molecular mechanism of how Dlx2 regulates chondrocytes differentiation.

Based on those previous findings, we hypothesized that *Dlx2* overexpression might have an effect on chondrocyte differentiation. In this study, we examined TMC23 chondrocytes to investigate the effects of *Dlx2* overexpression on chondrogenesis and the mechanisms underlying these effects. We first found that forced

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overexpression of *Dlx2* in TMC23 cells enhanced accumulation of aggrecan and type II collagen, one of the major component of cartilage. Unexpectedly, both mRNA and protein levels of aggrecan and type II collagen were not significantly affected by *Dlx2* overexpression. Importantly, further investigation on MMP13, collagenase-3, revealed that both protein level of MMP13 in culture medium and cellular transcription level of MMP13 were decreased by *Dlx2* overexpression in TMC23 chondrocytes. MMP13, expressed and secreted by chondrocytes, not only degrades type II collagen but also cleaves aggrecan, playing a critical role in extracellular matrix (ECM) destruction during late stages of chondrongenic differentiation [8]. Moreover, we identified two Dlx2-response elements in *MMP13* promoter and provided evidence that forced overexpression of *Dlx2* inhibited *MMP13* expression by directly binding to its promoter, via luciferase-reporter assay and chromatin-immunoprecipitation (ChIP) assay. Based on these observations, we propose that forced overexpression of *Dlx2* enhances early chondrocyte differentiation by increasing accumulation of type II collagen and aggrecan, but interferes later stages of chondrocyte differentiation through inhibiting *MMP13* expression.

2. Materials and methods

2.1. Cell cultures

A TMC23 cell line was a gift from Professor Steve E. Harris from University of Texas Health Science Center at San Antonio, an HEK 293 T cell line from American Type Culture Collection (Rockville, Md, USA). TMC23 cells were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) supplied with 10% fetal calf serum. To induce terminal differentiation, TMC23 cells were treated with 4 mM β-glycerol phosphate (Sigma-Aldrich Corp (St. Louis, MO, USA)) and 50 μg/ml ascorbic acid (Sigma-Aldrich), as described previously [5]. HEK 293 T cells were cultured as described previously [9].

2.2. Lentivirus construction and transduction

The lentiviral expression system for Dlx2 overexpression was named as Lenti-DLX2 OE. The open reading frame of Dlx2 in mice (NM_010054) was cloned into pL/IRES/GFP vector (Novobio, Shanghai, China), generating pL/IRES/GFP-DLX2. The control lentiviral expression system was named as Lenti-CTRL and used as mock control. HEK 293 T cells were transfected with vectors listed above. Both transfection and lentivirus transduction were conducted as described previously [5].

2.3. Alcian blue staining, reverse transcription quantitative real-time PCR (RT-qPCR) and western blot analysis

Alican blue staining was performed as described previously [10]. Briefly, cells were first fixed with 100% methanol, and a solution containing 0.1% Alcian blue 8GX was applied to the cells for 2 h followed by washing three times with phosphate-buffered saline. RT-qPCR and western blot analysis was conducted as described previously [10]. Total mRNA was extracted from cultured cells using TRIzol RNA isolation reagent (Takara, Tokyo, Japan). cDNA was amplified using a STEP ONE PLUS real-time PCR system (Applied Biosystems, San Diego, CA). All the primers used in this study were listed in Table S1. As for western blotting, anti-aggrecan (1:1000; Abcam), anti-α-tubulin (1:2000; Abcam), anti-type II collagen (1:1000; Abcam) and anti-Dlx2 (1:1000; Abcam) antibodies were used for detection of aggrecan, α-tubulin, type II collagen and Dlx2, respectively.

2.4. Luciferase-reporter assay

The promoter region of MMP13 was synthesized and cloned into the pGL3-basic vector (Promega), thus generating pGL3-MMP13. To produce constructs containing mutations in MMP13 promoter, we performed site-direct mutagenesis assay as described previously [11]. The open reading frame of Dlx2 (NM_010054) was cloned into pCMV-FLAG, thus generating pCMV-Dlx2-FLAG for overexpressing Dlx2. Transfection and luciferase assay was conducted according to previous study [11].

2.5. ChIP, confocal laser-scanning microscopy and immunocytochemistry

ChIP assay was performed in accordance with a standard protocol [12]. Purified DNA was analyzed by qPCR, and the primers used in ChIP-qPCR was listed in Table S1. The confocal laser-scanning microscopy was carried out according to a previous immunofluorescence protocol [13]. Anti-Dlx2 antibody (1:250 dilution in blocking buffer, Abcam) and anti-MMP13 (1:250 dilution in blocking buffer, Abcam) antibodies were used for detection of Dlx2 and MMP13. An antifade reagent with DAPI (Thermo Fisher) was used for detection of nucleus. Cells on coverslips were examined with FV1000 confocal microscope (Olympus). Immunocytochemistry procedure for localizing type II collagen are identical to a previous study [14]. Briefly, cells were seeded on coverslips for 48 h followed by fixed with cold acetone. After rinsing in TBS for three times, cells were incubated antibody against anti-type II collagen (1:100 dilution, Abcam) for 1 h. An HRP-conjugated anti-mouse antibody was added to visualize type II collagen. To visualize the nuclei, cells were counterstained with hematoxylin.

2.6. ELISA

The production of MMP13 in cell culture was determined using commercially available ELSIA kits, according to the previous study [15].

3. Results

3.1. Endogenous Dlx2 expression in TMC-23 cells

To evaluate *Dlx2* expression during terminal differentiation, we treated TMC23 cells with chondrogenic induction medium. The presence of both *Dlx2* mRNA and protein was measured in TMC23 cells with RT-qPCR and western blot analysis. RT-qPCR results showed that *Dlx2* expression was rapidly induced by chondrogenic treatment, with a 3.1-fold induction occurring at 6 h after the treatment. However, after 1, 3 and 7 days of culture in chondrogenic-induction medium, *Dlx2* levels was not different from that in cells cultured in normal culture medium (Fig. 1A). Consistently, western blot analysis demonstrated that protein level of Dlx2 increased at 1 h following chondrogenic treatment, and decreased after 1 day of culture in chondrogenic-induction medium (Fig. 1B). These findings revealed that *Dlx2* was upregulated in TMC23 cells during terminal differentiation.

3.2. Transduction of TMC23 with Dlx2-overexpressing lentivirus

To investigate the effect of *Dlx2* in chondrocyte differentiation, we utilized a clonal chondroblastic cell line, TMC23 [16]. Cultured TMC23 cells were transduced with Lenti-DLX2 OE lentivirus for stable *Dlx2* overexpression. Parallel TMC23 cells were also transduced with Lenti-CTRL lentivirus as mock control. RT-qPCR analysis revealed that Lenti-DLX2 OE-transduced TMC23 cells displayed a

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