Am J Transl Res 2024;16(4):1454-1467
www.ajtr.org /ISSN:1943-8141/AJTR0156167

Original Article

Ddx5 participates in regulation of *Col10a1* expression and chondrocyte hypertrophic differentiation *in vitro*

Tiaotiao Han[1,2], Tianxiang Zhu[1,2], Huiqin Bian[1,2], Jinnan Chen[1,2], Yaojuan Lu[1,2,3], Junxia Gu[1,2], Tong-Chuan He[5], Longwei Qiao[4], Qiping Zheng[1,2,3,5]

[1]Department of Laboratory Medicine, School of Medicine, Jiangsu University, Zhenjiang 212013, Jiangsu, China; [2]Department of Hematological Laboratory Science, Jiangsu Key Laboratory of Medical Science and Laboratory Medicine, School of Medicine, Jiangsu University, Zhenjiang 212013, Jiangsu, China; [3]Shenzhen Walgenron Bio-Pharm Co., Ltd., Shenzhen 518118, Guangdong, China; [4]The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou 215008, Jiangsu, China; [5]The Molecular Oncology Laboratory, Department of Orthopaedic Surgery and Rehabilitation Medicine, The University of Chicago Medical Center, Chicago, IL 60637, USA

Received February 27, 2024; Accepted March 27, 2024; Epub April 15, 2024; Published April 30, 2024

Abstract: Background and aims: The type X collagen gene (*Col10a1*), is a specific molecular marker of hypertrophic chondrocytes during endochondral ossification. *Col10a1* expression is known to be influenced by many regulators. In this study, we aim to investigate how DEAD-box helicase 5 (DDX5), a potential binding factor for *Col10a1* enhancer, may play a role in *Col10a1* expression and chondrocyte hypertrophic differentiation *in vitro*. Methods: The potential binding factors of the 150-bp *Col10a1* cis-enhancer were identified with the hTFtarget database. The expression of DDX5 and COL10A1 was detected by quantitative real-time PCR (qRT-PCR) and Western blot in chondrogenic ATDC5 and MCT cell models with or without *Ddx5* knockdown or overexpression. Dual-luciferase reporter assay and chromatin immunoprecipitation (ChIP) were performed to determine the interaction between DDX5 and the *Col10a1* enhancer. The effect and mechanism of DDX5 on chondrocyte differentiation and maturation was evaluated by alcian blue, alkaline phosphatase (ALP), and alizarin red staining in ATDC5 cell lines with stable knockdown of *Ddx5*. Results: DDX5 was identified as a potential binding factor for the *Col10a1* enhancer. The expression of DDX5 in hypertrophic chondrocytes was higher than that in proliferative chondrocytes. Knockdown of *Ddx5* decreased, while overexpression of *Ddx5* slightly increased COL10A1 expression. DDX5 promotes the enhancer activity of *Col10a1* as demonstrated by dual-luciferase reporter assay, and the ChIP experiment suggests a direct interaction between DDX5 and the *Col10a1* enhancer. Compared to the control (NC) group, we observed weaker alcian blue and ALP staining intensity in the *Ddx5* knockdown group of ATDC5 cells cultured both for 7 and 14 days. Whereas weaker alizarin red staining intensity was only found in the *Ddx5* knockdown group of cells cultured for 7 days. Meanwhile, knockdown of *Ddx5* significantly reduced the level of runt-related transcription factor 2 (RUNX2) in related ATDC5 cells examined. Conclusions: Our results suggest that DDX5 acts as a positive regulator for *Col10a1* expression and may cooperate with RUNX2 together to control *Col10a1* expression and promote the proliferation and maturation of chondrocytes.

Keywords: Chondrocyte differentiation and hypertrophy, *Ddx5*, *Col10a1*, *Runx2*, ATDC5 cells

Introduction

Mammalian bone tissue is formed through two distinct processes: intramembranous and endochondral bone formation [1]. Endochondral bone formation is the main process which forms most mammalian bones, including the skull, longitudinal and appendage bones [1-3]. The differentiation and maturation of chondrocytes play a key role in the process of endochondral bone formation, in which chondrocytes promote longitudinal growth through proliferation, extracellular matrix secretion, and hypertrophy [4]. Subsequently, chondrocytes undergo apoptosis, blood vessels and osteoblasts invade the growing cartilage, and eventually bone is formed [4-6]. Therefore, chondrocyte hypertrophy is a terminal stage of chondrocyte differentiation, mediating the transformation of cartilage into bone [7]. Hypertrophic chondrocytes express specific markers, including osteopontin, matrix metalloproteinase-9/13

https://doi.org/10.62347/ZDB03541

(MMP9/13), vascular endothelial growth factor (VEGF), to prepare for osteogenesis [8-10]. In addition to their apoptotic fate, hypertrophic chondrocytes can also re-enter the cell cycle and differentiate into osteoblasts and other mesenchymal cells [7, 11, 12]. Such properties of hypertrophic chondrocytes make them very important targets for understanding bone growth and development.

As a specific marker of the hypertrophic chondrocytes, *Col10a1* normal expression is essential for the mineralization process [13]. Multiple studies have found that mutations and abnormal expression of *Col10a1* are often accompanied by abnormal hypertrophy of chondrocytes, which is common in many diseases [14-16]. Schmid metaphyseal chondrodysplasia (SMCD) is an autosomal dominantly inherited skeletal disorder caused by human *COL10A1* gene mutation [14, 17-19]. SMCD is characterized by short stature, irregular growth plate, and hip valgus [20, 21]. Osteoarthritis (OA) is a multifactorial disease characterized by progressive degeneration of articular cartilage. Subchondral bone pathological changes, such as osteophyte formation, sclerosis, and cyst formation are typical features of OA [22, 23]. During the progression of OA, articular chondrocytes undergo abnormal hypertrophy and the expression of *Col10a1* is abnormally increased [24-27]. Therefore, regulators controlling *Col10a1* expression during chondrocyte hypertrophy are expected to play crucial roles in skeletal development and bone-related diseases.

Multiple regulatory factors, including SRY-box transcription factor 9 (SOX9), RUNX2, tafazzin (TAZ), etc. [28-30] have been shown to control the specific expression of the *Col10a1* gene in hypertrophic chondrocytes. SOX9 is a known negative *Col10a1* regulator, where RUNX2 positively regulates *Col10a1* expression by directly binding to its promoters [31], and that *Col10a1* expression is not detectable in *Runx2* deficient mice [32]. At the same time, SOX9 has been shown to inhibit the expression of RUNX2 in chondrocytes, and thus coordinate bone formation [33, 34]. We have previously localized the mouse *Col10a1* cis-enhancer to a 150 bp distal promoter and found that RUNX2 binding to this enhancer is required but not sufficient for cell specific *Col10a1* enhancer activity [29, 35, 36]. Further bioinformatics analysis of this 150 bp *Col10a1* enhancer identified and validated many potential binding factors, including distalless homeobox5 (DLX5), myocyte enhancer factor 2A (MEF2A), signal transducer and activator of transcription 5A (STAT5A), etc. [37-39]. Interestingly, we also found potential DDX5 binding sites within the *Col10a1* cis-enhancer which are adjacent to the known RUNX2 binding sites. However, whether DDX5 plays a role in the regulation of *Col10a1* expression has never been elucidated.

DDX5, also called p68, is a member of the DEAD-box RNA helicase protein superfamily [40]. It is an ATP-dependent RNA helicase that is involved in many biological processes, including mRNA processing, ribosomal RNA maturation, and transcriptional regulation [41, 42]. DDX5 is known to participate in genesis and development, and has been recognized as a potential biomarker and therapeutic target for various cancers, including breast cancer, gastric cancer, and osteosarcoma [43]. As the interacting proteins of myogenic regulator (MyoD), DDX5 is required for normal differentiation of skeletal muscle cells and promotes formation of transcription initiation complex and the assembly of proteins required for chromatin remodeling [44]. Notably, DDX5 acts as a transcriptional co-activator of *Runx2*, an essential transcription factor for osteoblast differentiation and development, to promote osteogenic differentiation [45-47]. DDX5 was also shown to inhibit osteogenic differentiation of progenitor cells, suggesting the diverse mechanism of DDX5 in regulation of osteoblast differentiation [45]. Interestingly, the expression level of DDX5 was found higher in the hypertrophic zone than in the proliferative zone of equine fetal growth cartilage [48]. Combined with our finding that there is a potential DDX5 binding site (adjacent to the RUNX2 site) within the *Col10a1* enhancer, we speculate that DDX5 may coordinate with RUNX2 together to play a role in the regulation of *Col10a1* expression and chondrocyte hypertrophy differentiation during endochondral ossification.

Materials and methods

Bioinformatics analysis of the Col10a1 cis-enhancer

The potential binding factors of the 150 bp *Col10a1* cis-enhancer were predicted by the TRAP program as previously described

Table 1. Primers for real-time PCR

Gene	Forward (5'-3')	Reverse (5'-3')
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Col10a1	TCTGTGAGCTCCATGATTGC	GCAGCATTACGACCCAAGATC
Ddx5	CGGGATCGAGGGTTTGGTG	GCAGCTCATCAAGATTCCACTTC
Runx2	ATGCTTCATTCGCCTCACAAA	GCACTCACTGACTCGGTTGG
Sox9	GAGCCGGATCTGAAGAGGGA	GCTTGACGTGTGGCTTGTTC
Mmp13	CTTCTTCTTGTTGAGCTGGACTC	CTGTGGAGGTCACTGTAGACT
Col2a1	GGGAATGTCCTCTGCGATGAC	GAAGGGGATCTCGGGGTTG

(http://trap.molgen.mpg.de/cgi-bin/trap_form.cgi) [35, 49]. The human transcription factor database (http://bioinfo.life.hust.edu.cn/hTFtargetprediction) was also utilized to predict and confirm the transcription factors and their binding sites of target genes for the same 150 bp *Col10a1* enhancer [50].

Cell culture

Three cell lines were used in this study. ATDC5 cells were isolated from mouse teratocarcinoma cells, and after adding insulin to the medium, chondroid cell aggregates were formed to express chondrocyte-specific proteoglycans and type II collagen [51]. These cells may differentiate into hypertrophic chondrocytes and express type X collagen with significantly increased alkaline phosphatase activity [52, 53]. After continued culture, the cell culture mineralizes and can be visualized by alizarin red staining [52]. These properties make ATDC5 cells an excellent *in vitro* model to study the mechanism of chondrocyte differentiation during endochondral bone formation [54]. The ATDC5 cells used in this experiment were donated by professor Teng of Nanjing University. ATDC5 cells were cultured at 37°C in DMEM/F-12 (1:1, Hyclone, USA) medium containing 5% fetal bovine serum (FBS, BI, Israel) and in a humidified incubator containing 5% CO_2. Hypertrophy of ATDC5 cells were induced by 1× insulin-transferrin-sodium selenite (ITS, Sigma, USA). MCT cells were obtained by immortalization of mouse chondrocytes with temperature-sensitive SV40 large T antigen [55]. When cultured at 32°C, MCT cells proliferate and express type II collagen and aggrecan, whereas when transferred to 37°C, MCT cells stop growing, enter a hypertrophic state, and begin to express hypertrophic chondrocyte-specific marker genes, including Col10a1 [55]. MCT cells were originally donated by the laboratory of Dr. de Crombrugghe, MD Anderson (Houston, USA). MCT cells were cultured at 32°C in Dulbecco's Modified Eagle Medium (DMEM, Hyclone, USA) containing 8% FBS with 8% CO_2 humidification. After growth to sub-confluence, MCT cells were switched to 37°C for 2-3 days to induce hypertrophy. 293T cells were cultured at 37°C and maintained in DMEM with 10% FBS in a humidified incubator containing 5% CO_2. The 293T cells were provided by professor Shao of Jiangsu University.

RNA isolation and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted from the cells using the Trizol Reagent (Vazyme, Nanjing, China) and cDNA was synthesized from total RNA using the PrimerScript™ RT kit (TaKaRa, Japan). The qRT-PCR was performed with the cDNA as a template using the SYBR Mixture (Tacara, Dalian, China) according to the manufacturer's instructions. Mouse β-actin was used as the internal control to normalize gene expression and the results were analyzed by the $2^{-\Delta\Delta Ct}$ method [56]. All primer sequences used in this study are listed in **Table 1**.

Western blot

Cellular proteins were extracted from cells harvested and lysed with RIPA buffer containing protease inhibitors (Beyotime, Jiangsu, China). Equal amounts of protein samples were separated by polyacrylamide gel and then transferred to PVDF membrane. Subsequently, the membrane was blocked in Tris buffered saline with 0.1% Tween-20 (TBS/T) containing 5% non-fat milk at room temperature for 1 hour, and then incubated with the primary antibodies at 4°C overnight. On the following day, the membranes were washed three times using TBS/T buffer, then incubated with secondary

antibodies (goat anti-rabbit or goat anti-mouse IgG) for 1 hour at room temperature. Finally, protein bands were detected via ECL system (Vazyme, Nanjing, China). Primary antibodies include anti-Collagen X (1:1000, Abcam, UK), anti-DDX5 (1:1000, HuaBio, China), anti-RUNX2 (1:1000, Abcam, UK), and anti-β-actin (1:1000, Beyotime, China) was used as an internal control.

Immunohistochemistry (IHC) analysis

The hind limbs of C57BL/6 mice were collected and fixed with 4% paraformaldehyde, decalcified with 10% EDTA, paraffin embedded in tissue, and finally the paraffin blocks were sliced. The slices were dewaxed and hydrated. They were then heated with citric buffer (PH 6.0) at 95°C for 20 min. In addition, the slices were soaked in 3% hydrogen peroxide solution and incubated for 25 min at room temperature to block endogenous peroxidase. After the slices were sealed with goat serum, the primary antibody was incubated overnight at 4°C, and the corresponding secondary antibody, HRP-labeled streptavidin and DAB solution were incubated on the second day successively. The sections were washed and stained with hematoxylin, then slices were sealed with neutral glue and the staining results were observed under a microscope (Nikon, Japan).

siRNA and plasmids transfection

The small interfering RNA sequences of mouse *Ddx5* are as follows: si*Ddx5*-1 (sense 5'-GGAUCAAAUAAGACCUGAUTT-3', antisense 5'-AUCAGGUCUUAUUUGAUCCTT-3'); si*Ddx5*-2 (sense 5'-CACUUUCUUUACACCUAAUTT-3', antisense 5'-AUUAGGUGUAAAGAAAGUGTT-3'); si*Ddx5*-3 (sense 5'-GCACAAUGGUAUGAACCAATT-3', antisense 5'-UUGGUUCAUACCAUUGUGCTT-3'); and scrambled sequence of negative control (sense 5'-UUCUCCGAACGUGUCACGUTT-3', antisense 5'-ACGUGACACGUUCGGAGAATT-3'). The interference sequences were designed and synthesized by GenePharma (Shanghai, China) and the plasmids, including pcDNA3.1/*Ddx5* and pcDNA3.1, were purchased from Suzhou Biology (Suzhou, China). Subsequently, si*Ddx5* and scrambled siRNA were transfected into ATDC5 and MCT cells using the Lipofectamine RNAiMax Reagent (Thermo Fisher Scientific, USA) according to the manufacturer's instructions. For plasmid transfection, cells were transfected with lipofectamine 3000 transfection reagent (Thermo Fisher Scientific, USA) and 1 μg of pcDNA3.1/*Ddx5* or pcDNA3.1 plasmid. After transfection, MCT cells were cultured with DMEM containing 8% FBS at 32°C for 6 h and then transferred to 37°C for another 2-3 days. ATDC5 cells were cultured in DMEM/F-12 containing 1% ITS for up to 7 days at 37°C. Cells were collected for subsequent experiments.

Dual-luciferase reporter assay

293T cells reaching approximately 70% confluence were plated into a 24-well plate and transfected using the liposome 3000 transfection kit (Thermo Fisher Scientific, USA). In each transfection assay, *Ddx5* overexpression plasmid or pcDNA3.1 and *Col10a1* promoter-luciferase reporter vector or pGL3 vector and pRL-TK were added according to the manufacturer's instructions. Cells were harvested after 48-h's transfection, and luciferase activities were measured via the Dual-Luciferase reporter system (Promega, Madison, WI, USA).

Chromatin immunoprecipitation (ChIP) assay

The ChIP experiments were performed using the One-Day Chromatin Immunoprecipitation Kits (Merck, USA). Approximately 1×10^7 ATDC5 cells were fixed with 1% formaldehyde solution, and cross-link of protein with DNA was according to the manufacturer's instructions. The chromatin fragments were cut into appropriate size using an ultrasonic cell crusher (JingXin, China). The chromatin complexes were treated with anti-DDX5 antibody (HuaBio, China) or normal mouse IgG. Subsequently, the immunoprecipitated DNA was purified. Finally, the purified DNA samples were used as templates for PCR and for agarose gel electrophoresis. The specific primer pairs of the 150-bp *Col10a1* enhancer sequences are as follows: sense 5'-CCTTCATAAAGTCACAGACCAGT-3'; antisense 5'-ATTGTAGAATCAGAGTATTTGCT-3'.

Lentiviral infection

shRNA-*Ddx5* and shRNA-NC were designed and constructed by OBiO (OBiO, China). Cells were seeded into 24-well plates, and viral infection was performed the next day when cell confluence reached 30%-40%. According to the pre-experimental protocol, 8 μl of virus were added

to each well. After 72 hours of viral infection, cells were observed under a fluorescence microscope to determine how efficiently the lentivirus infected the target cells. The cells were then screened with a concentration of 8 μg/ml of puromycin. Subsequently, the cells were grown in a medium containing 4 μg/ml of puromycin for subsequent experiments.

Alcian blue, ALP and alizarin red staining

ATDC5 cells were cultured and induced with ITS for 7 and 14 days and then stained. For alcian blue staining, ATDC5 cells were fixed with methanol at -20°C for 2 min, and then 0.1% alcian blue solution (Bomei, China) was added overnight at room temperature. Subsequently, the cells were washed with ddH_2O and air dry. ALP staining was performed with the alkaline phosphatase staining cAKP kit (Jiancheng, Nanjing, China). In brief, cells were fixed for 2-5 min, and then the dye solution was dropped sequentially, followed by regular ddH_2O wash and air dry. For alizarin red staining, cells were fixed with 95% ethanol for 10 min, they were then stained with 1% Alizarin Red (Solarbio, China) for 10 min and then washed with ddH_2O and air dried. The staining images were observed and taken with a Nikon Eclipse 80i microscope (Nikon Instruments Inc., NY, USA) at designated magnifications.

Statistical analysis

Statistical analysis of the data was performed by GraphPad Prism software version 6.0 using Student's t-test or one-way analysis of variance (ANOVA). The gray scale of protein bands and the intensity of staining results were normalized using Image J, and then analyzed by Student's t-test using GraphPad Prism software version 6.0. All the experiments were carried out with at least three independent replicates. $P < 0.05$ was considered statistically significant.

Results

Bioinformatic prediction

We previously reported the potential transcription factors and their binding sites within the mouse 150 bp *Col10a1* enhancer as predicted by the TRAP program [35, 49]. Here, we also searched for transcription factors and their binding sites within the same 150 bp *Col10a1* enhancer using the hTFtarget database. After input the sequences in FASTA format into the "prediction" box, we obtained a series of candidate transcription factors with *p*-values much less than 0.05 (**Figure 1A**, **1B**). Notably, one of the candidate TF DDX5, which we chose for this study, was previously shown to be higher in the hypertrophic zone than in the proliferative zone of equine fetal growth cartilage [48].

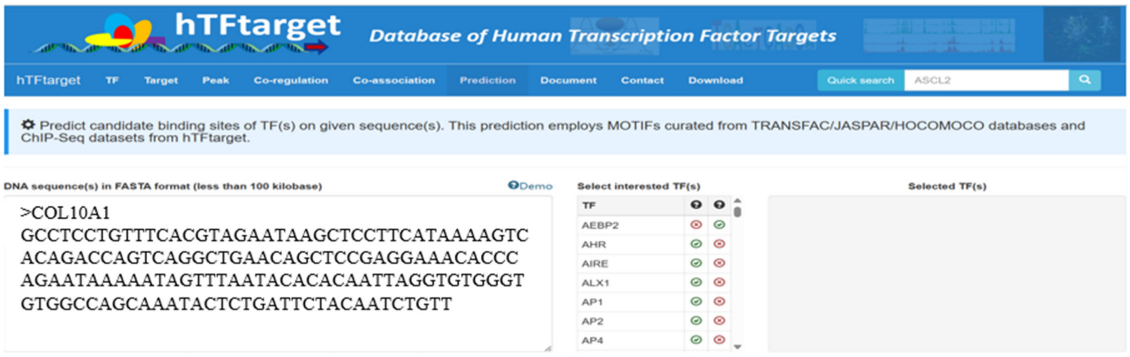
Basal expression of Ddx5 and Col10a1 in chondrocytes

Two chondrogenic cell models, ATDC5 and MCT cells were selected for the analyses [51, 52, 55]. ATDC5 cells were cultured in medium containing 1% ITS to induce hypertrophic differentiation. With the increase of ITS induction time, the mRNA and protein expression levels of COL10A1 were up-regulated and reached a peak on 14th day (**Figure 2A**). Meanwhile, qRT-PCR and Western blot showed that the mRNA and protein levels of DDX5 were also up-regulated and the trend was consistent with that of COL10A1 (**Figure 2A**). MCT cells were cultured at 32°C and switched to 37°C to induce the hypertrophic differentiation process *in vitro* [55]. The expression changes of DDX5 and COL10A1 were then examined at two temperatures by qRT-PCR and Western blot. The qRT-PCR results showed that the mRNA levels of *Col10a1* and *Ddx5* were up-regulated in hypertrophic MCT cells compared with proliferating MCT cells (**Figure 2B**). The Western blot results also showed that the expression of COL10A1 and DDX5 were increased in hypertrophic MCT cells (**Figure 2B**). IHC analysis indicated that COL10A1 and DDX5 showed positive staining in hypertrophic chondrocytes compared with proliferative chondrocytes (**Figure 2C**). The above results suggested that the expression of *Ddx5* is higher in hypertrophic chondrocytes than in the proliferative chondrocytes, which corresponds well with *Col10a1* expression in these chondrogenic cell models examined.

DDX5 up-regulates Col10a1 expression in vitro

To investigate the effect of DDX5 on *Col10a1* expression, we transfected pcDNA3.1/*Ddx5* plasmid and *Ddx5* small interference fragments in ATDC5 and MCT cells for validation.

A



B

TF	Source	Sequence name	Start	Stop	Strand	Score	P value	Q value	Matched motif
SPI1	hTFtarget	COL10A1	112	127	-	11.8714	3.31E-05	0.00892	TGCTGGCCACACCCAC
STAT5A	hTFtarget	COL10A1	109	120	-	12.0132	3.31E-05	0.00892	CACACCCACACC
MEF2A	hTFtarget	COL10A1	80	93	-	12.0746	3.32E-05	0.00799	AACTATTTTTATTC
SPI1	hTFtarget	COL10A1	112	127	+	11.4211	3.40E-05	0.00914	GTGGGTGTGGCCAGCA
E2F4	hTFtarget	COL10A1	113	123	-	11.5526	3.40E-05	0.00954	GGCCACACCCA
EGR1	hTFtarget	COL10A1	105	126	-	10	3.43E-05	0.00877	GCTGGCCACACCCACACCTAAT
E2F4	hTFtarget	COL10A1	112	123	+	11.1184	3.50E-05	0.0096	GTGGGTGTGGCC
BRD3	hTFtarget	COL10A1	109	124	-	11.597	3.55E-05	0.00957	TGGCCACACCCACACC
BRD2	hTFtarget	COL10A1	89	104	+	10.4868	3.56E-05	0.00907	TAGTTTAATACACACA
E2F4	hTFtarget	COL10A1	112	123	-	11.4342	3.59E-05	0.01	GGCCACACCCAC
ELK3	hTFtarget	COL10A1	112	127	+	11.0658	3.62E-05	0.00944	GTGGGTGTGGCCAGCA
SPI1	hTFtarget	COL10A1	112	127	-	10.4342	3.64E-05	0.0098	TGCTGGCCACACCCAC
NFATC1	hTFtarget	COL10A1	112	120	-	12.5263	3.66E-05	0.0102	CACACCCAC
ESR1	hTFtarget	COL10A1	49	60	+	11.9848	3.67E-05	0.0101	GTCAGGCTGAAC
SPI1	hTFtarget	COL10A1	111	126	-	11.3429	3.68E-05	0.00992	GCTGGCCACACCCACA
DDX5	hTFtarget	COL10A1	105	120	-	11.6494	3.72E-05	0.00962	CACACCCACACCTAAT
SPI1	hTFtarget	COL10A1	111	126	+	11.5658	3.73E-05	0.01	TGTGGGTGTGGCCAGC
HDAC6	hTFtarget	COL10A1	96	104	+	12.1974	3.79E-05	0.0103	ATACACACA
RELA	hTFtarget	COL10A1	66	80	+	11.7143	3.79E-05	0.0101	CGAGGAAACACCCAG
ZBTB33	hTFtarget	COL10A1	112	127	-	10.4211	3.80E-05	0.0103	TGCTGGCCACACCCAC
RUNX2	hTFtarget	COL10A1	106	121	-	11.8608	3.85E-05	0.00939	CCACACCCACACCTAA

Figure 1. Prediction of the candidate transcription factor binding sites by hTFtarget. A. hTFtarget analysis of the 150 bp *Col10a1* cis-enhancer. B. Candidate transcription factors and their binding sites identified by hTFtarget (*P*-values much less than 0.05 were recorded).

The results showed that transfection with *Ddx5*-siRNA into ATDC5 and MCT cells significantly down-regulated the mRNA and protein levels of COL10A1 (**Figure 3A**, **3B**). Meanwhile, the mRNA and protein levels of COL10A1 were significantly upregulated in the groups of ATDC5 and MCT cells with *Ddx5* overexpression compared to the control group transfected with pcDNA3.1 (**Figure 4A**, **4B**). These results suggested that DDX5 promotes *Col10a1* expression in hypertrophic chondrocytes *in vitro.*

Interaction between DDX5 and the Col10a1 enhancer

To determine the interaction between DDX5 and the *Col10a1* enhancer, we performed dual-luciferase reporter assay and ChIP experiment. As shown in **Figure 5A**, the mRNA and protein levels of DDX5 were significantly increased in 293T cells transfected with the pcDNA3.1/*Ddx5* plasmid. The enhancer activity of *Col10a1* was increased in cells transfected with pcDNA3.1/*Ddx5* and *Col10a1* enhanc-

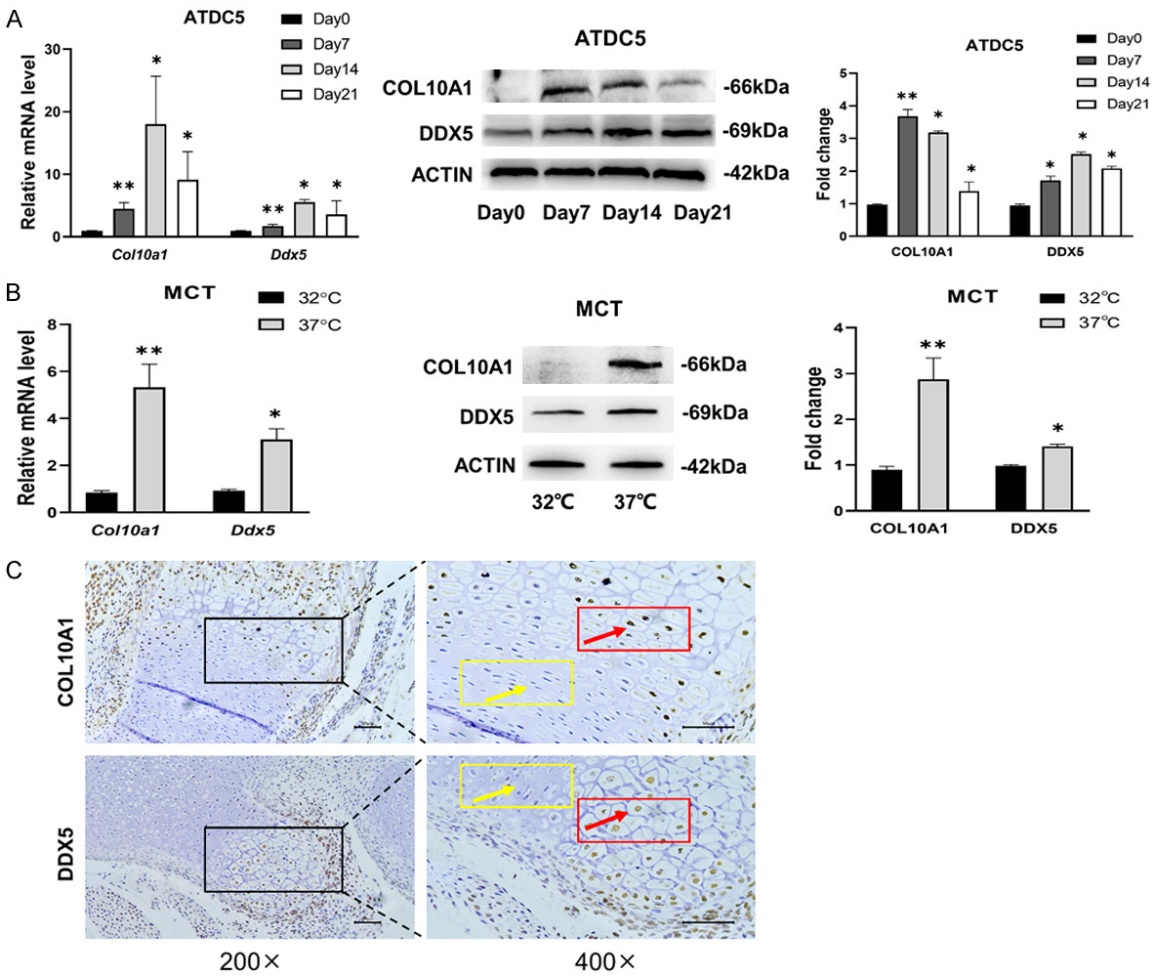


Figure 2. Basal expression of *Ddx5* and *Col10a1* in mouse chondrogenic cell models and in the growth plate. A. ATDC5 cells were cultured in medium with addition of 1% ITS for 0, 7, 14, and 21 days respectively. The mRNA levels of *Col10a1* and *Ddx5* were measured by qRT-PCR. Protein levels of COL10A1 and DDX5 were determined by Western blot. β-actin was used as an internal control and the densitometry analysis of the western results were as illustrated. B. MCT cells were cultured either in the proliferative phase at 32°C or further incubated in the hypertrophic phase at 37°C for an additional 2-3 days. The mRNA levels of *Col10a1* and *Ddx5* were measured by qRT-PCR. Protein levels of COL10A1 and DDX5 were determined by Western blot. β-actin was used as an internal control and the right panel shows the densitometry analysis results of the western blot. C. Immunohistochemical analysis detected the expression of COL10A1 and DDX5 in growth plate chondrocytes. Red arrows point to the positive staining cells, yellow arrows point to the negative staining cells, magnification =400×, scale bar =50 μm. $*P < 0.05$, $**P < 0.01$.

er luciferase reporter compared to cells transfected with pcDNA3.1 and the reporter control (**Figure 5B**). The binding of DDX5 with the *Col10a1* enhancer was further confirmed by ChIP experiment. The DNA fragments obtained by immunoprecipitation were amplified using PCR, and the amplified products were then subjected to agarose gel electrophoresis. Obvious PCR amplified bands can be seen in the precipitates with the anti-DDX5 antibody but not in the group with the control IgG (**Figure 5C**). These results indicated that DDX5 directly interacted with 150 bp *Col10a1* enhancer to promote *Col10a1* gene expression.

Effects of DDX5 on chondrocytes differentiation and maturation in vitro

Stable cell lines with knockdown of *Ddx5* were established by transfection of sh*Ddx5* into ATDC5 cells and cells with appropriate transfection efficiency were selected for further experiments (**Figure 6A**). The results of qRT-PCR and Western blot indicated that COL10A1 expression was decreased when *Ddx5* was knocked down (**Figure 6B**). ATDC5 stable cells lines were cultured in a medium containing 1% ITS for 7 and 14 days, and then the effect of DDX5 on chondrocyte differentiation and matu-

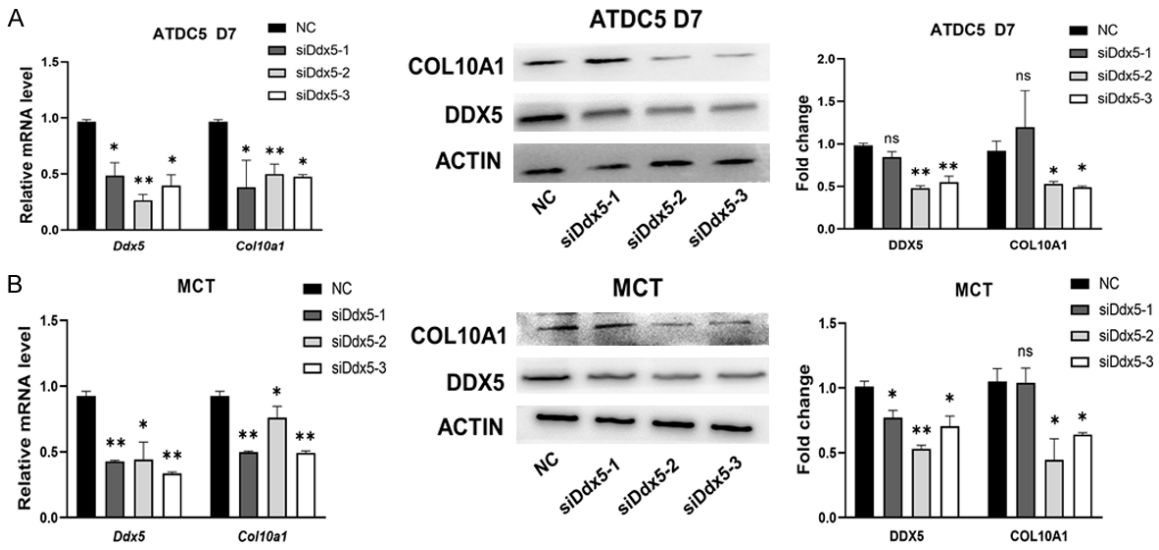


Figure 3. Knockdown of *Ddx5* decreased *Col10a1* expression *in vitro*. A. *Ddx5*-siRNA was transiently transfected into ATDC5 cells and induced in a medium containing 1% ITS for 7 days. The mRNA and protein levels of COL10A1 were detected by qRT-PCR and western blot when the expression of *Ddx5* was knocked down. β-actin was used as an internal control. B. The *Ddx5*-siRNA was transfected into MCT cells and then cultured at 37 °C for 2-3 days. The mRNA and protein levels of DDX5 and COL10A1 were detected by qRT-PCR and western blot. β-actin was used as an internal control. **P* < 0.05, ***P* < 0.01.

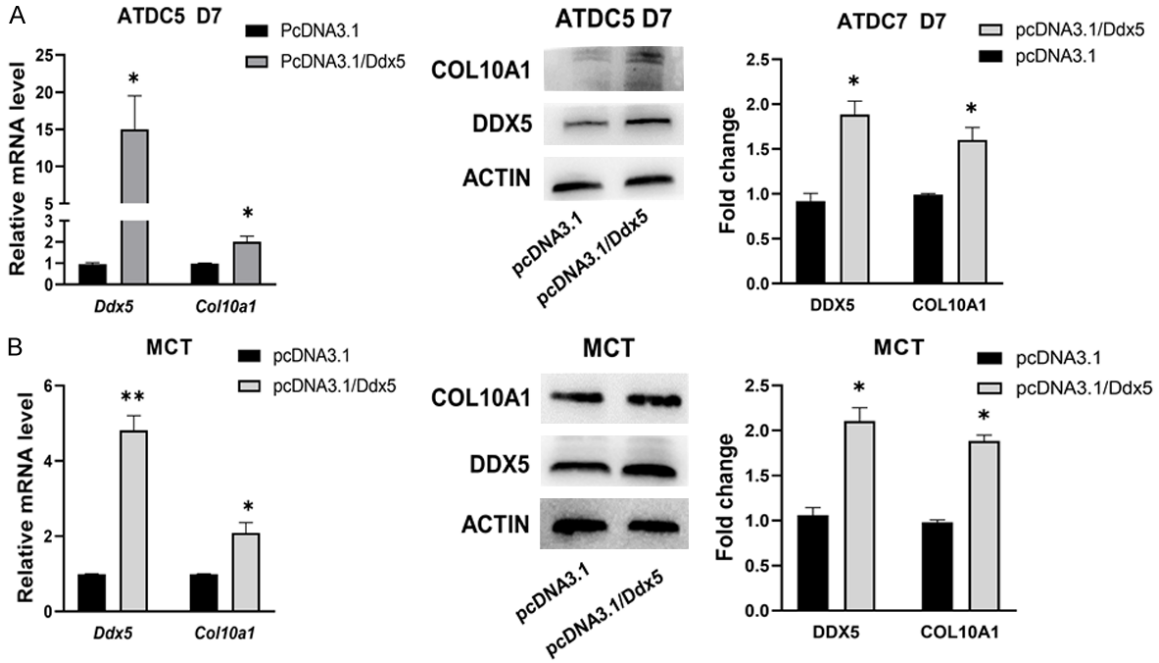


Figure 4. Overexpression of *Ddx5* increased *Col10a1* expression *in vitro*. A. ATDC5 cells were transfected with pcDNA3.1 or pcDNA3.1/*Ddx5* plasmids and then induced in a medium containing 1% ITS for 7 days. The mRNA and protein levels of COL10A1 were detected by qRT-PCR and western blot when DDX5 was overexpressed. β-actin was used as an internal control. B. The pcDNA3.1 or pcDNA3.1/*Ddx5* plasmids were transiently transfected into MCT cells and cultured at 37 °C for 2-3 days. The mRNA and protein levels of COL10A1 were detected by qRT-PCR and western blot when DDX5 was overexpressed. β-actin was used as an internal control. **P* < 0.05, ***P* < 0.01.

ration *in vitro* were evaluated by Alcian blue, ALP, and Alizarin red staining. The staining results showed that after 7 and 14 days of culture with induction, the intensity of Alcian blue

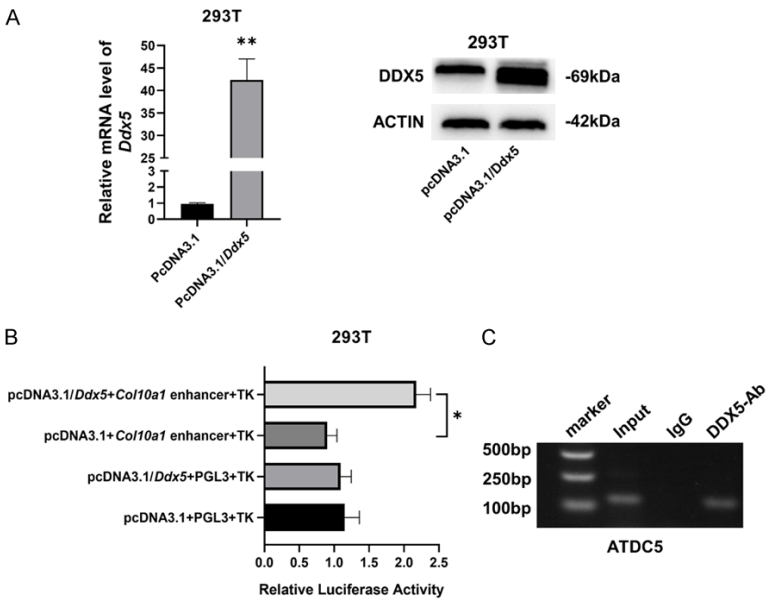


Figure 5. Mechanism of DDX5 regulating *Col10a1* expression. A. The qRT-PCR and western blot showed that the mRNA and protein levels of DDX5 were increased in the 293T cells transfected with the pcDNA3.1/*Ddx5* plasmid compared with that with the pcDNA3.1 control. β-actin was used as an internal control. B. The relative fluorescence activity of 293T cells was determined by dual luciferase reporter assay. C. The associated DNA fragments bound by anti-DDX5 antibody were detected by PCR and agarose gel electrophoresis. $*P < 0.05$, $**P < 0.01$.

and ALP staining of the ATDC5 cells with *Ddx5* knockdown was significantly reduced compared with the control (NC) group (**Figure 6C**). Whereas weaker alizarin red staining intensity was only found in the *Ddx5* knockdown group of cells cultured for 7 days, and there was no significant difference in the staining intensity between the two groups on the 14th day (**Figure 6C**).

Potential mechanism of DDX5 on regulation of Col10a1 expression

We next evaluated the correlation between *Ddx5* and the expression of some marker genes, including *Runx2*, *Sox9*, *Mmp13* and type II collagen gene (*Col2a1*) that are relevant to chondrocyte proliferation, hypertrophy, as well as matrix mineralization, in ATDC5 cells. The qRT-PCR and Western blot results showed that with stable knockdown of *Ddx5* in ATDC5 cells, when these cells were cultured with ITS induction for 7 and 14 days, both *Col10a1* and *Runx2* expression levels were significantly down-regulated compared to controls, while other relevant marker genes (*Sox9*, *Mmp13* etc.) did not change significantly (**Figure 7A**, **7B**). These results suggest a possible mechanism of Ddx5 on regulation of Col10a1 expression and, possibly on chondrocyte differentiation and maturation, which may be coordinated with RUNX2.

Discussion

We have been studying the regulation of *Col10a1* expression in hypertrophic chondrocytes both *in vitro* and *in vivo* for many years. Based on previous studies, we obtained a range of potential binding factors for the 150 bp *Col10a1* enhancer sequence through a Web-based hTFtarget database. Among these candidate binding factors, DDX5 has been shown to be more highly expressed in the hypertrophic zone than in the proliferative zone of horse fetal growth cartilage [48]. Therefore, in this study, we explored the potential roles and mechanisms of *Ddx5* in the regulation of *Col10a1* expression and chondrocyte hypertrophy using the above ATDC5 and MCT chondrogenic cell models.

The results showed that the expression of DDX5 and COL10A1 significantly increased when ATDC5 and MCT cells were induced to enter the hypertrophic state. Furthermore, IHC analysis of the mouse hindlimb revealed that the expression levels of COL10A1 and DDX5 were higher in the hypertrophic zone than in the proliferative zone. We also found that knockdown of *Ddx5* reduced the expression of *Col10a1* in chondrocyte models, while overexpression of *Ddx5* upregulated *Col10a1* expression. These results suggest that DDX5 is positively correlated with the expression of COL10A1, and thus plays a role in chondrocyte hypertrophic differentiation. To investigate the potential mechanism of DDX5 regulation of *Col10a1*, we performed dual-luciferase reporter assay and CHIP experiments and the results showed that DDX5 could enhance the activity of the *Col10a1* enhancer, possibly via direct interaction with this enhancer. Subsequently, we established the ATDC5 cell line with stable

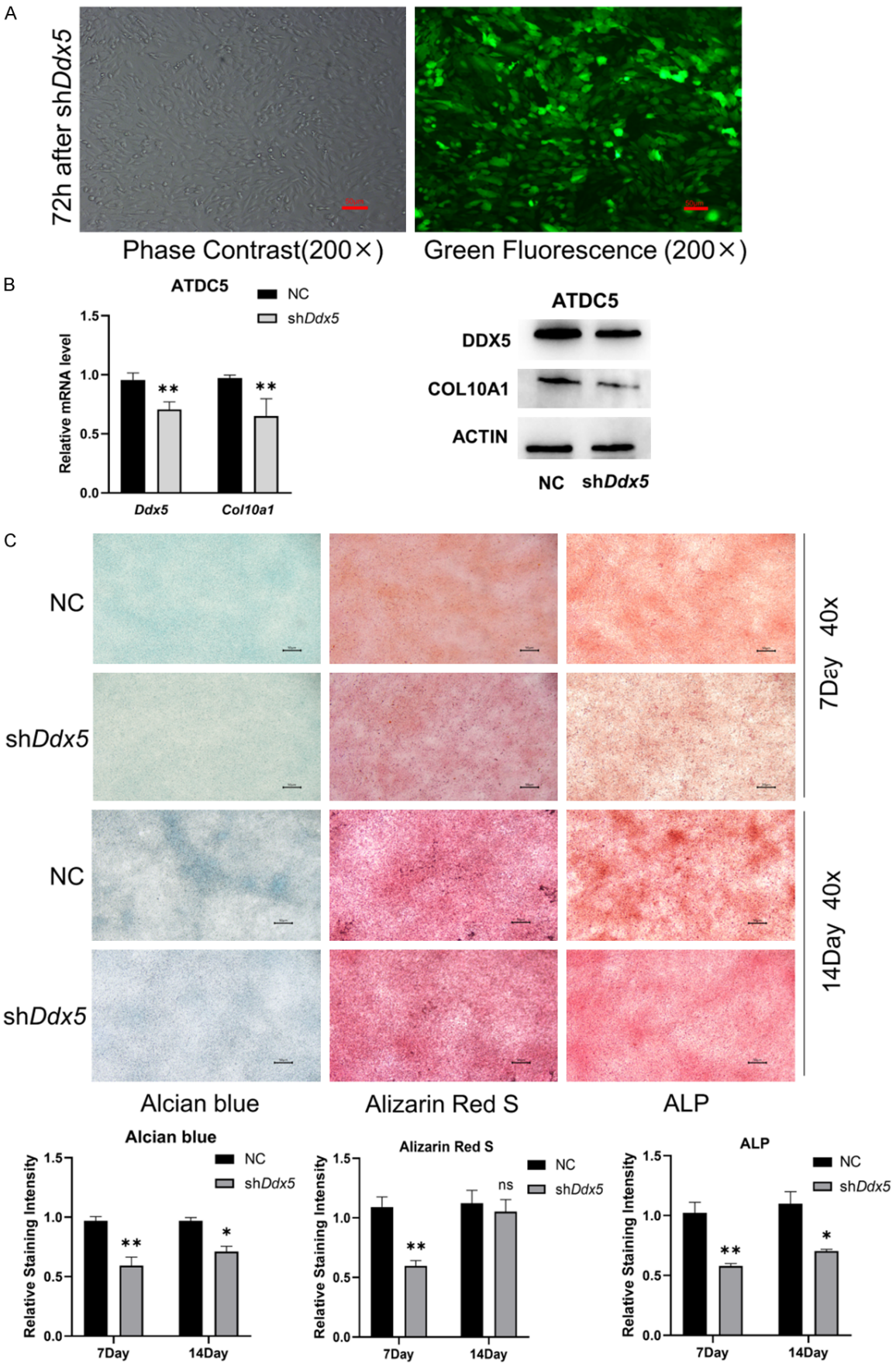

A
72h after shDdx5
Phase Contrast(200×)
Green Fluorescence (200×)
B
ATDC5
NC
shDdx5
Relative mRNA level
1.5
1.0
0.5
0.0
**
**
Ddx5
Col10a1
ATDC5
DDX5
COL10A1
ACTIN
NC
shDdx5
C
NC
shDdx5
NC
shDdx5
7Day 40x
14Day 40x
Alcian blue
Alizarin Red S
ALP
Alcian blue
Relative Staining Intensity
NC
shDdx5
**
*
7Day
14Day
Alizarin Red S
Relative Staining Intensity
NC
shDdx5
**
ns
7Day
14Day
ALP
Relative Staining Intensity
NC
shDdx5
**
*
7Day
14Day

Figure 6. Effects of DDX5 on chondrocyte differentiation and maturation *in vitro.* A stable cell line with *Ddx5* knockdown was constructed by transfection of sh*Ddx5* into ATDC5 cells. A. The transfection efficiency was observed under a fluorescence microscope. Magnification =200×, scale bar =50 μm. B. The mRNA and protein levels of COL10A1 in ATDC5 cells with Ddx5 stably knocked-down were detected by qRT-PCR and western blot. β-actin was used as an internal control. C. Stable cell lines with knockdown of *Ddx5* were induced in medium containing 1% ITS for 7 and 14 days, and stained with alcian blue, ALP, and alizarin red staining. Densitometry analyses of the staining intensity were shown underneath. Magnification =40×, scale bar =50 μm. **P* < 0.05, ***P* < 0.01.

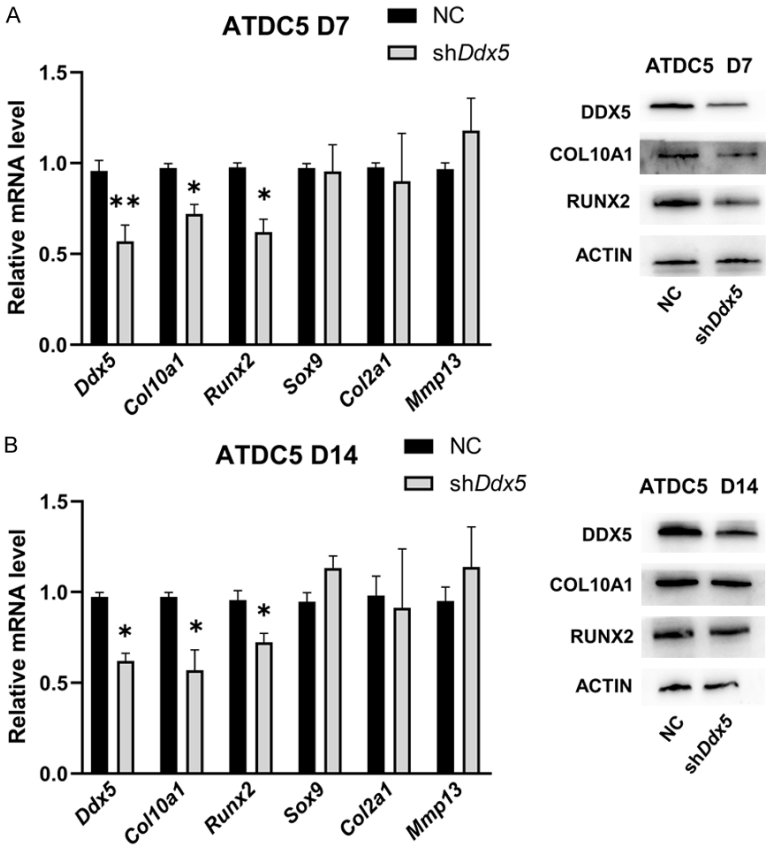


Figure 7. Potential impact and mechanism of *Ddx5* on chondrocyte differentiation. ATDC5 cells with *Ddx5* stably knocked-down were induced in medium containing 1% ITS for 7 and 14 days. The mRNA and protein levels of genes involved in chondrocyte differentiation and maturation were determined by qRT-PCR and western blot at day 7 (A) and day 14 (B). β-actin was used as an internal control. The results showed that both COL10A1 and RUNX2 expression was down-regulated when *Ddx5* was knocked-down. **P* < 0.05, ***P* < 0.01.

knockdown of *Ddx5*, and further explored the potential effects of DDX5 on chondrocyte differentiation *in vitro* by Alcian blue, ALP, and Alizarin red staining. The results support a positive role of DDX5 in chondrocyte proliferation and differentiation *in vitro*, but with only moderate effect on matrix mineralization. Interestingly, we have analyzed the expression of some marker genes, including *Runx2*, *Sox9*, *Mmp13*, etc. and found that *Runx2* expression was downregulated in ATDC5 cells when *Ddx5* was knocked down.

It is well established that RUNX2 is a known transcription factor that plays an essential role in osteoblast differentiation and chondrocyte maturation [57, 58]. In *Runx2*-deficient mice, bone formation was completely lacking, and the maturation of chondrocytes was severely disturbed [59]. *Runx2* can also promote the activity of alkaline phosphatase and matrix calcification in chondrocytes, thus affecting chondrocyte differentiation [60]. As to *Col10a1* expression, we have shown that Runx2 was an essential regulator of *Col10a1*, acting by binding directly to the *Col10a1* enhancer [29]. Previous studies have shown that *Runx2*-null mice had cartilaginous bone, but with severely reduced expression of *Col10a1* and disordered chondrocyte maturation [32, 58]. Meanwhile, abnormal expression of *Runx2* was also found in many bone-related diseases, such as OA and skeletal dysplasia, etc. [61-63]. Notably, multiple studies have shown that DDX5 and RUNX2 interact and co-activate to promote osteogenic differentiation, and are related to osteocalcin [45-47]. In addition, inhibition of DDX5 in C2C12 cells, which are myocyte and osteoblast progenitor cells, could accelerate osteoblast differentiation [45]. These findings suggest that DDX5 plays multiple roles in *Col10a1* expression, chondrocyte and osteoblast differentiation, and the underly-

ing mechanism is likely related to RUNX2 expression in these cell models.

In summary, our results support that DDX5 promotes *Col10a1* expression by enhancing the activity of the *Col10a1* cis-enhancer. DDX5 may cooperate with RUNX2 together to regulate the expression of *Col10a1* and promote the differentiation and maturation of chondrocytes during endochondral ossification *in vitro*.

Acknowledgements

This study was supported by the grant from the Jiangsu provincial key research and development program (#BE2020679 to Q.Z.) and the Science and Research Innovation Program for Graduate Students in Jiangsu Province (KYCX23_3761 to T.H.).

Disclosure of conflict of interest

None.

Address correspondence to: Qiping Zheng, Department of Hematological Laboratory Science, Jiangsu Key Laboratory of Medical Science and Laboratory Medicine, School of Medicine, Jiangsu University, Zhenjiang 212013, Jiangsu, China. E-mail: qp_zheng@hotmail.com; Longwei Qiao, The Affiliated Suzhou Hospital of Nanjing Medical University, Suzhou 215008, Jiangsu, China. E-mail: qiaolongwei1@126.com; Tong-Chuan He, The Molecular Oncology Laboratory, Department of Orthopaedic Surgery and Rehabilitation Medicine, The University of Chicago Medical Center, Chicago, IL 60637, USA. E-mail: tche@uchicago.edu

References

[1] Long F and Ornitz DM. Development of the endochondral skeleton. Cold Spring Harb Perspect Biol 2013; 5: a008334.
[2] Berendsen AD and Olsen BR. Bone development. Bone 2015; 80: 14-18.
[3] Kronenberg HM. Developmental regulation of the growth plate. Nature 2003; 423: 332-336.
[4] Mackie EJ, Tatarczuch L and Mirams M. The skeleton: a multi-functional complex organ: the growth plate chondrocyte and endochondral ossification. J Endocrinol 2011; 211: 109-121.
[5] Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS and Mirams M. Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol 2008; 40: 46-62.
[6] Gawlitta D, Farrell E, Malda J, Creemers LB, Alblas J and Dhert WJ. Modulating endochondral ossification of multipotent stromal cells for bone regeneration. Tissue Eng Part B Rev 2010; 16: 385-395.
[7] Hallett SA, Ono W and Ono N. The hypertrophic chondrocyte: to be or not to be. Histol Histopathol 2021; 36: 1021-1036.
[8] Gerber HP, Vu TH, Ryan AM, Kowalski J, Werb Z and Ferrara N. VEGF couples hypertrophic cartilage remodeling, ossification and angiogenesis during endochondral bone formation. Nat Med 1999; 5: 623-628.
[9] Wu CW, Tchetina EV, Mwale F, Hasty K, Pidoux I, Reiner A, Chen J, Van Wart HE and Poole AR. Proteolysis involving matrix metalloproteinase 13 (collagenase-3) is required for chondrocyte differentiation that is associated with matrix mineralization. J Bone Miner Res 2002; 17: 639-651.
[10] Hojo H, Ohba S, Yano F and Chung UI. Coordination of chondrogenesis and osteogenesis by hypertrophic chondrocytes in endochondral bone development. J Bone Miner Metab 2010; 28: 489-502.
[11] Yang L, Tsang KY, Tang HC, Chan D and Cheah KS. Hypertrophic chondrocytes can become osteoblasts and osteocytes in endochondral bone formation. Proc Natl Acad Sci U S A 2014; 111: 12097-12102.
[12] Chung UI. Essential role of hypertrophic chondrocytes in endochondral bone development. Endocr J 2004; 51: 19-24.
[13] Kwan AP, Cummings CE, Chapman JA and Grant ME. Macromolecular organization of chicken type X collagen in vitro. J Cell Biol 1991; 114: 597-604.
[14] Warman ML, Abbott M, Apte SS, Hefferon T, McIntosh I, Cohn DH, Hecht JT, Olsen BR and Francomano CA. A type X collagen mutation causes Schmid metaphyseal chondrodysplasia. Nat Genet 1993; 5: 79-82.
[15] Ikegawa S, Nishimura G, Nagai T, Hasegawa T, Ohashi H and Nakamura Y. Mutation of the type X collagen gene (COL10A1) causes spondylometaphyseal dysplasia. Am J Hum Genet 1998; 63: 1659-1662.
[16] Zheng Q, Sebald E, Zhou G, Chen Y, Wilcox W, Lee B and Krakow D. Dysregulation of chondrogenesis in human cleidocranial dysplasia. Am J Hum Genet 2005; 77: 305-312.
[17] Ain NU, Makitie O and Naz S. Autosomal recessive chondrodysplasia with severe short stature caused by a biallelic COL10A1 variant. J Med Genet 2018; 55: 403-407.
[18] Wallis GA, Rash B, Sweetman WA, Thomas JT, Super M, Evans G, Grant ME and Boot-Handford RP. Amino acid substitutions of conserved residues in the carboxyl-terminal do-

main of the alpha 1(X) chain of type X collagen occur in two unrelated families with metaphyseal chondrodysplasia type Schmid. Am J Hum Genet 1994; 54: 169-178.
[19] Goyal M, Gupta A, Choudhary A and Bhandari A. Schmid type metaphyseal chondrodysplasia with a novel COL10A1 mutation. Indian J Pediatr 2019; 86: 183-185.
[20] Richmond CM and Savarirayan R. Schmid metaphyseal chondrodysplasia. In: Adam MP, Everman DB, Mirzaa GM, Pagon RA, Wallace SE, Bean LJH, Gripp KW, Amemiya A, editors. GeneReviews(®). Seattle (WA): University of Washington, Seattle Copyright © 1993-2023, University of Washington, Seattle. GeneReviews is a registered trademark of the University of Washington, Seattle. All rights reserved.; 1993.
[21] Al Kaissi A, Ghachem MB, Nabil NM, Kenis V, Melchenko E, Morenko E, Grill F, Ganger R and Kircher SG. Schmid's type of metaphyseal chondrodysplasia: diagnosis and management. Orthop Surg 2018; 10: 241-246.
[22] Glyn-Jones S, Palmer AJ, Agricola R, Price AJ, Vincent TL, Weinans H and Carr AJ. Osteoarthritis. Lancet 2015; 386: 376-387.
[23] Weinans H, Siebelt M, Agricola R, Botter SM, Piscaer TM and Waarsing JH. Pathophysiology of peri-articular bone changes in osteoarthritis. Bone 2012; 51: 190-196.
[24] Zhong L, Huang X, Karperien M and Post JN. Correlation between gene expression and osteoarthritis progression in human. Int J Mol Sci 2016; 17: 1126.
[25] Rim YA, Nam Y and Ju JH. The role of chondrocyte hypertrophy and senescence in osteoarthritis initiation and progression. Int J Mol Sci 2020; 21: 2358.
[26] Lian C, Wang X, Qiu X, Wu Z, Gao B, Liu L, Liang G, Zhou H, Yang X, Peng Y, Liang A, Xu C, Huang D and Su P. Collagen type II suppresses articular chondrocyte hypertrophy and osteoarthritis progression by promoting integrin β1-SMAD1 interaction. Bone Res 2019; 7: 8.
[27] Lamas JR, Rodríguez-Rodríguez L, Vigo AG, Alvarez-Lafuente R, López-Romero P, Marco F, Camafeita E, Dopazo A, Callejas S, Villafuertes E, Hoyas JA, Tornero-Esteban MP, Urcelay E and Fernández-Gutiérrez B. Large-scale gene expression in bone marrow mesenchymal stem cells: a putative role for COL10A1 in osteoarthritis. Ann Rheum Dis 2010; 69: 1880-1885.
[28] Leung VY, Gao B, Leung KK, Melhado IG, Wynn SL, Au TY, Dung NW, Lau JY, Mak AC, Chan D and Cheah KS. SOX9 governs differentiation stage-specific gene expression in growth plate chondrocytes via direct concomitant transactivation and repression. PLoS Genet 2011; 7: e1002356.
[29] Li F, Lu Y, Ding M, Napierala D, Abbassi S, Chen Y, Duan X, Wang S, Lee B and Zheng Q. Runx2 contributes to murine Col10a1 gene regulation through direct interaction with its cis-enhancer. J Bone Miner Res 2011; 26: 2899-2910.
[30] Li Y, Yang S, Qin L and Yang S. TAZ is required for chondrogenesis and skeletal development. Cell Discov 2021; 7: 26.
[31] Zheng Q, Zhou G, Morello R, Chen Y, Garcia-Rojas X and Lee B. Type X collagen gene regulation by Runx2 contributes directly to its hypertrophic chondrocyte-specific expression in vivo. J Cell Biol 2003; 162: 833-842.
[32] Inada M, Yasui T, Nomura S, Miyake S, Deguchi K, Himeno M, Sato M, Yamagiwa H, Kimura T, Yasui N, Ochi T, Endo N, Kitamura Y, Kishimoto T and Komori T. Maturational disturbance of chondrocytes in Cbfa1-deficient mice. Dev Dyn 1999; 214: 279-290.
[33] Yamashita S, Andoh M, Ueno-Kudoh H, Sato T, Miyaki S and Asahara H. Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes. Exp Cell Res 2009; 315: 2231-2240.
[34] Zhou G, Zheng Q, Engin F, Munivez E, Chen Y, Sebald E, Krakow D and Lee B. Dominance of SOX9 function over RUNX2 during skeletogenesis. Proc Natl Acad Sci U S A 2006; 103: 19004-19009.
[35] Gu J, Lu Y, Li F, Qiao L, Wang Q, Li N, Borgia JA, Deng Y, Lei G and Zheng Q. Identification and characterization of the novel Col10a1 regulatory mechanism during chondrocyte hypertrophic differentiation. Cell Death Dis 2014; 5: e1469.
[36] Ding M, Lu Y, Abbassi S, Li F, Li X, Song Y, Geoffroy V, Im HJ and Zheng Q. Targeting Runx2 expression in hypertrophic chondrocytes impairs endochondral ossification during early skeletal development. J Cell Physiol 2012; 227: 3446-3456.
[37] Chen J, Chen F, Wu X, Bian H, Chen C, Zhang X, Hei R, XiaotongYang, Yuan H, Wang Q, Lu Y, Qiao L and Zheng Q. DLX5 promotes Col10a1 expression and chondrocyte hypertrophy and is involved in osteoarthritis progression. Genes Dis 2023; 10: 2097-2108.
[38] Chen C, Wu X, Han T, Chen J, Bian H, Hei R, Tang S, Li Z, Lu Y, Gu J, Qiao L and Zheng Q. Mef2a is a positive regulator of Col10a1 gene expression during chondrocyte maturation. Am J Transl Res 2023; 15: 4020-4032.
[39] Wu X, Chen C, Han T, Bian H, Chen J, Hei R, Meng Y, Wu C, Lu Y, Gu J, Qiao L and Zheng Q. Stat5a promotes Col10a1 gene expression during chondrocyte hypertrophic differentiation. Am J Transl Res 2023; 15: 4006-4019.

[40] Linder P and Jankowsky E. From unwinding to clamping - the DEAD box RNA helicase family. Nat Rev Mol Cell Biol 2011; 12: 505-516.
[41] Hamm J and Lamond AI. Spliceosome assembly: the unwinding role of DEAD-box proteins. Curr Biol 1998; 8: R532-534.
[42] Xing Z, Ma WK and Tran EJ. The DDX5/Dbp2 subfamily of DEAD-box RNA helicases. Wiley Interdiscip Rev RNA 2019; 10: e1519.
[43] So KKH, Huang Y, Zhang S, Qiao Y, He L, Li Y, Chen X, Sham MH, Sun H and Wang H. seRNA PAM controls skeletal muscle satellite cell proliferation and aging through trans regulation of Timp2 expression synergistically with Ddx5. Aging Cell 2022; 21: e13673.
[44] Caretti G, Schiltz RL, Dilworth FJ, Di Padova M, Zhao P, Ogryzko V, Fuller-Pace FV, Hoffman EP, Tapscott SJ and Sartorelli V. The RNA helicases p68/p72 and the noncoding RNA SRA are coregulators of MyoD and skeletal muscle differentiation. Dev Cell 2006; 11: 547-560.
[45] Jensen ED, Niu L, Caretti G, Nicol SM, Teplyuk N, Stein GS, Sartorelli V, van Wijnen AJ, Fuller-Pace FV and Westendorf JJ. p68 (Ddx5) interacts with Runx2 and regulates osteoblast differentiation. J Cell Biochem 2008; 103: 1438-1451.
[46] Fuller-Pace FV and Ali S. The DEAD box RNA helicases p68 (Ddx5) and p72 (Ddx17): novel transcriptional co-regulators. Biochem Soc Trans 2008; 36: 609-612.
[47] Li X, Decker M and Westendorf JJ. TEThered to Runx: novel binding partners for runx factors. Blood Cells Mol Dis 2010; 45: 82-85.
[48] Ayodele BA, Mirams M, Pagel CN and Mackie EJ. The vacuolar H(+) ATPase V(0) subunit d(2) is associated with chondrocyte hypertrophy and supports chondrocyte differentiation. Bone Rep 2017; 7: 98-107.
[49] Zheng Q, Keller B, Zhou G, Napierala D, Chen Y, Zabel B, Parker AE and Lee B. Localization of the cis-enhancer element for mouse type X collagen expression in hypertrophic chondrocytes in vivo. J Bone Miner Res 2009; 24: 1022-1032.
[50] Zhang Q, Liu W, Zhang HM, Xie GY, Miao YR, Xia M and Guo AY. hTFtarget: a comprehensive database for regulations of human transcription factors and their targets. Genomics Proteomics Bioinformatics 2020; 18: 120-128.
[51] Atsumi T, Miwa Y, Kimata K and Ikawa Y. A chondrogenic cell line derived from a differentiating culture of AT805 teratocarcinoma cells. Cell Differ Dev 1990; 30: 109-116.
[52] Shukunami C, Ishizeki K, Atsumi T, Ohta Y, Suzuki F and Hiraki Y. Cellular hypertrophy and calcification of embryonal carcinoma-derived chondrogenic cell line ATDC5 in vitro. J Bone Miner Res 1997; 12: 1174-1188.
[53] Shukunami C, Shigeno C, Atsumi T, Ishizeki K, Suzuki F and Hiraki Y. Chondrogenic differentiation of clonal mouse embryonic cell line ATDC5 in vitro: differentiation-dependent gene expression of parathyroid hormone (PTH)/PTH-related peptide receptor. J Cell Biol 1996; 133: 457-468.
[54] Yao Y and Wang Y. ATDC5: an excellent in vitro model cell line for skeletal development. J Cell Biochem 2013; 114: 1223-1229.
[55] Lefebvre V, Garofalo S and de Crombrugghe B. Type X collagen gene expression in mouse chondrocytes immortalized by a temperature-sensitive simian virus 40 large tumor antigen. J Cell Biol 1995; 128: 239-245.
[56] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 2001; 25: 402-408.
[57] Komori T. Molecular mechanism of Runx2-dependent bone development. Mol Cells 2020; 43: 168-175.
[58] Kim IS, Otto F, Zabel B and Mundlos S. Regulation of chondrocyte differentiation by Cbfa1. Mech Dev 1999; 80: 159-170.
[59] Enomoto H, Enomoto-Iwamoto M, Iwamoto M, Nomura S, Himeno M, Kitamura Y, Kishimoto T and Komori T. Cbfa1 is a positive regulatory factor in chondrocyte maturation. J Biol Chem 2000; 275: 8695-8702.
[60] Enomoto-Iwamoto M, Enomoto H, Komori T and Iwamoto M. Participation of Cbfa1 in regulation of chondrocyte maturation. Osteoarthritis Cartilage 2001; 9 Suppl A: S76-84.
[61] Chen D, Kim DJ, Shen J, Zou Z and O'Keefe RJ. Runx2 plays a central role in Osteoarthritis development. J Orthop Translat 2019; 23: 132-139.
[62] Jaruga A, Hordyjewska E, Kandzierski G and Tylzanowski P. Cleidocranial dysplasia and RUNX2-clinical phenotype-genotype correlation. Clin Genet 2016; 90: 393-402.
[63] Lee KE, Seymen F, Ko J, Yildirim M, Tuna EB, Gencay K and Kim JW. RUNX2 mutations in cleidocranial dysplasia. Genet Mol Res 2013; 12: 4567-4574.