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Original Article

Mef2a is a positive regulator of *Col10a1* gene expression during chondrocyte maturation

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Abstract: Background: The type X collagen gene (*Col10a1*) is a signature gene of hypertrophic chondrocytes that are known as the main engine of long bone growth. Multiple transcription factors (TFs), including myocyte enhancer factor 2A (Mef2a), have previously been identified by *in silico* analysis as potential *Col10al* gene regulators. Objectives: In this study, we aimed to investigate the correlation between Mef2a and Col10a1 expression and the possible effects on chondrocyte proliferation and hypertrophic differentiation *in vitro*. Methods: First, Mef2a expression in proliferating and hypertrophic chondrocytes were detected by quantitative real-time PCR (qRT-PCR) and Western blotting in two chondrocytic models, ATDC5 and MCT cells, as well as in mouse chondrocytes *in situ*. Transfection with Mef2a small interfering fragments or Mef2a overexpression plasmids in the above chondrocytic models were performed to determine how Mef2a knockdown or overexpression may influence Col10a1 expression. The binding between Mef2a and its putative binding site within the 150 bp *Col10a1* cis-enhancer which was evaluated by the dual luciferase reporter assay. The effect of Mef2a on chondrocyte differentiation was determined by examining the chondrogenic marker gene expression by qRT-PCR and by alcian blue, alkaline phosphatase (ALP), and alizarin red staining of the ATDC5 cells stably knocked down by Mef2a. Results: The expression of Mef2a in hypertrophic chondrocytes was significantly higher than that in proliferative chondrocytes in both chondrocytic models as well as in mouse chondrocytes *in situ*. Interference with Mef2a caused decreased Col10a1 expression, while overexpression of Mef2a upregulated Col10a1. The result of the dual luciferase reporter assay showed that Mef2a enhanced Col10a1 gene enhancer activity via its putative Mef2a binding site. For the staining of ATDC5 stable cell lines, although no significant differences were seen in ALP staining, significantly weaker alcian blue staining intensity was noticed in Mef2a knockdown stable cell lines compared to the control cells at day 21, while slightly weaker alizarin red staining was seen in the stable cell lines at days 14 and 21. Correspondingly, we detected decreased runt-related transcription factor 2 (*Runx2*), increased SRY-box transcription factor 9 (*Sox9*), as well as differential expression of other chondrogenic markers in ATDC5 stable cell lines compared with the controls. Conclusions: In conclusion, our results support that Mef2a upregulates Col10a1 expression possibly by interaction with its cis-enhancer. Altered levels of Mef2a affects the expression of chondrogenic marker genes, such as Runx2 and Sox9, but may only play an insignificant role during chondrocyte proliferation and maturation.

Keywords: Mef2a, Col10a1, gene enhancers, chondrocyte hypertrophy, endochondral bone formation

Introduction

In vertebrates, most of the skeleton is formed by endochondral ossification [1]. Endochondral bone formation involves mesenchymal cell condensation, chondrocyte proliferation and hypertrophy, and mineralization, as well as gradual replacement of the original cartilage by bone tissue containing osteoblasts and osteoclasts [2-5]. In this complex process, hypertrophic chondrocytes are the primary regulators of bone growth, producing factors such as vascular endothelial growth factor to attract blood vessels and chondroclasts and directing adjacent perichondrial cells to become osteoblasts [6].

The type X collagen gene (*Col10a1*) plays a key role in promoting and regulating endochondral osteogenesis in articular cartilage and is a

stage-specific marker of the hypertrophic stage [7, 8]. Type X collagen helps to compartmentalize matrix components to the hypertrophic zone of growth cartilage, increases vascular supply, and promotes normal initiation of mineralization by altering matrix organization [8]. Previous studies have found that mutations and abnormal expression of *COL10A1* are strongly associated with diseases such as chondrodysplasia and osteoarthritis [9-12]. Human *COL10A1* mutation causes Schmid-type metaphyseal chondrodysplasia (SMCD), an autosomal dominant disorder in which patients present with short stature and bent legs, including other symptoms of skeletal dysplasia [9, 11, 13]. In addition, premature differentiation of focal chondrocytes into hypertrophic cells in osteoarthritis (OA) leads to increased Col10a1 synthesis in OA chondrocytes [14]. Therefore, it is crucial to elucidate the regulatory mechanisms of Col10a1 to comprehend human diseases and injury responses associated with cartilage development.

In the past decade, multiple transcriptional regulators have been shown to be involved in *Col10a1* gene regulation, including runt-related transcription factor 2 (Runx2), myocyte enhancer factor 2A (Mef2c), t-box transcription factor 5 (Tbx5), SRY-box transcription factor 9 (Sox9), tafazzin (TAZ), etc. [15-20]. It was found that Runx2 is an essential regulator for Col10a1 expression, while Sox9 negatively regulates Col10a1 expression during chondrocyte differentiation [20-23]. We have previously localized a 150 bp *Col10a1* cis-enhancer element within its distal promoter (-4296 to 4147 bp) that is sufficient to direct its hypertrophic chondrocyte-specific expression *in vivo* [24]. We also showed data that Runx2 interaction with this cis-enhancer is essential but not sufficient for cell-specific Col10a1 expression, suggesting the existence of additional Col10a1 regulatory factors [17, 18, 25, 26]. Indeed, several web-based bioinformatics analytical tools, such as TRAP program, PROMO3.0, and MATCH program, have been utilized to screen out many candidate TFs with their potential binding sites within the 150 bp *Col10a1* cis-enhancer [17, 25]. These TFs are potential Col10a1 regulators. Notably, myogenic enhancer factor 2A (*Mef2a*) was one of such candidate binding factors that are differentially expressed in proliferating and hypertrophic chondrocytes. However, no research to date has verified whether Mef2a is involved in the regulation of Col10a1 expression.

Mef2a is a TF belonging to the MEF2 (myocyte enhancer factor-2) family, which also comprises broadly expressed TFs that control numerous developmental pathways in embryos as well as pleiotropic responses in adults [27, 28]. There are four *MEF2* genes in vertebrates, namely *MEF2A*, *-B*, *-C*, and *-D*. These genes are essential for the development of muscle, neurons, immune cells, and bone, and are also involved in many tumorigenesis [27, 29-31]. *Mef2c* was shown to be an important early regulator of chondrogenesis that controls chondrocyte hypertrophy and bone development [15, 32]. *Mef2c* gene deletion or mutation can inhibit chondrocyte hypertrophy and cartilage angiogenesis in mice [15]. In the femur, *MEF2C* had the highest expression level, followed by *MEF2A* and *D* [33]. MEF2A, C, and D knockdown prevents osteoblasts from expressing endogenous sclerostin (SOST) [33]. Moreover, the deletion of *Mef2c* in osteoblasts was shown to lead to an increase in bone mass [34]. Through a genome-wide association study (GWAS) of anatomy of the cerebral vault in 4419 European individuals, Jasmien Roosenboom et al. identified 32 suggestive loci and several candidate genes at these loci, including neuroligin (*NLG)*, *MEF2A*, *SOX9* and *SOX11*, suggesting that these genes may play a role in cranial development [35]. Interestingly, Mef2a was found to regulate osteoclast differentiation and was sex-related, as osteoporosis was found only in Mef2a conditional knockout female rats but not males [36]. In a study of soft tissue tumors and osteosarcomas, Mef2a was found to be a gene partner for ubiquitin specific peptidase 6 (USP6) fusions indicating a role of Mef2a in the pathogenesis of bone tumors [37]. These studies suggest a potential role for *Mef2a* during skeletal development. Given the putative Mef2a binding site identified within the *Col10a1* cis-enhancer, here we investigate whether Mef2a transactivates *Col10a1* and plays a significant role in chondrocyte hypertrophy.

Methods

Cell culture and chondrogenic differentiation

MCT cells were originally from the laboratory of Dr. de Crombrugghe, MD Anderson (Houston,

Table 1. Primers designed for qRT-PCR

Gene	RefSeqID	Sense Primer (5'-3')	Antisense Primer (3'-5')	Amplicon (bp)
Mef2a	NM_001033713.1	GGGGTGACTTCCATTCTCCA	CATGTGTCCATCCTCATGCG	94
Col10a1	NM_007742	GCAGCATTACGACCCAAGATC	TCTGTGAGCTCCATGATTGC	138
β-actin	NM_007393.5	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT	154
Runx2	NM_001145920	ACCCAGCCACCTTTACCTAC	TATGGAGTGCTGCTGGTCTG	150
Sox9	NM_011448	TTCATGAAGATGACCGACGA	ATGCACACGGGGAACTTATC	200
Alp	NM_007431.2	GTGAGCGCAGCCACAGAGC	GTGTGGCGTGGTTCACCCGA	134
Col2a1	NM_001113515	CCTCCGTCTACTGTCCACTGA	ATTGGAGCCCTGGATGAGCA	121
Mmp13	NM_008607.2	CTTCTTCTTGTTGAGCTGGACTC	CTGTGGAGGTCACTGTAGACT	173

USA). Cells were cultured in DMEM medium containing 8% FBS (Gibco, New Zealand) and cultured at 32°C under 8% CO_2. When cells reached 70%-80% confluence, they were switched to 37°C and continued to be cultured for 2-3 days to induce hypertrophy. Professor Teng of Nanjing University donated the ATDC5 cell line, which was cultured in a DMEM/F12 (1:1) mixed medium with 5% FBS (Gibco, New Zealand) at 37°C and 5% CO_2. 1% insulin-transferrin-selenium (ITS, Sigma) was given to the cells to stimulate chondrogenesis when they were 70%-80% confluent. Cells were continually cultured for 7, 14, and 21 days, with medium changes once every two days. The day of induction was recorded as day 0 (Not add ITS). The ATDC5 cells that had been lentivirus transduced had puromycin (4 μg/ml) added to their media. 293T cells were provided by Professor Shao of Jiangsu University and were cultured in DMEM media with 10% FBS (Gibco, New Zealand) at 37°C and 5% CO_2.

Isolation of total RNA, reverse transcription-PCR, and quantitative real-time PCR

We selected one-day-old C57BL/6 mice, removed their muscle tissue, and isolated the ribs under the microscope. Chondrocytes in proliferative and hypertrophic areas were distinguished and enriched according to the color difference of bone and cartilage. Microdissected tissue containing hypertrophic and proliferative areas was immediately placed into an EP tube with Trizol reagent (Vazyme, Nanjing, China) added in advance. RNA from mouse costal cartilage tissues and cells were extracted with Trizol reagent and cDNA reverse transcription was performed using the PrimeScript™ RT kit with gDNA eraser (Takara, Dalian, China) according to the manufacturer's instructions. The quantitative real-time PCR (qRT-PCR) reaction was then conducted using the cDNA sample as the template. **Table 1** lists the unique primer sequences. qRT-PCR was carried out on a StepOnesoftwarev 2.3 using SYBR Mixture (CWBIO, Beijing, China), and the housekeeping gene *β*-actin was used to standardize the data. The $2^{-\Delta\Delta Ct}$ method was used to determine the relative level of mRNA expression.

siRNA transfection and plasmid transfection

siRNA sequences for Mef2a were synthesized by GenePharma (Shanghai, China). Sequence information is as follows: siRNA: 5'-3'CAUUCUGCUGAAUUAUUUATT; 3'-5'UAAAUAAUUCAGCAGAAUGUG. Negative control: 5'-3'UUCUCCGAACGUGUCACGUTT; 3'-5'ACGUGACACGUUCGGAGAATT.

The plasmids overexpressing Mef2a were purchased from YouBio (Hunan, China), and the negative control was pDONR223. For transient transfection, cells cultured in 6-well plates and grown at 70%-80% confluence were employed. To downregulate Mef2a expression, 10 μM siRNA was transfected into the cells by Lipofectamine RNAiMax Reagent (Thermo Fisher Scientific, USA) in accordance with the manufacturer's instructions. MCT cells were continually cultured for another 6 hours at 32°C, and then for 2-3 days at 37°C, while ATDC5 cells were cultured in ITS-supplemented medium the next day and harvested after 7 days. Following the manufacturer guidelines, the cells were transfected with 2500 ng Mef2a expression plasmid via Lipofectamine3000 transfection Reagent (Thermo Fisher Scientific, USA). Six hours following transfection, MCT cells were replaced with fresh medium and switched to 37°C for continual culturing, while ATDC5 cells were replaced with complete medium and ITS was added the next day.

Lentiviral infection

Mef2a-shRNA was obtained from HANBIO (Shanghai, China). ATDC5 cells were infected with shMef2a lentivirus to establish Mef2a knockdown cell lines. When the cells reached 60%-70% confluence, the lentivirus infection was carried out by 1/2 small volume infection method, and the infection efficiency was confirmed by observing the fluorescence after 72 hours. Then 8 μg/ml of puromycin (Solarbio, Beijing, China) was used to screen successfully infected cells. After 48 hours, cells were cultured in medium containing 4 μg/ml puromycin for subsequent experiments.

Western blot

Harvested cells were lysed in a proteinase inhibitor-containing 1× RIPA buffer. After centrifugation, the protein extract was collected from the supernatant and the concentration was determined. Then the Blue Loading Buffer was diluted to 1× in advance and boiled for 5 min was added. 1,100 μg of protein of the samples were run on 10% SDS-PAGE gels and PVDF membrane was used to transfer the Blots. The membrane was then incubated with specific primary antibodies overnight at 4°C after being blocked with 5% nonfat dry milk for 1 h at room temperature: anti-Collagen X (1:1000, Abclonal), anti-*β*-actin (1:1000, Beyotime), anti-Mef2a (1:500, WanLeiBio). After three TBST washes for 10 min each, the membrane was incubated for 1 h with the matching HRP-linked secondary antibodies: goat anti-mouse IgG (1:2000, Beyotime), goat anti-rabbit IgG (1:2000, Beyotime). Membrane immunoreactivity was detected by the ECL system (Vazyme, Nanjing, China).

Immunohistochemistry (IHC) analysis

4% PFA was used to fix the femurs of C57BL/6 mice for half an hour, then they were embedded and sliced into 5-μm-thick tissue sections. Antibodies to Mef2a and Col10a1 were used to evaluate their expression. Rabbit Antibody Immunohistochemistry (SP Method) Kit was purchased from WanleiBio (Shenyang, China). Briefly, sections were dewaxed in xylene and dehydrated in gradient alcohol, then heated for 18 min at 95°C in citrate buffer (PH 6.0) to repair antigens. Sections were incubated with 30% H_2O_2 at RT and protected from light for 15 min. After washing, sections were covered with goat serum blocking solution (WanleiBio, China) for 15 min, and incubated with antibodies to Mef2a or Col10a1 overnight at 4°C. Next, in accordance with the manufacturer's instructions, slides were incubated with the secondary antibody and the tertiary antibody (HRP-labeled streptavidin) and detected with the use of DAB (3,3-Diaminobenzidine). Hematoxylin re-stained sections were then observed under a microscope (Nikon, Japan) after sealing with neutral gum.

Putative Mef2a binding site and dual luciferase activity assay

The putative Mef2a binding site within the 150 bp Col10a1 cis-enhancer was previously predicted by the TRAP (http://trap.molgen.mpg.de/cgi-bin/trap_form.cgi) and PROMO (http://alggen.lsi.upc.es/cgibin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) programs as previously reported [24, 38, 39]. Interestingly, the binding sites predicted by both programs are very similar, so we conservatively selected the longer sequence covering another possible site as the hypothetical binding site for Mef2a and cloned it into the pGL6 vector (RiboBio, Guangzhou, China). Renilla luciferase was used as the internal reference. 24-well plates were used to culture 293T cells. When 60%-70% fusion was achieved, transfection was performed according to the ratio of Mef2a expression plasmid: firefly-reporter gene pGL6: TK-Renilla = 20:10:1 using lipofectamine3000 transfection reagent. The luciferase activity was detected after 48 h using the luciferase reporter assay kit (Promega, Madison, USA).

Alcian blue, alkaline phosphatase (ALP), and alizarin red staining

Day 7, Day 14, and Day 21 ATDC5 cells were harvested for cell staining. With the media removed, the cells were washed with PBS twice. For Alcian blue staining, methanol was used to fix ATDC5 cells for 2 min at -20°C, then 0.1% Alcian blue was added to each well and stained overnight at RT. The Alcian stain was gently rinsed off with ddH_2O and observed under 10× microscope after air dry. For alkaline phosphatase (ALP) staining, we used the Cell alkaline phosphatase stain cAKP kit (Jiancheng, Nanjing, China) in accordance with the manufacturer's instructions. In short, reagent 1 was

used to fix cells for 2-5 min, then the pre-configured matrix solution dropwise was added, followed by 15 min of dark incubation at 37°C. The dye solution dropwise was added in sequence and washed with distilled water, and then analyzed under the 20× microscope after being air dried. For Alizarin red staining, cells were stained with 1% Alizarin Red for 10 min at RT after being fixed in 95% ethanol for 10 min [40]. Next they were washed with ddH_2O and then observed under 10× microscope after being air dried.

Statistical analysis

At least 3 independent replications of each experiment were conducted. qRT-PCR results were analyzed using GraphPad Prism 8 software. The $2^{-\Delta\Delta Ct}$ methodology was used to determine the relative mRNA level. No more than two experimental groups were compared using the Student's t-test. The differences between two or more groups of data were compared using one-way analysis of variance (ANOVA). Statistical significance was set at $P < 0.05$.

Results

Expression analysis of Mef2a in chondrocytes in vitro and in situ

First, we examined Col10a1 and Mef2a expression in two in vitro chondrocytic models, MCT and ATDC5 cells. MCT cells are temperature-sensitive mouse chondrocytes that undergo simian virus 40 large tumor antigen-immortalization [41]. Cells grew continuously in monolayer culture at 32°C, but stopped growing and began hypertrophy while highly expressing the marker gene Col10a1 at a non-permissive temperature of 37°C to 39°C [41]. Therefore, we examined the expression of Mef2a in hypertrophic (37°C) and proliferative (32°C) MCT cells. As expected, Col10a1 expression in MCT cells cultured at 37°C for 2-3 days increased significantly (**Figure 1A**), indicating that MCT cells were in a hypertrophic state. Meanwhile, the mRNA expression of *Mef2a* was 3-fold higher in hypertrophic MCT cells compared to proliferative MCT cells (**Figure 1A**). Western blotting showed the similar results as expected (**Figure 1C**). ATDC5 cells have been utilized as a good cell model for studying the molecular mechanisms of chondrocyte differentiation *in vitro* [42, 43]. It was shown that this cell line has the property of replicating chondrocyte differentiation in the presence of insulin [42, 43]. ATDC5 cells were grown for 7, 14, and 21 days in media with 1% ITS to induce chondrogenic differentiation. It was found that *Col10a1* mRNA in ATDC5 cells increased with the cell induction and culturing time and reached the highest level at 14 days of culture (**Figure 1B**). Interestingly, the mRNA level of *Mef2a* also reached the highest on day 14, which was about 9 times higher than that on day 0 (**Figure 1B**). Additionally, Western blotting revealed that Col10a1 and Mef2a protein expression levels at day 14 are significantly higher than day 0 (**Figure 1D**). Next, we analyzed the expression of Col10a1 and Mef2a in primary mouse chondrocytes as well as in hind limb bone tissue. The mouse rib cartilage was separated under a dissecting microscope, with the gray part being the hypertrophic area and the white part being the proliferative area (**Figure 1E**). The qRT-PCR results showed that the mRNA level of *Col10a1* in the tissues of the hypertrophic zone was much higher than those of the proliferative zone. Furthermore, the mRNA expression of *Mef2a* was also 3-fold higher than that of the proliferative zone (**Figure 1F**). We also examined the expression of Col10a1 and Mef2a at the histochemical level. As shown in **Figure 1G**, either Col10a1 or Mef2a showed stronger positive staining in cells in the hypertrophic zone. The above results suggested that Mef2a may correlate with Col10a1 expression.

Up-regulation or down-regulation of Mef2a affected Col10a1 expression

To further verify whether Mef2a affects the expression of Col10a1, Mef2a was overexpressed or knocked down in both MCT and ATDC5 cells, and we then examined the changes of Col10a1 expression. We successfully knocked down the expression of Mef2a in two cell lines by transient transfection of Mef2a small interference fragment siRNA (**Figure 2A-D**). As expected, the expression of Col10a1 also showed a significant downregulation both at the mRNA level and protein level (**Figure 2A-D**). To overexpress Mef2a, the pDONR223/Mef2a plasmid was transfected into ATDC5 and MCT cells. Col10a1 mRNA and protein levels in the Mef2a overexpression group were considerably higher than those in the pDONR223 group (**Figure 2E-H**). In summary,

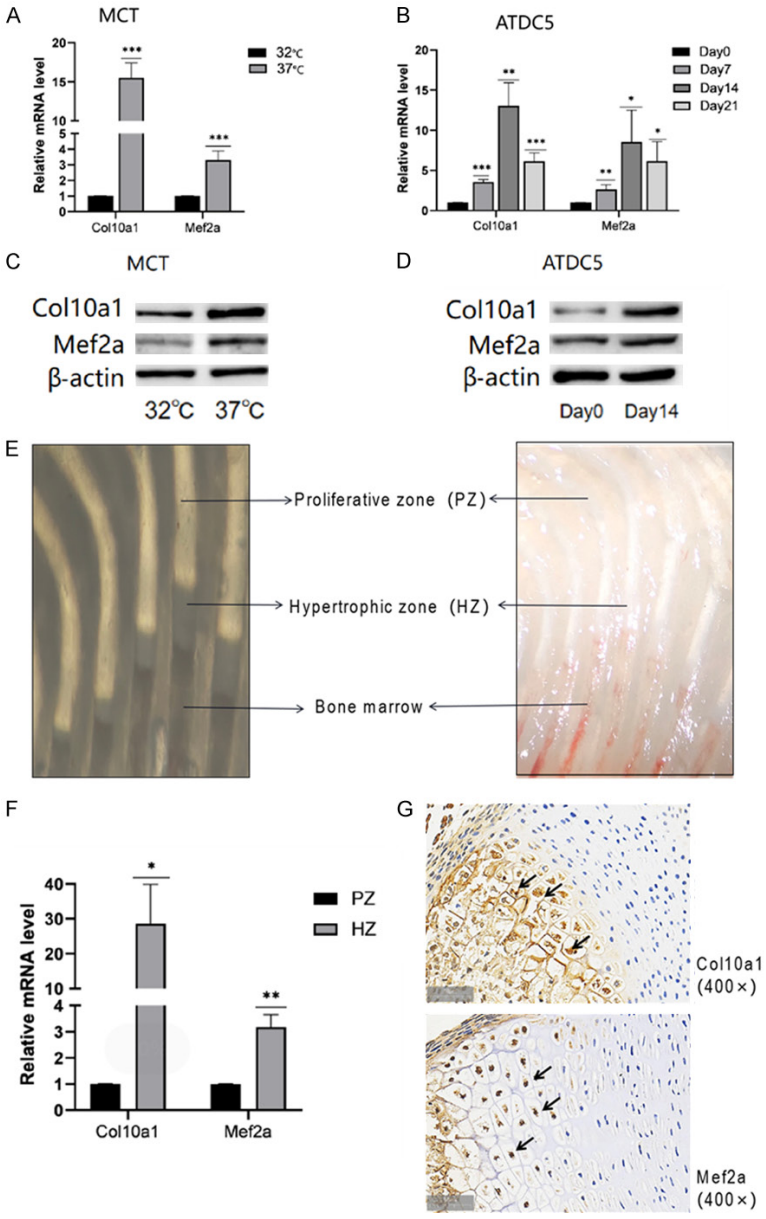

Figure 1. Basal expression of Mef2a in chondrocytes in *vitro* and in *situ*. A. MCT cells were grown at 32°C and then at 37°C for 2-3 days to stimulate hypertrophy, qRT-PCR results showed that the mRNA levels of *Mef2a* and *Col10a1* were much higher in hypertrophic MCT cells (37°C) than in proliferative MCT cells (32°C). B. ATDC5 cells were grown in complete media containing 1% ITS for 0, 7, 14, and 21 days. qRT-PCR results showed that the mRNA levels of *Col10a1* and *Mef2a* changed with the induction and culturing time and reached highest on day 14. C. WB results showed that the protein levels of Mef2a and Col10a1 in hypertrophic MCT cells (37°C) were significantly higher than in proliferative MCT cells (32°C). D. WB results showed that the protein levels of Col10a1 and Mef2a at day 14 were significantly higher than day 0. E. Microscopic view of ribs of 1-day-old C57BL/6 mice. The white area is the proliferative zone, and the gray area is the hypertrophic zone. F. In comparison with the proliferative zone, the mRNA levels of *Col10a1* and *Mef2a* were significantly higher in tissues of the rib cartilage indicating the hypertrophic zone. G. Immunohistochemical analysis showed that Col10a1 and Mef2a were strongly expressed in hypertrophic chondrocytes, while no significant expression was detected in chondrocytes of the proliferative zone, magnification = 400×, scale bar = 50 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

the findings imply that *Mef2a* may be involved in the regulation of the *Col10a1* expression.

Mef2a strengthens the activity of the Col10a1 gene enhancer

The TRAP and PROMO programs successfully predicted two putative Mef2a binding sites, which were very similar within the 150 bp *Col10a1* cis-enhancer (**Figure 3A** and **3B**). To explore whether Mef2a acts by binding to its binding sites, a dual luciferase reporter gene assay was performed. Among the two predicted binding sites, we conservatively selected the longer sequence (78 bp-94 bp) as the hypothetical binding site and cloned it into the pGL6 vector. Western blot results revealed that the protein level of Mef2a was dramatically increased in 293T cells transfected with pDONR223/Mef2a (**Figure 3C**). In fact, the luciferase activity was nearly 4-fold higher in the group cotransfected with pDONR/Mef2a and *Col10a1* enhancers compared to the group cotransfected with either pDONR223 or *Col10a1* enhancers (**Figure 3D**). Therefore, we conclude that Mef2a promotes *Col10a1* expression by binding to the corresponding site within the *Col10a1* cis-enhancer.

Knockdown of Mef2a expression in ATDC5 cells by lentiviral transduction of shRNA

To construct a stable cell line with Mef2a knockdown, we transfected shMef2a in ATDC5 cells. Transfection efficiency was roughly estimated to be around 70% based on

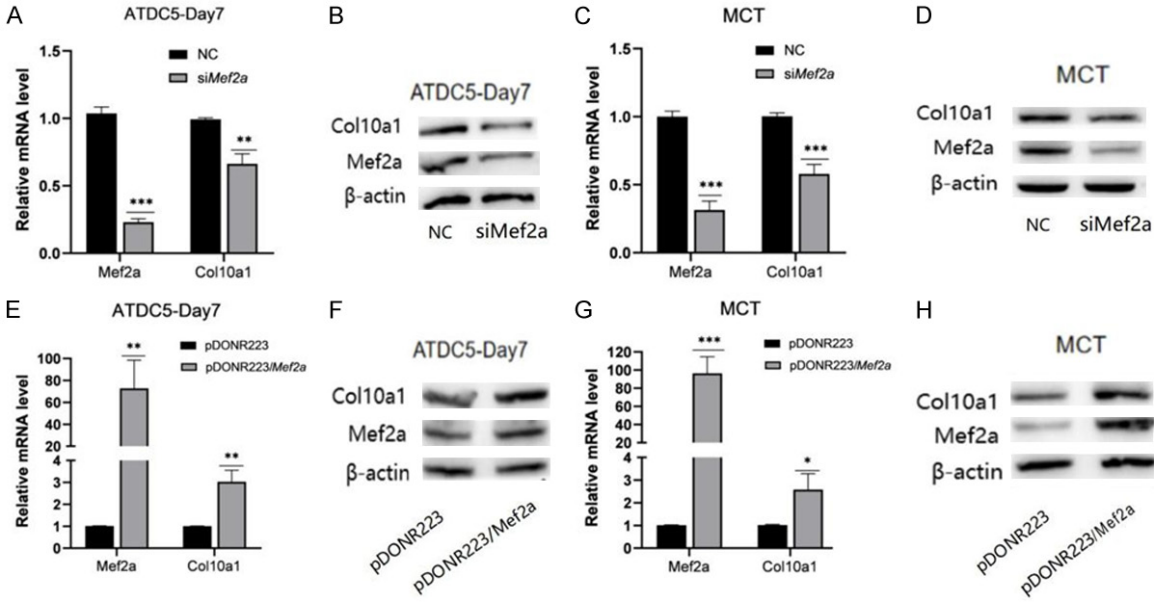

Figure 2. Mef2a affects the expression of Col10a1. A. ATDC5 cells with Mef2a knocked down were cultured with complete medium containing 1% ITS for 7 days. The mRNA levels of *Col10a1* were also correspondingly reduced. B. WB results showed the protein level of Mef2a was successfully knocked down in ATDC5 cells, and Col10a1 was also downregulated in these cells. C. qRT-PCR results showed that *Mef2a* was successfully knocked down in MCT cells, while the mRNA level of *Col10a1* was correspondingly reduced in these cells. D. WB results showed the protein level of Mef2a was significantly reduced in the Mef2a knockdown group of MCT cells, and Col10a1 was also downregulated in these cells. E. ATDC5 cells transfected with pDONR223/Mef2a or pDONR223 were cultured in complete medium containing 1% ITS for 7 days. *Mef2a* was successfully overexpressed, and the mRNA levels of *Col10a1* were obviously increased in these cells. F. WB results showed the protein levels of Mef2a and Col10a1 were increased in MCT cells transfected with pDONR223/Mef2a compared to the group with pDONR223. G. qRT-PCR results showed that *Mef2a* was overexpressed about 90-fold in MCT cells, and the mRNA level of *Col10a1* was significantly increased in these cells. H. WB results showed that Mef2a was successfully overexpressed at the protein level in ATDC5 cells after 7 days of induction, and the protein level of Col10a1 was also correspondingly increased in these cells. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

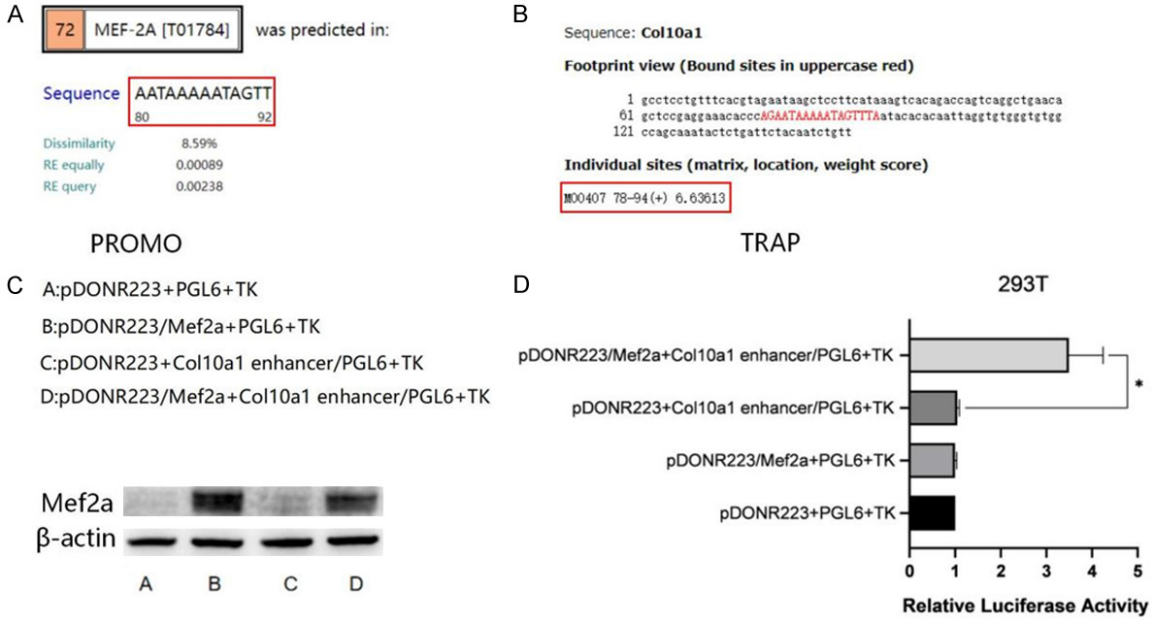

Figure 3. Bioinformatics prediction and validation of transcription factor binding sites. A. Binding sites predicted by the PROMO program. B. Binding sites predicted by the TRAP program. C. Mef2a protein levels were significantly increased in 293T cells transfected with pDONR223/Mef2a. D. The relative fluorescence activity of 293T cells was examined by dual luciferase reporter gene assay, the luciferase activity was nearly 4-fold higher in the group cotransfected with pDONR/Mef2a and *Col10a1* enhancers compared to the group cotransfected with either pDONR223 or *Col10a1* enhancers. $^{*}P < 0.05$.

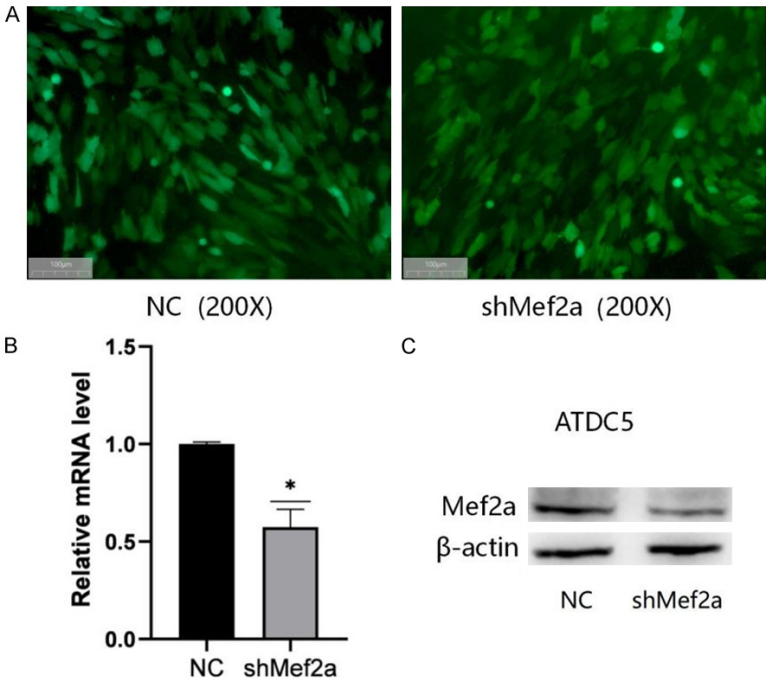

Figure 4. Construction of a stable cell line with Mef2a knockdown by transfection of shMef2a into ATDC5 cells. A. Transfection efficiency was roughly estimated to be around 70% based on fluorescence intensity, magnification = 200×, scale bar = 100 μm. B. Detection of *Mef2a* mRNA levels in ATDC5 cell lines with stable Mef2a knockdown by qRT-PCR, and the results showed that Mef2a expression decreased by half. C. The protein levels of Mef2a in ATDC5 cell lines with Mef2a stably knocked-down by western blot analysis, and the results showed that Mef2a expression was obviously decreased. $^{*}P < 0.05$.

fluorescence intensity (**Figure 4A**). After two days of 8 μg/ml puromycin screening, we used qRT-PCR for the quantification of *Mef2a* mRNA expression to evaluate the effect of *Mef2a* knockdown. As seen in **Figure 4B**, *Mef2a* expression in the shMef2a lentivirus-infected group decreased by half. Western blotting also verified the same results at the protein level (**Figure 4C**).

Effects of Mef2a on proliferation, differentiation and maturation of chondrocytes

ATDC5 stable cell lines were grown with ITS for 7, 14, and 21 days before being harvested for Alcian blue, ALP, and Alizarin red staining to evaluate the function of Mef2a in chondrocyte differentiation *in vitro*. For Alcian blue staining, no significant difference was found in the staining intensity of Mef2a knockdown stable cell lines compared to controls at days 7 and 14, but weaker staining intensity than controls was observed at day 21, suggesting that Mef2a may promote chondrocyte proliferation *in vitro* (**Figure 5A**). For ALP staining, no significant staining differences were observed at days 7, 14, and 21, suggesting a moderate role for Mef2a in chondrocyte differentiation and matrix mineralization *in vitro* (**Figure 5B**). Finally, in the Alizarin red staining results, we found that stable cell lines with Mef2a knockdown at days 14 and 21 exhibited weaker staining intensity than the control cells, indicating that Mef2a may promote matrix mineralization during the late stages of osteogenesis *in vitro* (**Figure 5C**).

Preliminary analysis of markers of chondrogenesis in Mef2a knockdown cells

We know that several regulatory genes are closely associated with chondrocyte differentiation, maturation and mineralization, including *Sox9*, *ALP*, matrix metallopeptidase 13 (*Mmp13*), *Col2a1* and *Runx2*. Here, to examine the impact of Mef2a on the expression of these relevant marker genes, we initially analyzed the mRNA levels of these genes in ATDC5 stable cell lines at 7, 14, and 21 days, and compared them with controls. qRT-PCR results showed that *Runx2* was significantly decreased in ATDC5 stable cell lines with knockdown Mef2a at day 21 of induction, while *Sox9* had a significant increase at days 14 and 21. As expected,

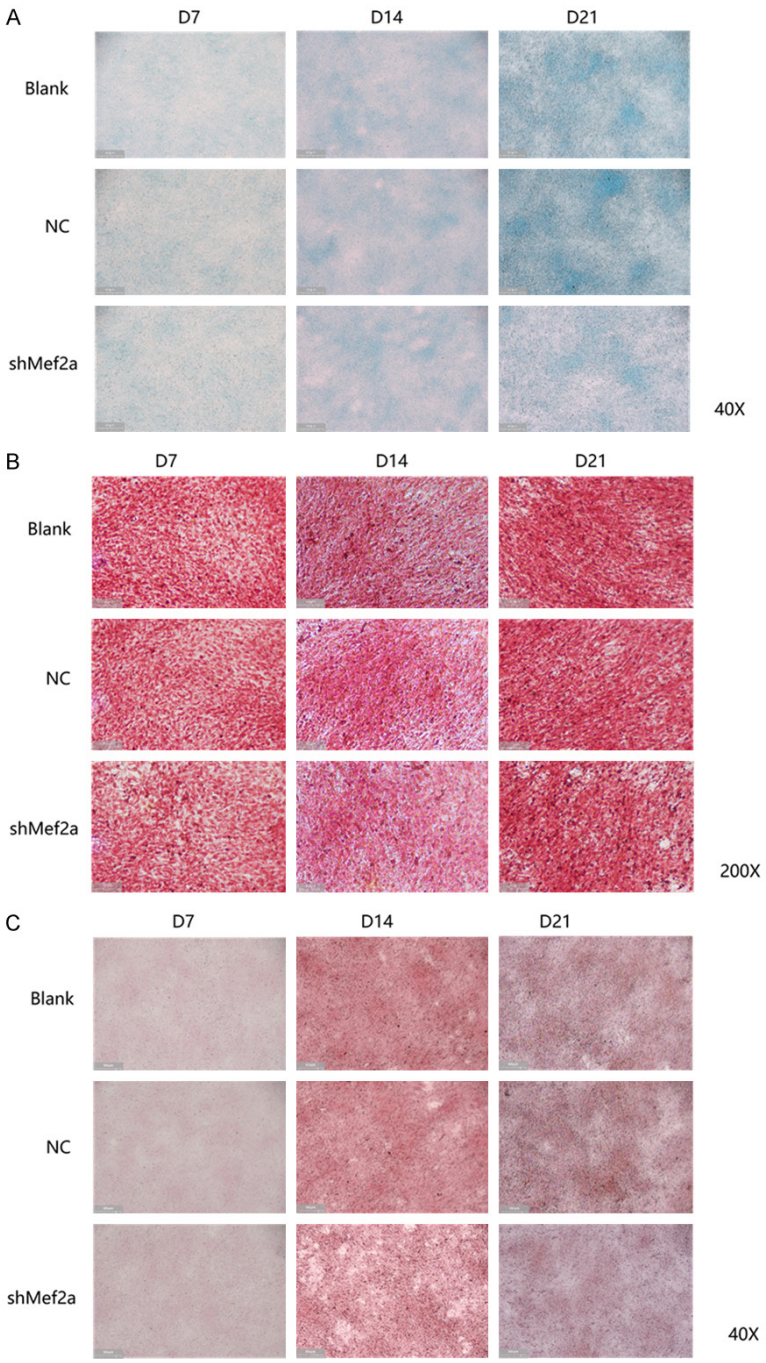

Figure 5. Effects of Mef2a on proliferation, differentiation and maturation of chondrocytes. A. At day 21 of culture, weaker Alcian blue staining was observed in Mef2a knockout cell lines than in control cells, but there was no significant difference on days 7 and 14, magnification = 40×, scale bar = 500 μm. B. No significant difference in ALP staining was observed in the three time periods, magnification = 200×, scale bar = 100 μm. C. Stronger alizarin red staining was observed in control cells on days 14 and 21, although there was no difference on days 7, magnification = 40×, scale bar = 500 μm.

Col10a1 expression was reduced in stable cell lines at days 7, 14 and 21 (**Figure 6A-C**). Therefore, our findings imply that Mef2a might play an integral role in the chondrogenesis of ATDC5 cells *in vitro*.

Discussion

The *Col10a1* gene is a known marker of hypertrophic chondrocytes during osteogenesis within cartilage [8]. Our previous *in silico* study suggested that Mef2a is a candidate *Col10a1* gene regulator [25]. In this research, we examined the correlation of *Mef2a* with the *Col10a1* gene expression and its potential effects on chondrocyte proliferation, differentiation and maturation *in vitro*.

We detected significantly increased Mef2a in two chondrogenic cell models, MCT and ATDC5 cells, when they were induced to reach hypertrophic states. The same results were verified *in situ* chondrocytes from mice. We also found that knockdown of Mef2a decreased Col10a1 expression, while overexpression of Mef2a promoted Col10a1 expression, suggesting a positive correlation between Mef2a and Col10a1. To determine the potential mechanism by which Mef2a regulates Col10a1, the putative Mef2a binding site within the 150 bp *Col10a1* cis-enhancer was identified and demonstrated functional by a dual luciferase assay, suggesting that Mef2a promotes Col10a1 expression by binding to the *Col10a1* cis-enhancer.

In order to further investigate the function of Mef2a in chondrocyte differentiation *in vitro*, we established a stable ATDC5 cell line with Mef2a knockdown and stained with Alcian

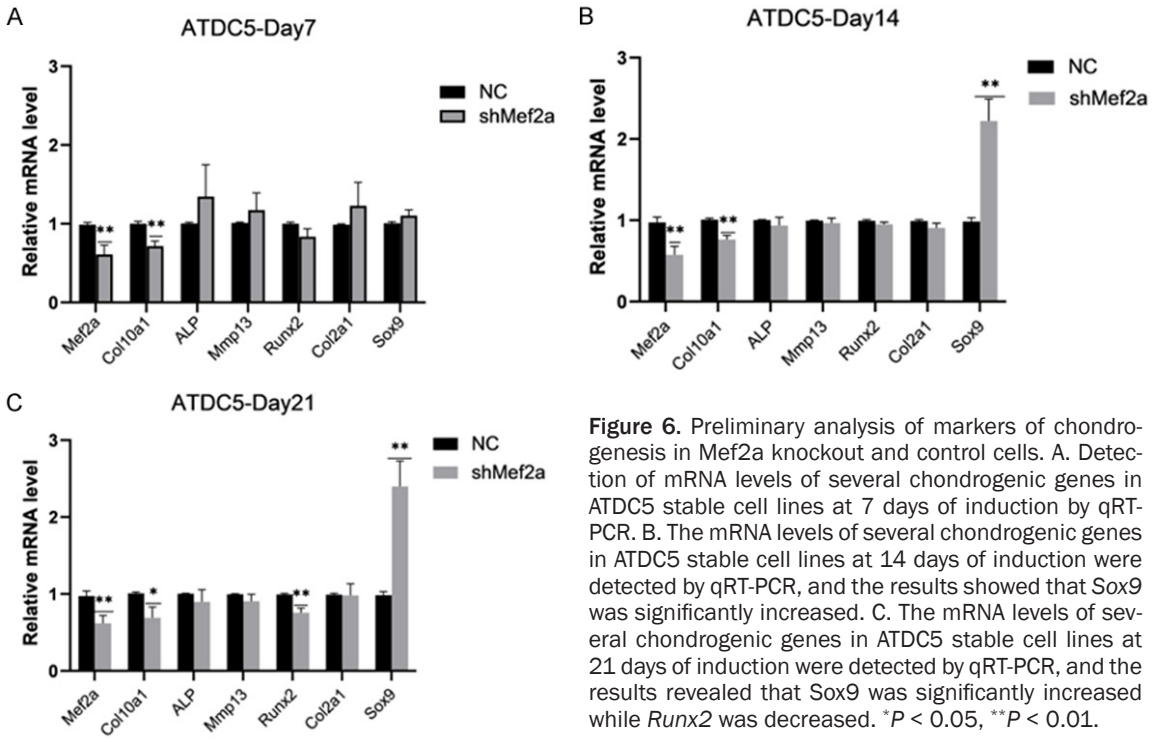

Figure 6. Preliminary analysis of markers of chondrogenesis in Mef2a knockout and control cells. A. Detection of mRNA levels of several chondrogenic genes in ATDC5 stable cell lines at 7 days of induction by qRT-PCR. B. The mRNA levels of several chondrogenic genes in ATDC5 stable cell lines at 14 days of induction were detected by qRT-PCR, and the results showed that *Sox9* was significantly increased. C. The mRNA levels of several chondrogenic genes in ATDC5 stable cell lines at 21 days of induction were detected by qRT-PCR, and the results revealed that Sox9 was significantly increased while *Runx2* was decreased. $^{*}P < 0.05$, $^{**}P < 0.01$.

blue, ALP, and Alizarin red. ATDC5 cells can mimic the procedure of chondrocyte differentiation and hypertrophy *in vitro*, and in the presence of insulin, express abundant hypertrophic chondrocyte marker gene *Col10al* with prolonged culture, making ATDC5 an optimal cell model for studying endochondral ossification *in vitro* [42, 43]. The results supported a role of Mef2a in chondrocyte proliferation and mineralization *in vitro* but had little influence on matrix maturation. Moreover, we have performed preliminary expression profiling of the following chondrogenic marker genes *Col2a1*, *Sox9*, *ALP*, and *Runx2* in stable cell lines. Consistent with the ALP staining results, knockdown of Mef2a did not affect the expression of ALP. However, we detected increased Sox9 expression and decreased Runx2 expression in ATDC5 stable cells with Mef2a knockdown. As we all know, Runx2 is a transcription factor essential for osteoblast differentiation and chondrocyte maturation [44-46]. Runx2$^{-/-}$ mice have no osteoblasts at all, their skeleton is made of cartilage, chondrocyte maturation is inhibited in most bones, and no vascular invasion occurs [47, 48]. However, the skeletally restricted fraction of Runx2$^{-/-}$ mice also detected terminally differentiated chondrocytes, suggesting that chondrocyte maturation also requires the involvement of other transcription factors [49]. The promoter region of Ihh has been discovered to have several Runx2 binding motifs and that Runx2 directly regulates Ihh expression, suggesting that Runx2 also regulates chondrocyte proliferation through Ihh [49, 50]. Sox9, a gene recognized to be a marker for early chondrocyte differentiation, is critical for the formation of limb mesenchymal cartilage blastomere, proliferation and differentiation of chondrocytes in the embryonic growth plate, and regulation of cartilage-specific genes [21]. Studies in transgenic mice showed that misexpression of Sox9 in hypertrophic chondrocytes impairs bone growth and terminal differentiation of hypertrophic chondrocytes [22]. Additionally, our previous research has shown that appropriate temporal and spatial expression of Sox9 is required for adults to maintain normal hypertrophic cartilage homeostasis, and that abnormal expression of Sox9 may result in the development of spontaneous osteoarthritis [51]. Thus, the differential expression of *Runx2* and *Sox9* in Mef2a knockdown ATDC5 stable cell lines and control cells suggested that Mef2a affects the expression of these genes and might play a crucial role endochondral ossification *in vitro*.

In summary, our results demonstrate that Mef2a positively regulates *Col10a1* gene

expression and may be involved in promoting chondrocyte proliferation and maturation *in vitro*. Although more research is required to fully understand the function and mechanisms of Mef2a *in vivo* during skeletal, especially long bone development involving growth inhibition.

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Disclosure of conflict of interest

None.

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