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Original Article

Stat5a promotes *Col10a1* gene expression during chondrocyte hypertrophic differentiation

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Abstract: Objectives: Multiple transcription factors (TFs) have previously been shown to control hypertrophic chondrocyte-specific mouse type X collagen gene (*Col10a1*) expression via interaction with *Col10a1* promoters. This study aims to investigate the role and mechanism of the potential binding factor signal transduction and transcription activator 5a (Stat5a) of *Col10a1* cis-enhancer, in controlling *Col10a1* gene expression and chondrocyte hypertrophic differentiation. Methods: The potential *Col10a1* regulator was predicted by the transcription factor affinity prediction (TRAP) analysis of the 150-bp *Col10a1* cis enhancer. Stat5a was screened and verified by qRT-PCR, western blot and IHC analyses. Transfection of Stat5a siRNA or expression plasmid into MCT and ATDC5 cells was performed to either knockdown or over-express Stat5a and to investigate the influence of Stat5a on *Col10a1* gene expression during the chondrocyte hypertrophy. Dual-luciferase reporter assay was performed to explore the mechanism of Stat5a affecting *Col10a1* transcription. Alcian blue, alkaline phosphatase, and alizarin red staining, as well as qRT-PCR analyses of related marker genes were performed to investigate the effect and possible mechanism of Stat5a on chondrocyte differentiation. Results: The potential binding factor of *Col10a1* cis-enhancer Stat5a and Col10a1 were both highly expressed and positively correlated within hypertrophic chondrocytes *in vitro* and *in situ*. Knockdown of Stat5a reduced Col10a1 expression, while overexpression of Stat5a enhanced Col10a1 expression in hypertrophic chondrocytes, suggesting Stat5a as a positive Col10a1 regulator. Mechanistically, Stat5a was shown to potentiate the reporter activity mediated by *Col10a1* promoter/enhancer. In addition, Stat5a increased the intensity of alkaline phosphatase staining of ATDC5 cells and the expression of relevant hypertrophic marker genes including Runx2, which was consistent with the expression of Stat5a and Col10a1. Conclusions: Our results support that Stat5a promoted Col10a1 expression and chondrocyte hypertrophic differentiation, possibly via interaction with the 150-bp *Col10a1* cis-enhancer.

Keywords: Stat5a, *Col10a1* gene regulation, chondrocyte hypertrophy, transcription factors, chondrogenic cell models

Introduction

Over 80% of the skeleton, including the appendicular skeleton, the axial skeleton and part of the skull, are formed by endochondral ossification [1, 2]. During endochondral bone formation, chondrocytes undergo proliferation, hypertrophic differentiation, maturation and apoptosis, followed by blood vessels invasion, matrix mineralization, and are eventually replaced by bone [3, 4]. In this process, hypertrophic chondrocytes are critical for longitudinal bone growth and the signaling molecules secreted may affect other cell types that are involved in endochondral ossification [5, 6]. The type X collagen gene (*Col10a1*), which is specifically expressed in hypertrophic chondrocytes, has previously been demonstrated to play a significant role both in bone development and in skeletal diseases. It was reported that Col10a1 facilitated matrix mineralization through interaction with matrix vesicles and the compartmentalization of matrix components during endochondral ossification [7]. Col10a1 is also

needed for hematopoiesis in endochondral ossification and the immune response. Abnormal expression of Col10a1 has been reported in many skeletal diseases [8]. Alteration of human *COL10A1* expression has been associated with Schmid metaphyseal chondrodysplasia (SMCD) and cleidocranial dysplasia (CCD) [9, 10]. Enhanced *COL10A1* expression and aberrant chondrocyte hypertrophy have been widely observed in cartilage of osteoarthritis (OA), a degenerative joint disease highly associated with age [11-15]. Undesirable hypertrophic differentiation along with *COL10A1* expression and tissue calcification after ectopic transplantation remains the major barrier limiting application of mesenchymal stem cells (MSCs) in regeneration therapy for impaired cartilage tissue [16, 17]. Therefore, further investigation of *Col10a1* gene regulation would lead to better understanding of the molecular mechanisms underlying endochondral bone formation and help identification of potential therapeutic targets for *Col10a1*-related skeletal diseases.

For the past decades, more and more transcription factors, including Runx2, Mef2c and TAZ etc., that have been demonstrated to promote *Col10a1* gene expression and chondrocyte hypertrophy, while Sox9, Nkx3.2/Bapx1 and HDAC4 show an opposite effect [18-23]. In our previous studies, we have localized a 150-bp *Col10a1* cis-enhancer element (-4296 to -4147 bp) that is critical to mediate cell-specific *Col10a1* expression *in vivo* [24]. We then showed that Runx2 interaction with this cis-enhancer, is essential but not sufficient for hypertrophic chondrocyte-specific *Col10a1* expression *in vivo*, suggesting requirement of additional regulators [25]. Indeed, further investigation from ours and other groups have identified some additional regulators including Cox-2, Dlx5, and Tbx5 etc., that either positively or negatively control *Col10a1* expression [26-28]. Interestingly, these TFs (Cox-2, Dlx5, and Tbx5) have been identified by the TRAP program, which can predict putative binding factors of the 150-bp *Col10a1* cis-enhancer with a *P*-value <0.05 based on affinity-ranking [26-28].

Given the above complicated regulatory processes, we hypothesize that candidate factor, signal transducer and activator of transcription 5A (Stat5a), which show binding affinity with the cis-enhancer within the range of $0.05<P<0.1$, may also participate in regulation of *Col10a1* gene expression and chondrocyte hypertrophic differentiation. The family of STATs has previously been shown to play a significant role during chondrogenesis [29]. STAT2 was demonstrated to express in OA but not normal human articular cartilage [30]. STAT3 activation might promote chondrogenic differentiation of MSCs *in vitro* [31]. STAT1 and STAT5 were also shown to affect the phenotypic severity, suppression of chondrocyte proliferation, and differentiation into prehypertrophic chondrocytes in FGFR3-related chondrodysplasias [32, 33]. A recent study found that the inhibition of IL-15/JAK3/STAT5 signaling by supplement of Sirt6 could slow down chondrocyte aging [34]. Moreover, a study of cDNA microarrays showed that FGF treatment of rat chondrosarcoma (RCS) chondrocyte facilitated chondrocyte hypertrophy and an increased expression of Stat5a [35]. Noticeably, STAT5 has been shown to promote MSC osteogenic differentiation by interaction with RUNX2, a critical TF known to control mouse *Col10a1* gene expression via direct interaction with its cis-enhancer [25]. STAT5 can be divided into isoforms Stat5a and Stat5b, which share over 90% identity and have some redundant functions. Activation of Stat5b was shown to stimulate chondrogenesis, whereas STAT5a and STAT5b null mice showed obvious phenotypic differences indicating their distinct roles [36, 37]. STAT5A was also shown to play a crucial role in osteoblast differentiation of BMSCs and bone healing of murine femur fractures [38]. However, whether Stat5a plays a significant role in *Col10a1* gene expression during chondrocyte maturation remains elusive.

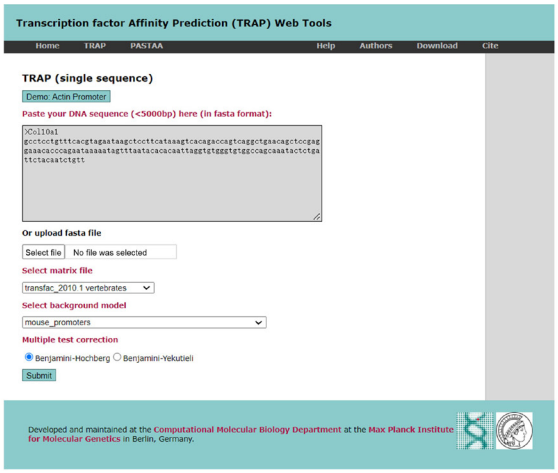
In this study, based on the results of TRAP analysis of the 150-bp *Col10a1* cis-enhancer and literature review of the effects of STAT family members on bone and cartilage formation, we investigated the function and possible mechanism of Stat5a in the regulation of cell-specific *Col10a1* gene expression and chondrocyte hypertrophic differentiation using multiple chondrogenic cell models.

Materials and methods

Bioinformatics analysis of Col10a1 cis-enhancer

A bioinformatics analysis of the 150-bp mouse *Col10a1* cis-enhancer (-4296 to -4147 bp) was

A



B

Affinity-based ranking of transcription factors

Rank	P-value	Corrected p-value	Matrix ID	Matrix name	Rank	P-value	Corrected p-value	Matrix ID	Matrix name	Rank	P-value	Corrected p-value	Matrix ID	Matrix name
49	0.0509	0.737	M00641	V$HSF_Q6	68	0.0661	0.737	M01659	V$CDX2_Q5_01	87	0.0879	0.737	M00478	V$CDC5_01
50	0.0518	0.737	M01429	V$HOMEZ_01	69	0.0661	0.737	M00302	V$NFAT_Q6	88	0.0887	0.737	M01297	V$HOXA13_02
51	0.0556	0.737	M01239	V$RELBP52_01	70	0.0695	0.737	M00241	V$NKX25_02	89	0.0896	0.737	M00619	V$ALX4_01
52	0.0559	0.737	M01365	V$EN1_02	71	0.0696	0.737	M01249	V$HIF2A_01	90	0.0898	0.737	M00131	V$HNF3B_01
53	0.0566	0.737	M00774	V$NFKB_Q6_01	72	0.0697	0.737	M00243	V$EGR1_01	91	0.0912	0.737	M00378	V$PAX4_03
54	0.0569	0.737	M00052	V$NFKAPPAB65_01	73	0.0704	0.737	M01401	V$LBX2_01	92	0.0913	0.737	M01314	V$LHX61_01
55	0.0589	0.737	M01455	V$EN2_01	74	0.0704	0.737	M01444	V$PMX2A_01	93	0.0914	0.737	M01387	V$OTX2_01
56	0.061	0.737	M00105	V$CDPCR3_01	75	0.0715	0.737	M00457	V$STAT5A_01	94	0.0918	0.737	M01334	V$NKX11_01
57	0.0613	0.737	M01399	V$HB24_01	76	0.0729	0.737	M00941	V$MEF2_Q6_01	95	0.092	0.737	M01650	V$PNR_01
58	0.0614	0.737	M01325	V$LH2_01	77	0.0748	0.737	M01655	V$P53_05	96	0.0927	0.737	M01068	V$UF1H3BETA_Q6
59	0.063	0.737	M01403	V$OTX3_01	78	0.0765	0.737	M01341	V$MSX3_01	97	0.0935	0.737	M00624	V$DBP_Q6
60	0.063	0.737	M01448	V$HOXD1_01	79	0.0796	0.737	M01412	V$MSX1_02	98	0.0938	0.737	M01474	V$ESX1_01
61	0.0631	0.737	M01382	V$GBX2_01	80	0.0808	0.737	M00232	V$MEF2_03	99	0.0969	0.737	M01415	V$SHOX2_01
62	0.0631	0.737	M01355	V$ALX3_01	81	0.0808	0.737	M01264	V$TBX15_02	100	0.0972	0.737	M00137	V$OCT1_03
63	0.0633	0.737	M00081	V$EVI1_04	82	0.0818	0.737	M01279	V$IRF3_Q3	101	0.0981	0.737	M01376	V$S8_02
64	0.0639	0.737	M01384	V$RHOX11_02	83	0.0831	0.737	M01252	V$E2F6_01	102	0.0981	0.737	M00257	V$RREB1_01
65	0.0641	0.737	M01330	V$HOXB3_01	84	0.0836	0.737	M00312	V$BEL1_B	103	0.0989	0.737	M00260	V$HLF_01
66	0.0653	0.737	M00251	V$XBP1_01	85	0.0839	0.737	M01347	V$RHOX11_01	104	0.0992	0.737	M00394	V$MSX1_01
67	0.0659	0.737	M00420	V$MEIS1AHOXA9_01	86	0.0878	0.737	M00248	V$OCT1_07	105	0.0996	0.737	M00791	V$HNF3_Q6

Figure 1. Candidate transcription factors prediction by TRAP. A. The search page and criteria of the TRAP program. The known 150-bp mouse *Col10a1* cis-enhancer sequence was submitted in fasta format. B. The bioinformatics analysis of candidate TFs in TRAP. The candidate TFs with a *P*-value between 0.05 and 0.1 were sorted from lowest to highest *P*-value based on their binding affinity.

performed using the transcription factor affinity prediction (TRAP) web tools as previously described (http://trap.molgen.mpg.de/cgi-bin/trap_form.cgi) [39]. The matrix file was selected for the transfac_2010.1 vertebrate, and the background model was set for mouse promoters, and 48 putative TFs with strong binding affinity and a *P*-value less than 0.05 were selected and as previously reported [39]. We still inputted the 150-bp *Col10a1* cis-enhancer sequence in fasta format to the input box and submit it for analysis. Then, the putative binding factors with affinity ranking of a *P* value between 0.05 and 0.1 were as displayed (**Figure 1**). Stat5a was one of the candidate regulators with a *P*-value at 0.0715.

Cell culture

MCT cells are mouse chondrocytes which were immortalized with a temperature-sensitive simian virus 40 large tumor antigen [40]. MCT cells were cultured in DMEM (Hyclone, USA) with 8% fetal bovine serum (FBS, QuaCell, USA) in 8% CO_2 at 32°C. When cells grew until 70%-90% confluence, they were switched to 37°C for continual culture to undergo hypertrophic differentiation. The mouse teratocarcinoma-derived ATDC5 cells are from a common mouse chondrogenic cell line which simulates a sequential process of endochondral ossification after being cultured with chondrogenic induced medium contained ITS (insulin, trans-

Table 1. Primers used for qRT-PCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
β-actin	GGCTGTATTCCCCTCCATCG	CCAGTTGGTAACAATGCCATGT
Col10a1	GCAGCATTACGACCCAAGATC	TCTGTGAGCTCCATGATTGC
Homez	GATGCCAGCAGTCTCAACAG	GTAGCAGGTGCTCATTTCCAT
En1	CTAAGGCCCGATTTCGGTTG	GAGTGAACGGGGTCTCTACCT
Xbp1	AGCAGCAAGTGGTGGATTTG	GAGTTTTCTCCCGTAAAAGCTGA
Nkx2-5	GACAAAGCCGAGACGGATGG	CTGTCGCTTGCACTTGTAGC
Egr1	TCGGCTCCTTTCCTCACTCA	CTCATAGGGTTGTTCGCTCGG
Stat5a	CGCCAGATGCAAGTGTTGTAT	TCCTGGGGATTATCCAAGTCAAT
Tbx15	CTCCGTTGAAGCCTTGATCGG	AGACGCCAGGTCAGTGTGA
Irf3	GAGAGCCGAACGAGGTTCAG	CTTCCAGGTTGACACGTCCG
E2f6	CCATGAACAGATTGTCATTGCAG	GGTCCTTTGGTGCTCCTAATATG
Cdc5	ATTCTGAAAGCAGCGGTAATGA	GATCCAGCCATTCGTACCATC
Lhx6.1	CATTGAGAGTCAGGTACAGTGC	GGGCCGTCCAAATCAGCTT
Dbp	GGAAACAGCAAGCCCAAAGAA	CAGCGGCGCAAAAAGACTC
Shox2	CTTACGGCGTTCGTCTCCAAG	CTCTCTAGCACCTCCCGGTA
Rreb1	GGCAGTCAGGCGATTTGGA	AGTGGGTTATCTGAGTGGGTC
Hlf	CATCCCGTCTCCGAACTGTAT	GACTCGGTGTATTGCGGTTTG
Acan	CCTGCTACTTCATCGACCCC	AGATGCTGTTGACTCGAACCT
Col2a1	CCTCCGTCTACTGTCCACTGA	ATTGGAGCCCTGGATGAGCA
Sox9	TTCATGAAGATGACCGACGA	ATGCACACGGGGAACTTATC
Runx2	ACCCAGCCACCTTTACCTAC	TATGGAGTGCTGCTGGTCTG
Alp	CCAACTCTTTTGTGCCAGAGA	GGCTACATTGGTGTTGAGCTTTT
Mmp13	CTTCTTCTTGTTGAGCTGGACTC	CTGTGGAGGTCACTGTAGACT

ferrin, and sodium selenite) for a long period of time [41, 42]. ATDC5 cells were maintained in the growth medium DME/F-12 (Hyclone, USA) with 5% FBS in 5% CO_2 at 37°C. When the ATDC5 cells grew to 70%-90% confluence, the medium was swapped out for a chondrogenic-induced medium which is consist of the growth medium and 1× ITS (Sigma, USA). On every other day, the medium was replaced with fresh medium. 293T cells were maintained in DMEM with 10% FBS and 5% CO_2 at 37°C.

Total RNA isolation, reverse transcription, and quantitative real-time-PCR (qRT-PCR)

Total RNA was extracted from cells in culture dishes or tissues separated from mouse using TRIzol reagent (Vazyme, China). RNA was reverse transcribed into cDNA using the PrimerScript™ RT Master Mix (TaKaRa, Japan) according to the manufacturer's instructions. cDNA samples were utilized for qRT-PCR using UltraSYBR Mixture (CWBIO, China) to examine the expression levels of target genes. The primer sequences used in this study were all listed in **Table 1**. The relative mRNA expression level was normalized to β-actin level and calculated by the $2^{-\Delta\Delta Ct}$ method.

Western blot

Cells were lysed using RIPA buffer (Millipore, USA) containing proteinase inhibitor. The equal amount of protein samples (100 μg) was separated by 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and then blotted onto polyvinylidene fluoride (PVDF) membranes. After being blocked with 5% non-fat milk in Tris buffered saline with 0.1% Tween-20 (TBST) at room temperature (RT) for an hour, membranes were incubated respectively with primary antibodies overnight at 4°C as follows: anti-β-actin (Beyotime, China), anti-Col10a1 (Abclonal, China) or anti-Stat5a (Abcam, USA). Next day, the membranes were washed in TBST and then incubated in horseradish peroxidase conjugated secondary antibodies (goat anti-rabbit IgG antibody or goat anti-mouse IgG antibody, Beyotime, China) at RT for an hour. After being washed in TBST, the protein bands from western blot were seen using an enhanced chemiluminescence (ECL) kit (Vazyme, China). β-actin was used as an internal control.

Skeletal staining, hematoxylin and eosin (H&E) staining, and immunohistochemistry (IHC)

Mouse skeletons of C57BL/6 mice at postnatal day 1 (P1) were subjected to whole skeletal staining with 0.03% Alcian blue (Sigma, USA) and 0.03% Alizarin red (Sigma, USA) according to the published protocol with some modification [43, 44]. Mouse hind limbs at P1 were separated, fixed in 4% paraformaldehyde, series of ethanol for dehydration, paraffin embedding, and sectioning with 5 μm-thick sagittal sections. The sections were subjected to H&E staining (Solarbio, China) using a standard protocol. We also performed IHC (Wanleibio, China) for the sections, firstly, the paraffin-embedded sections were dewaxed and rehydrated. Next,

they were incubated with hot sodium citrate buffer (0.01 M, pH 6.0) lasting for 30 minutes heated for antigen retrieval. Subsequently, these slices were incubated with 3% hydrogen peroxide (H_2O_2) for 10 minutes at room temperature to inactivate the endogenous peroxidase, then they were blocked by goat serum at room temperature for 30 minutes and incubated with primary antibody at 4°C overnight. Next day, the slices were incubated with biotinylated secondary antibody, HRP-labeled streptavidin, and DAB solution successively. Then the slices were counterstained with hematoxylin and observed under the light microscope (Nikon, Japan).

Transfection of small interfering RNA (siRNA) and plasmids

Cells were seeded into 6-well plates. When the cells grew until 60-80% confluence, they were respectively transfected with si-Stat5a (sense 5'-CGAAAGCAGUUGACGGAUATT-3', antisense 5'-UAUCCGUCAACUGCUUUCGCA-3') and the scrambled sequence of negative control (NC, sense 5'-UUCUCCGAACGUGUCACGUTT-3', antisense 5'-ACGUGACACGUUCGGAGAATT-3') (GenePharma, China) using Lipofectamine RNAiMAX Reagent (Invitrogen, USA) or Stat5a expression plasmids and pcDNA3.1 vector (RiboBio, China) as a negative control using Lipofectamine 3000 Transfection Kit (Invitrogen, USA). After transfection, MCT cells were cultured at 32°C for 6 hours, then they were moved to 37°C and continued to culture for 48 hours. ATDC5 cells were maintained in the chondrogenic-induced medium containing ITS after transfection for 7 days.

Dual-luciferase reporter assay

293T cells were counted and seeded at a certain amount to 24-well plates. When the cells grew until 60-70% confluence, they underwent co-transfection with different reporter plasmids and TK-renilla (RiboBio, China) in different groups. After transfection for 48 h, the luciferase activity was detected using the Dual Luciferase Reporter Assay System (Promega, USA). For comparison, the firefly luciferase activity was normalized to the renilla luciferase activity.

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

The three rounds of staining were all performed on ATDC5 cells. For Alcian blue staining, ATDC5 cells were fixed with methanol for 2 minutes at -20°C and then stained with 0.1% Alcian blue overnight. For Alp staining, ATDC5 cells were fixed, incubated with substrate and stained following the manufacturer's instructions (Nanjing Jiancheng, Bioengineering Institute, China). For Alizarin red staining, ATDC5 cells were fixed with 95% ethanol for 10 minutes at room temperature, and followed by staining with 1% Alizarin red for 10 minutes. Then cells were observed and photographed under the light microscope.

Statistical analysis

All experiments in this study were performed in triplicate at least. GraphPad Prism 8.0 software was used for statistical analysis in this study. Data were analyzed using Student's t-test to determine statistically significant differences between the two groups, while using one-way analysis of variance (ANOVA) test for three groups or more. $P<0.05$ was considered to be statistically significant.

Results

Candidate transcription factors prediction by TRAP

We recently reported 48 potential TF binding sites within the known 150-bp mouse *Col10a1* cis-enhancer identified by TRAP program at the significance level (based on affinity ranking) of $P<0.05$. Here, we continued to predict and research the potential TFs with a *P*-value within the range at $0.05<P<0.1$. We set the search criteria as shown in **Figure 1A**. After inputting the known 150-bp mouse *Col10a1* cis-enhancer sequence in fasta format and submitting it for analyses, we got approximately 60 (57) candidate TFs with a *P*-value between 0.05 and 0.1, and they were sorted from lowest to highest *P*-value in the light of their binding affinity (**Figure 1B**).

Expression analysis of Col10a1 and candidate TFs in vitro and in situ

To characterize the candidate *Col10a1* regulators predicted by TRAP, we examined *Col10a1* and candidate TFs in MCT cells, ATDC5 cells, as well as primary chondrocytes extracted from growth plates of mouse ribs. MCT cells are in proliferative stage when cultured at 32°C, whereas they undergo growth arrest and hyper-

trophic differentiation at 37°C accompanied by significantly upregulated *Col10a1* expression. The qRT-PCR results showed that the mRNA level of *Col10a1* was notably increased when MCT cells were placed in the incubator whose temperature was set at 37°C instead of 32°C (**Figure 2A**). The mouse chondrogenic cell line ATDC5 mimics different stages of endochondral ossification under a long-term ITS induction. The results of qRT-PCR revealed that *Col10a1* mRNA levels were all elevated on day 7, 14 and 21 under ITS induction compared with day 0 (no ITS induction), and peaked on day 14 (**Figure 2B**). Then we performed Alcian blue and Alizarin red staining of the whole postnatal day 1 (P1) mouse skeleton to stain the cartilage blue and the bone red. The results revealed that a mass of bone tissue and cartilage tissue in the ribs are available for extraction (**Figure 2C**). Thus, we observed mouse rib growth plates to distinguish proliferative zone (PZ), hypertrophic zone (HZ) and bone marrow (BM), then extracted primary proliferative chondrocytes and hypertrophic chondrocytes *in situ* respectively under stereoscopic microscope (**Figure 2D**). qRT-PCR results showed a marked elevation of *Col10a1* mRNA level in HZ than PZ (**Figure 2E**). Overall, these findings demonstrated a significantly elevated *Col10a1* mRNA level in hypertrophic chondrocytes compared with proliferative chondrocytes *in vitro* and *in situ*.

Next, the mRNA levels of all the candidate TFs were detected in the above two cell models *in vitro* and primary chondrocytes of mouse ribs *in situ*. The results indicated that the mRNA levels of *Homez*, *Stat5a*, *Tbx15*, *Cdc5*, *Shox2* and *Rreb1* were upregulated in hypertrophic MCT cells (**Figure 2F**). When it comes to ATDC5 cells, the mRNA levels of *Stat5a*, *Tbx15* and *Cdc5* were upregulated after ITS induction for 14 days (**Figure 2G**). Moreover, we showed that the mRNA levels of *Stat5a* and *Cdc5* were upregulated in hypertrophic primary chondrocytes of mouse ribs (**Figure 2H**). The expression changes of these candidate TFs upon *Col10a1* upregulation in hypertrophic chondrocytes suggested that they were potential *Col10a1* regulators.

Stat5a expression is increased in hypertrophic chondrocytes

Stat5a is a candidate TF identified by the TRAP program and is increased not only in two hypertrophic cell models but also in hypertrophic primary chondrocytes of mouse ribs (**Figure 2F-H**). To further analyze the relevance between Stat5a and *Col10a1* expression, we examined *Stat5a* mRNA level during the whole ITS induction process. These findings indicated that *Stat5a* mRNA level corresponds well with *Col10a1* (**Figures 2B** and **3A**). Western blot results indicated that the protein levels of both Stat5a and Col10a1 were noticeably elevated after ITS induction for 14 days in ATDC5 cells (**Figure 3B**). As shown in **Figure 3C**, the same trend was observed in MCT cells. Then we performed H&E staining and IHC analysis on the sagittal sections of P1 mouse hind limbs. The growth plate was categorized into two different zones: proliferative zone (PZ) and hypertrophic zone (HZ), depending on the shape and size of the chondrocytes (**Figure 3D**). IHC analysis indicated that both Col10a1 and Stat5a expression were more abundant in hypertrophic chondrocytes than in proliferative chondrocytes (**Figure 3E**). In summary, these findings supported that Stat5a and Col10a1 expression in hypertrophic chondrocyte were positively correlated.

Stat5a promotes Col10a1 expression in vitro

To investigate the effect of Stat5a in regulating *Col10a1* expression *in vitro*, we knocked-down or overexpressed Stat5a in MCT and ATDC5 cells respectively. The findings revealed that the mRNA and protein levels of Col10a1 were markedly decreased when Stat5a was knocked-down by transient transfection of siRNA in MCT cells (**Figure 4A** and **4B**). Meanwhile, when Stat5a was overexpressed by transient transfection of Stat5a expression plasmids in MCT cells, we detected an upregulation of mRNA and protein levels of Col10a1 (**Figure 4C** and **4D**). Consistent with those findings, the mRNA and protein levels of Col10a1 were decreased by Stat5a knockdown while increased by Stat5a overexpression in ATDC5 cells (**Figure 4E-H**). Taken together, above findings indicated that Stat5a participated in Col10a1 gene regulation and could promote Col10a1 expression in hypertrophic chondrocytes.

Luciferase reporter assay of STAT5a transcriptional activity on Col10a1 promoter

To elucidate the mechanism by which Stat5a regulates Col10a1 expression, we performed

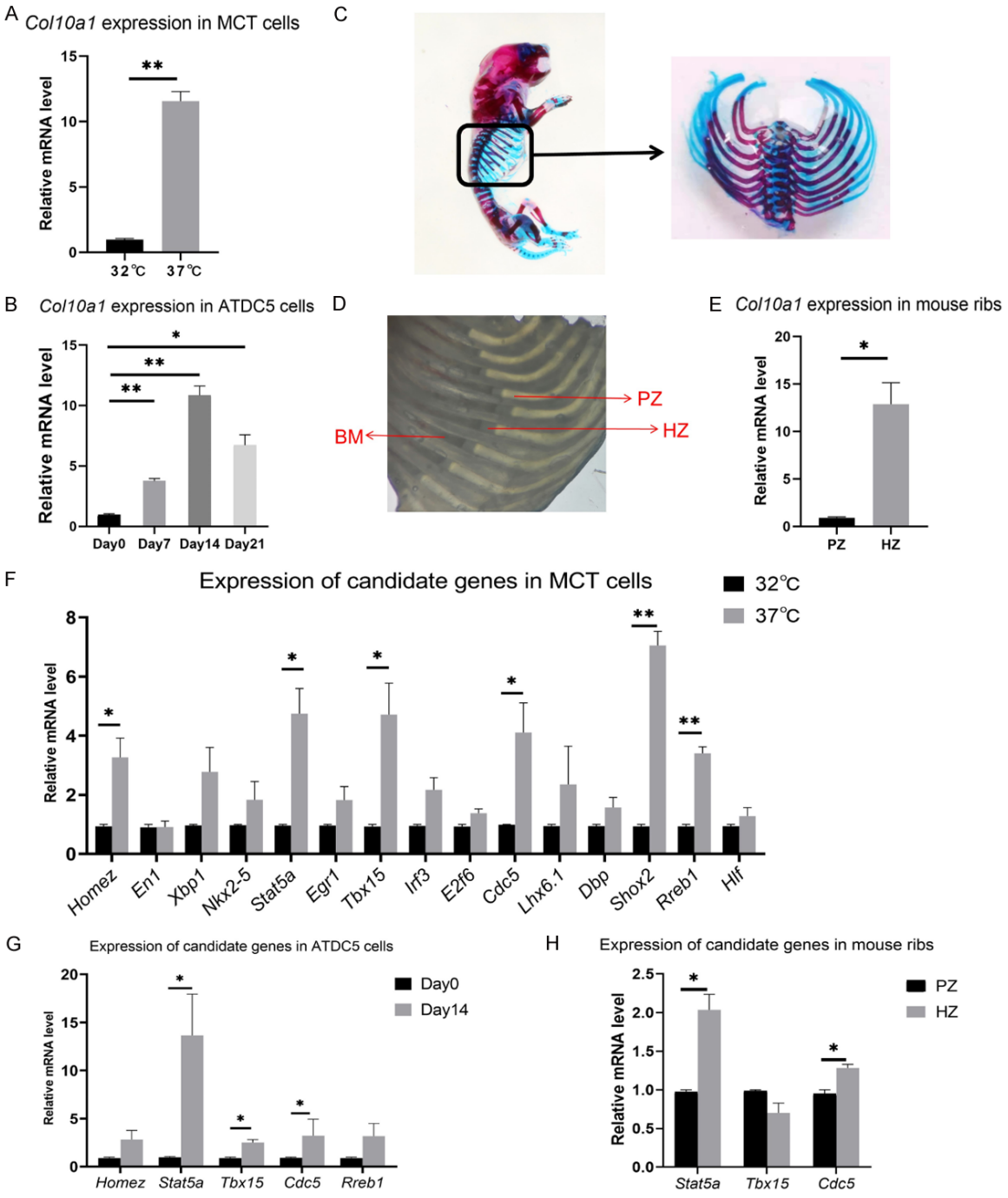


Figure 2. Expression analysis of *Col10a1* and candidate TFs *in vitro* and *in situ*. A. qRT-PCR showed the mRNA level of *Col10a1* was notably increased at 37 °C compared with 32 °C in MCT cells. B. qRT-PCR showed the relative mRNA level of *Col10a1* was increased on day 7, 14 and 21 under ITS induction compared with day 0 (no ITS induction), and peaked on day 14 in ATDC5 cells. C. Alcian blue and Alizarin red staining of the whole P1 mouse skeleton. D. The view of mouse rib growth plates under stereoscopic microscope. E. qRT-PCR showed the mRNA level of *Col10a1* in hypertrophic zone (HZ) was higher than proliferative zone (PZ) of mouse ribs. F. Relative mRNA expression of candidate TFs in MCT cells at 32 and 37 °C were detected by qRT-PCR. The mRNA levels of *Homez*, *Stat5a*, *Tbx15*, *Cdc5*, *Shox2* and *Rreb1* were upregulated at 37 °C. G. Relative mRNA expression of candidate TFs in ATDC5 cell line on day 0 and day 14 of ITS induction were detected by qRT-PCR. The mRNA levels of *Stat5a*, *Tbx15* and *Cdc5* were increased on day 14. H. Relative mRNA expression of candidate TFs for PZ and HZ of mouse ribs were detected by qRT-PCR. The mRNA levels of *Stat5a* and *Cdc5* were upregulated in hypertrophic primary chondrocytes. $^{*}P<0.05$, $^{**}P<0.01$.

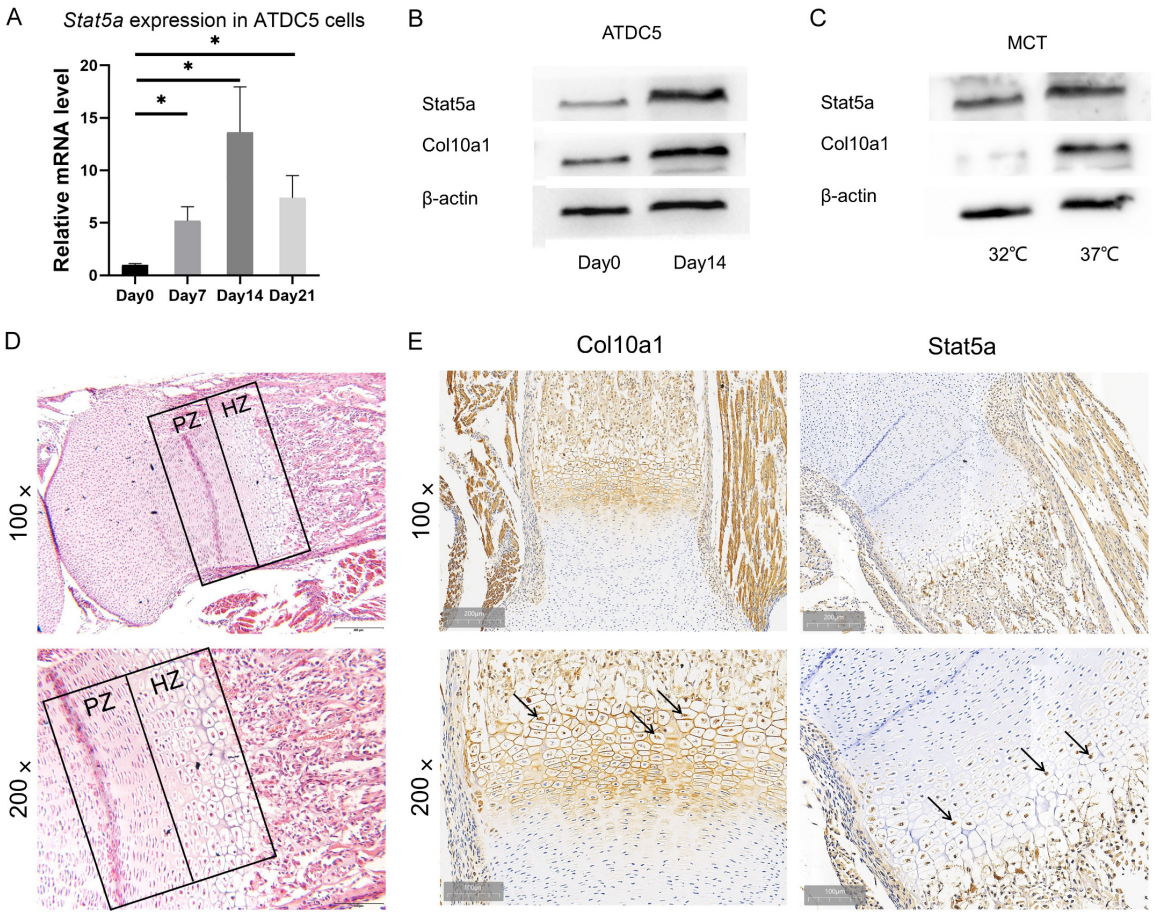


Figure 3. Stat5a expression is increased in hypertrophic chondrocyte. (A) qRT-PCR showed the mRNA level of *Stat5a* was upregulated on day 7, 14 and 21, and peaked on day 14 in ATDC5 cells during ITS induction. (B) Western blot showed the protein levels of both Stat5a and Col10a1 were noticeably increased after ITS induction for 14 days in ATDC5 cells. (C) Western blot showed the protein levels of both Stat5a and Col10a1 are elevated in MCT cells at 37°C compared with 32°C. (D) H&E staining and (E) IHC analysis of the sagittal sections of P1 mouse hind limbs. Col10a1 and Stat5a expression were more abundant in hypertrophic chondrocytes than in proliferative chondrocytes. The black arrows represent the positive cells. $^{*}P<0.05$.

dual-luciferase reporter assay to verify the interaction between Stat5a and the 150-bp *Col10a1* cis-enhancer. The findings revealed that co-transfection of pcDNA3.1/Stat5a plasmid and pGL3/Col10a1 enhancer plasmid in 293T cells increased the relative luciferase activity (**Figure 5A**). Western blot results verified the considerable overexpression effect of Stat5a after transfection with pcDNA3.1/Stat5a plasmid in 293T cells (**Figure 5B**). Overall, these findings demonstrated that Stat5a potentiated the activity of *Col10a1* cis-enhancer via direct interaction.

Effect of Stat5a on chondrocyte differentiation

To further explore the influence of Stat5a on chondrocyte development, we performed three kinds of staining experiments on ATDC5 cells and qRT-PCR on several relevant marker genes during chondrocyte differentiation in MCT and ATDC5 cells. As illustrated in **Figure 6A**, the intensity of Alp staining was weaker in the si-Stat5a group than in the NC group and stronger in the pcDNA3.1/Stat5a group compared with the pcDNA3.1 group, implying the positive role of Stat5a in chondrocyte differentiation and maturation. Nevertheless, no clear difference was observed in the intensity of Alcian blue and Alizarin red staining between experimental and control groups, indicating a limited role of Stat5a in chondrocyte proliferation at early stage and matrix mineralization at late stage of endochondral ossification (**Figure 6A**).

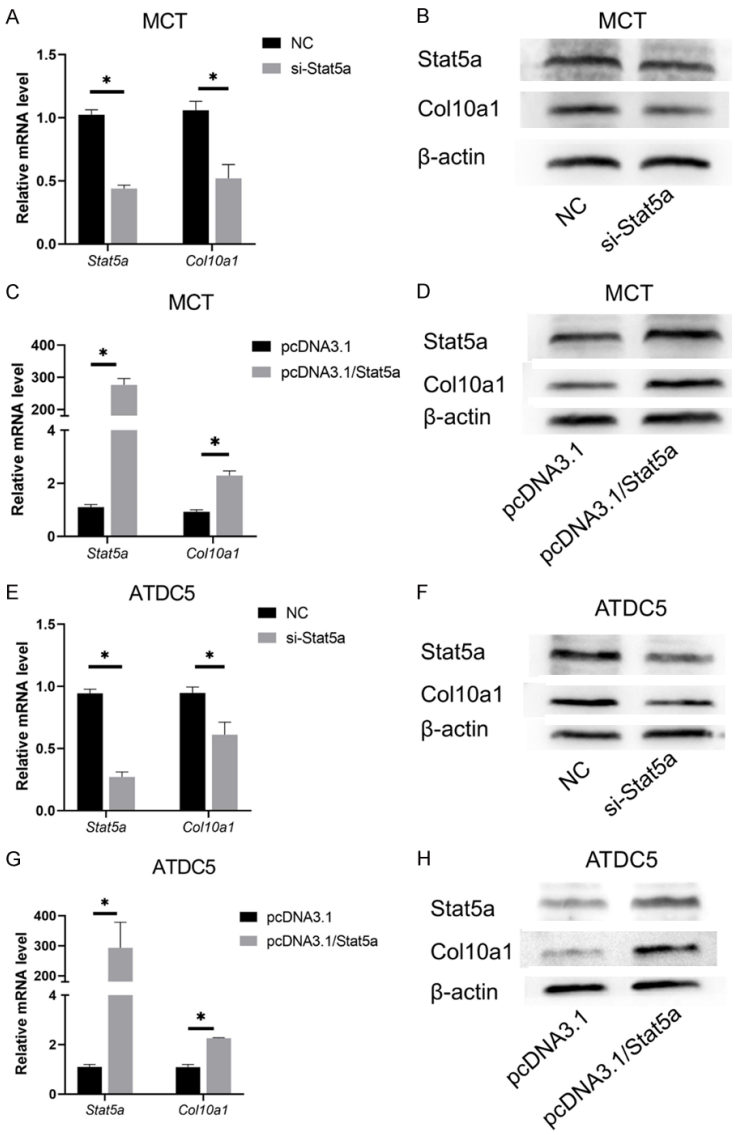


Figure 4. Stat5a promotes Col10a1 expression *in vitro*. (A) mRNA and (B) protein expression of Stat5a and Col10a1 are decreased after transient transfection of siRNA and negative control sequence in MCT cells. (C) mRNA and (D) protein expression of Stat5a and Col10a1 are increased after transient transfection of Stat5a expression plasmids and pcDNA3.1 vector as a control in MCT cells. (E) mRNA and (F) protein expression of Stat5a and Col10a1 are decreased after transient transfection of siRNA and scrambled control sequence in ATDC5 cells. (G) mRNA and (H) protein expression of Stat5a and Col10a1 are increased after transient transfection of Stat5a expression plasmids and pcDNA3.1 vector as a control in ATDC5 cells. $^{*}P<0.05$.

In MCT cells, we detected significantly reduced mRNA levels of *Runx2* and *Alp* in the Stat5a knockdown group while noticeable elevated mRNA level of *Runx2* in the Stat5a overexpression group (**Figure 6B** and **6C**). In ATDC5 cells, we observed decreased mRNA levels of *Runx2*, *Alp* and *Mmp13* in the Stat5a knockdown group while increased mRNA level of them in the Stat5a overexpression group, whereas genes *Acan*, *Col2a1* and *Sox9* showed no significant difference (**Figure 6D** and **6E**). The aforementioned genes have been linked to chondrocyte proliferation, differentiation and maturation. Overall, the diverse expression profiles of the relevant marker genes pointed to a limited but definite role of Stat5a during chondrocyte (hypertrophic) differentiation.

Discussion

For years, we have been working on the regulation of hypertrophic chondrocyte-specific *Col10a1* gene expression during endochondral ossification both *in vitro* and *in situ*. We sought to search the regulators which could bind to mouse *Col10a1* cis-enhancer and regulate *Col10a1* expression as well as affect chondrogenic differentiation. Previous studies have predicted and analyzed the transcription factors that may directly interact with the 150-bp *Col10a1* cis-enhancer by TRAP of $P<0.05$ [28]. Here, we tried to systematically research potential binding factors in $0.05<P<0.1$ predicted by TRAP for the first time. The TRAP web tools could predict the affinity of a TF to a given sequence and all potential binding sites within the sequence. The *P*-value in the TRAP web tools is a comparable statistical score which is defined to normalize an observed affinity, allowing a direct comparison of the binding affinity of different factors [39, 45]. In general, $P<0.05$ is statistically significant, giving a statistical meaning to the binding affinity prediction in TRAP. Considering the statistical *P*-value and significance level of 0.05 as just probability, the result of $P<0.05$ indicates that the aforementioned candidate TFs have a higher probability of binding to the 150-bp *Col10a1* cis-enhancer statistically, but the biological

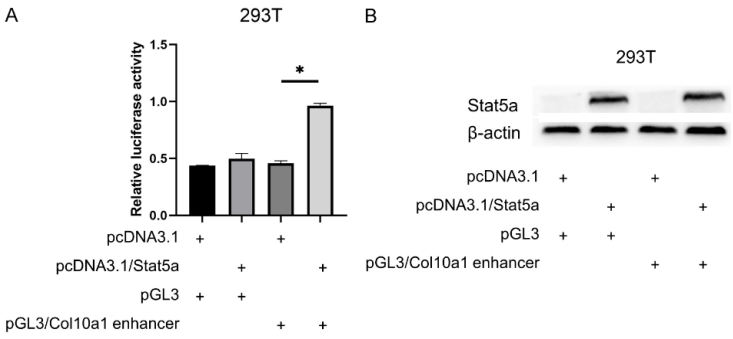


Figure 5. Luciferase reporter assay of STAT5a transcriptional activity on Col10a1 promoter. A. Dual-luciferase reporter assay results in 293T cells. Co-transfection of pcDNA3.1/Stat5a plasmid and pGL3/Col10a1 enhancer plasmid increased the relative luciferase activity compared with other groups. B. Western blot results of each group in dual-luciferase reporter assay showed transfection of pcDNA3.1/Stat5a plasmid significantly elevated the protein level of Stat5a. $^{*}P<0.05$.

function of them needs to be validated. Meanwhile, for the TFs predicted by TRAP at 0.05<*P*<0.1, they just represent a relatively lower binding probability statistically, again without biological evidence to follow. This implies that some TFs may also be engaged in *Col10a1* gene regulation although in 0.05< *P*<0.1. Indeed, there are several TFs in 0.05<*P*<0.1, such as XBP1, HIF2A, EGR1, FoxA2 (i.e. HNF3B) and Shox2, that have been demonstrated to play a positive role in the regulation of *Col10a1* gene expression and chondrocyte hypertrophy or maturation in previous studies [46-52]. Therefore, we attempted to perform an in-depth study of the candidate TFs in 0.05<*P*<0.1.

To research these factors, we examined *Col10a1* and candidate TFs gene expression condition in MCT cells, ATDC5 cells and mouse rib growth plates and found that Stat5a increased along with Col10a1 in hypertrophic chondrocyte. This finding caught our attention since Stat5a is an essential TF controlling cell differentiation and has already been demonstrated to play a vital role in chondrogenesis [35-38, 53, 54]. Subsequently, we investigated the function of Stat5a and found that Stat5a could promote Col10a1 expression *in vitro*. Then we carried out dual-luciferase reporter assay and found that relative luciferase activities of the plasmids containing the 150-bp mouse *Col10a1* cis-enhancer was increased in the presence of Stat5a, which indicates that Stat5a promoted the activity of *Col10a1* cis-enhancer via direct interaction. This result is consistent with the prediction of the TRAP program and endowed the statistical prediction with biological meaning. Previous studies have identified and characterized the *Col10a1* regulators by analysis of the 150-bp cis-enhancer adopting a mass of bioinformatics and proteomic methods, and eventually confirmed the interaction between Cox-2 and *Col10a1* cis-enhancer, following a study showed that Cox-2 promote Col10a1 expression and chondrocyte hypertrophic differentiation [26, 27].

In the qRT-PCR results of several relevant marker genes during chondrocyte differentiation in ATDC5 cells, we can see that *Acan*, *Col2a1* and *Sox9* showed no obvious change when Stat5a was knocked-down or overexpressed. These findings were consistent with the result of Alcian blue staining, which showed the content of cartilage-specific proteoglycan [55]. As is known to all, *Acan*, *Sox9* and *Col2a1* are relevant marker genes of chondrocyte proliferation at early stage of chondrogenesis [56]. These findings suggested an insignificant role of Stat5a in early chondrogenesis. *Runx2*, *Alp* and *Mmp13* are regarded as relevant marker genes for chondrocyte hypertrophy and maturation [57, 58]. The results of qRT-PCR and Alp staining on ATDC5 cells together showed that Stat5a promoted chondrocyte hypertrophic differentiation. It is noteworthy that the expression level of *Runx2* was stably consistent with Stat5a and Col10a1 whether knockdown or overexpression in both MCT and ATDC5 cells. Runx2 is an essential TF involved in mouse *Col10a1* gene regulation via direct interaction with its cis-enhancer [25]. This suggested that Stat5a may act in concert with Runx2 in chondrocytes to modulate the cell-specific *Col10a1* gene expression. Transcriptional activation function of STAT5 has been reported to require the cooperation with other transcription factors that bind tightly to target gene promoters and enhancers [59]. Indeed, a previous study has reported a STAT5-RUNX2 interaction and its favorable effects on osteoblast differentiation in MSC [53].

There are some limitations for this study. Our functional and mechanistic research was per-

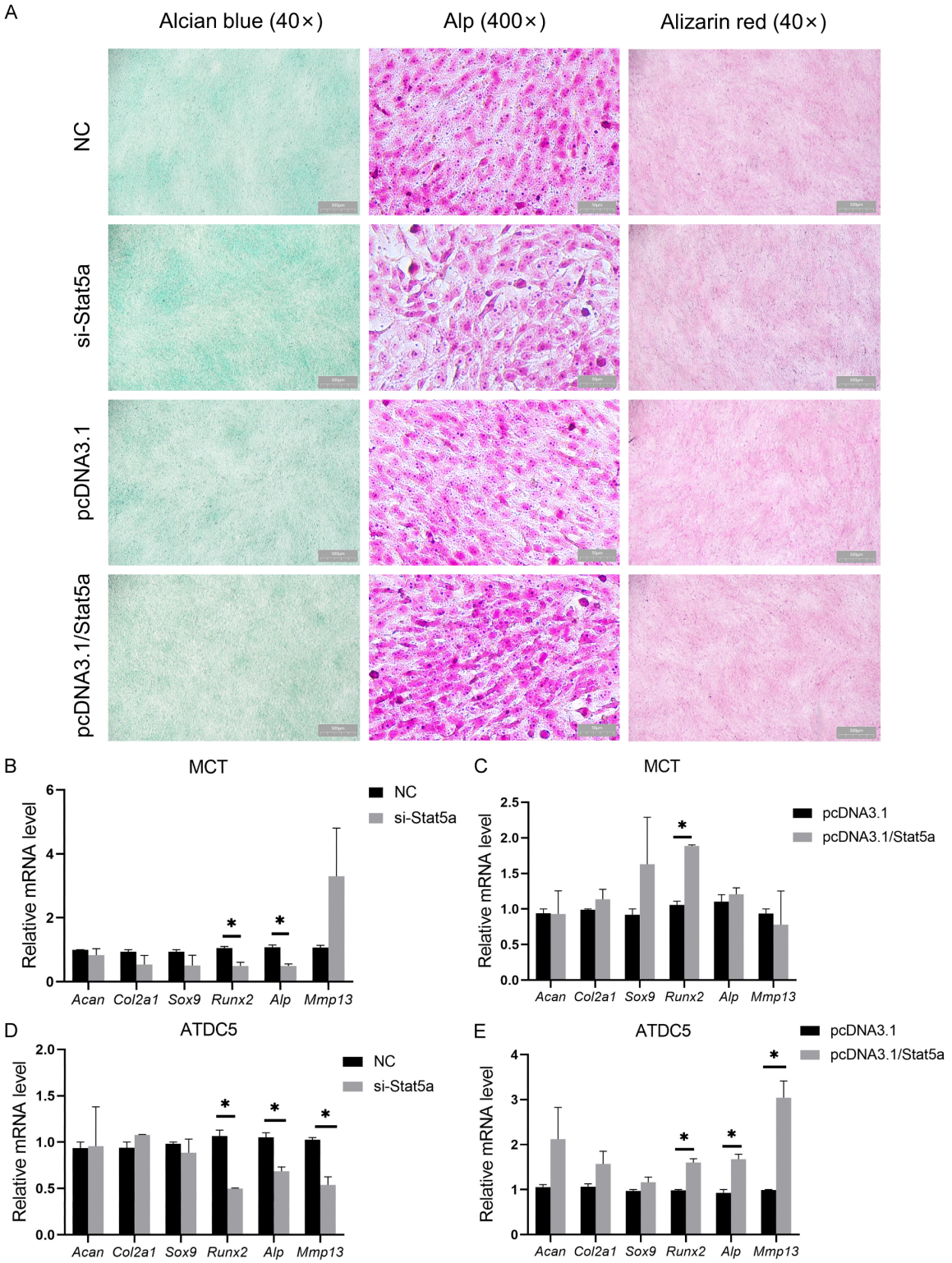


Figure 6. Effect of Stat5a on chondrocyte differentiation. A. Alcian blue (40×), Alp (400×) and Alizarin red staining (40×) of ATDC5 cells. The intensity of Alp staining was weaker at si-Stat5a group than NC group and stronger at pcDNA3.1/Stat5a group compared with pcDNA3.1 group. No clear difference was observed in the intensity of Alcian blue and Alizarin red staining between experimental and control groups. B. Relative mRNA expression of *Runx2* and *Alp* were decreased when Stat5a was knocked down by si-Stat5a in MCT cells. C. Relative mRNA expression of *Runx2* was increased when Stat5a was over expressed by pcDNA3.1/Stat5a plasmid in MCT cells. D. Relative mRNA expression of *Runx2*, *Alp* and *Mmp13* was decreased when Stat5a was knocked down by si-Stat5a in ATDC5 cells. E. Relative mRNA expression of *Runx2*, *Alp* and *Mmp13* was increased when Stat5a was over expressed by pcDNA3.1/Stat5a plasmid in ATDC5 cells. $^{*}P<0.05$.

formed *in vitro*, which means further *in vivo* studies are needed in the future. In addition, the detailed relationship and exact regulatory mechanism of Stat5a, Runx2 and Col10a1 in endochondral ossification requires further investigation.

In conclusion, we have demonstrated that Stat5a was increased along with Col10a1 in hypertrophic chondrocyte. Also that Stat5a promoted Col10a1 expression and chondrocyte hypertrophy through direct interaction with its binding site within the 150-bp *Col10a1* cis-enhancer. Taken together, these findings revealed Stat5a as a new regulator of endochondral bone formation as well as suggesting potential broad application prospect of it in skeletal development disorders.

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Disclosure of conflict of interest

None.

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