Published in final edited form as:

Exp Cell Res. 2009 August 1; 315(13): 2231–2240. doi:10.1016/j.yexcr.2009.03.008.

Sox9 directly promotes Bapx1 gene expression to repress Runx2 in chondrocytes

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Abstract

The transcription factor, Sry-related High Mobility Group (HMG) box containing gene 9 (Sox9), plays a critical role in cartilage development by initiating chondrogenesis and preventing the subsequent maturation process called chondrocyte hypertrophy. This suppression mechanism by Sox9 on late-stage chondrogenesis partially results from the inhibition of Runt-related transcription factor 2 (Runx2), the main activator of hypertrophic chondrocyte differentiation. However, the precise mechanism by which Sox9 regulates late chondrogenesis is poorly understood.

In the present study, the transcriptional repressor vertebrate homolog of *Drosophila* bagpipe (Bapx1) was found to be a direct target of Sox9 for repression of Runx2 expression in chondrocytes. We identified a critical Sox9 responsive region in the *Bapx1* promoter via a luciferase reporter assay. Analysis by chromatin immunoprecipitation and electrophoretic mobility shift assays indicated that Sox9 physically bound to this region of the *Bapx1* promoter. Consistent with the notion that Bapx1 and Sox9 act as negative regulators of chondrocyte hypertrophy by regulating Runx2 expression, transient knockdown of Sox9 or Bapx1 expression by shRNA in chondrocytes increased *Runx2* expression, as well as expression of the late chondrogenesis marker, *Col10a1*. Furthermore, while over-expression of Sox9 decreased *Runx2* and *Col10a1* expressions, simultaneous transient knockdown of Bapx1 diminished that Sox9 over-expressing effect.

Our findings reveal that the molecular pathway modulated by Bapx1 links two major regulators in chondrogenesis, Sox9 and Runx2, to coordinate skeletal formation.

Keywords

Sox9; Bapx1; Runx2; chondrocyte; chondrogenesis; shRNA

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Introduction

Chondrogenesis is executed in multiple steps during endochondral ossification. Mesenchymal cells first proliferate, condense, become immature chondroblasts, transform into prehypertrophic chondrocytes, and differentiate into hypertrophic chondrocytes [1]. In immature chondroblasts (i.e., during early chondrogenesis), extracellular matrix (ECM) proteins such as type II collagen alpha 1 (Col2a1), type XI collagen alpha 2 (Col11a2), and aggrecan 1 (Agc1) are specifically expressed. On the other hand, in prehypertrophic and hypertrophic chondrocytes (i.e., during late chondrogenesis), expression of these proteins is decreased, while type X collagen alpha 1 (Col10a1), Indian hedgehog (Ihh), and osteopontin expression is increased [2]. In addition, Sox9 is dominantly expressed in early chondrogenesis, whereas Runx2 dominates as the critical transcription factor in late chondrogenesis [2,3].

The transcription factor Sox9 is a critical regulator of chondrocyte-specific proteins, such as Col2a1, Col11a2, and Agc1, during cartilaginous development [4–6]. Analyses in genetically modified mice revealed that Sox9 promotes the early stage, but suppresses the late stage, of chondrogenesis [7–9]. On the contrary, the transcription factor Runx2 is a critical enhancer of chondrocyte maturation and osteoblast differentiation [10–14]. Although the relationship between Sox9 and Runx2 in chondrogenesis is poorly understood, Sox9 directly binds to Runx2 and inhibits its function [15].

Another transcription factor, Bapx1 (also known as Nkx3.2), plays a critical role during cartilage development. [16–18]. Bapx1 is a transcriptional repressor known to regulate Runx2 expression [19]. Interestingly, a recent study revealed that forced expression of Bapx1 throughout the cartilage template blocked chondrocyte hypertrophy, mirroring the Sox9 over-expression phenotype [20]. Furthermore, experiments using chicken somatic explants in culture have shown that Bapx1 and Sox9 are able to induce the expression of each other [21].

While Bapx1 is known to be a transcription factor crucial for chondrogenic differentiation [22], little is known about its transcriptional targets. Recently, Runx2 has been reported to be a target gene of Bapx1 [19], representing a critical transcription factor for osteoblast differentiation and also promoting hypertrophic chondrocyte differentiation in endochondral ossification [10–14,23]. Furthermore, Sox9 promotes the early stage, but suppresses the late stage, of chondrogenesis [9]. These results suggest that Bapx1 potentially links the two major regulators of chondrogenesis, Sox9 and Runx2.

In the present study, Bapx1 expression was up-regulated by Sox9 in chondrocytes, and luciferase reporter assays revealed that Sox9 activated the *Bapx1* promoter. Chromatin immunoprecipitation (ChIP) and electrophoretic mobility shift assays (EMSA) demonstrated that Sox9 physically bound to the *Bapx1* promoter in chondrocytes. Additionally, our gain- and loss-of-function experiments showed that the expression of *Runx2* and its target, *Col10a1*, was repressed by Sox9, coincident with increased Bapx1 expression. These data collectively demonstrate that Bapx1 is a direct Sox9 target in chondrocytes. Moreover, our findings strongly suggest that Sox9 represses Runx2 through Bapx1 regulation, resulting in suppression of hypertrophic chondrocyte differentiation.

Materials and Methods

Antibodies

Anti-Sox9 antibody (Chemicon) was used in western blots and electrophoretic mobility shift assays (EMSA). Anti-Bapx1 antibody (Abcam) was used in western blots. Anti-actin antibody (Sigma) was used as a control for quantitative determination of western blots. As secondary antibodies for western blots, horseradish peroxidase (HRP)-conjugated anti-rabbit

immunoglobulin G (IgG) and anti-mouse IgG (Sigma) were used. Normal rabbit IgG (Santa Cruz Biotechnology Inc.) was used as a negative control in chromatin immunoprecipitation (ChIP) and EMSA experiments.

Cell culture

C3H10T1/2 cells were maintained in DMEM containing 10% FBS at 37°C in an atmosphere of 5% CO₂. To induce chondrogenesis, 10 µl aliquots of a suspension containing 10⁷ cells/ml were plated into wells of a 24-well plate containing culture medium. After 2 hours, 100 ng of human bone morphogenetic protein 2 (BMP-2) (PeproTech)/ml was added to the culture. Media was changed every 2 days.

Ribs from embryonic day 16.5 mice were used in the preparation of chondrocytes by digestion with collagenase. Murine chondrocytes were maintained in DMEM containing 10% FBS at 37°C in an atmosphere of 5% CO₂.

Western blot

Cells were lysed with NP-40 lysis buffer (20 mM Tris-HCl (pH 7.6), 3 mM EDTA, 150mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM β-glycerophosphate, 5 mM Na₄P₂O₇ □ 10 H₂O, 1mM Na₃VO₄, 10 µg/ml PMSF) containing a Complete® protease inhibitor cocktail (Roche Diagnostics, Tokyo, Japan). Protein concentrations were measured using the DC Protein Assay kit (Bio-Rad Laboratories, CA, USA), and equal amounts of protein were subjected to SDS-polyacrylamide gel electrophoresis (PAGE). Separated proteins were electrically transferred to PVDF membrane, blocked with TBST (Tris-buffered saline containing 0.1% Tween-20) containing 5% skim milk, washed with TBST, and then incubated with primary antibody (appropriately diluted with blocking buffer) for either 1.5 hours at room temperature (RT) or overnight at 4°C. Subsequently, blots were washed with TBST, incubated with secondary antibody (diluted in blocking buffer) for 1 hour at RT, and washed with TBST. Proteins were detected using a chemiluminescence kit (Nacalai, Kyoto, Japan).

RT-Qualitative and quantitative PCR

Total RNA was extracted with ISOGEN (Nippon Gene) according to the manufacturer's protocol. Total RNA was reverse transcribed with Ready-To-Go You-Prime First-Strand Beads (GE Healthcare) with oligo-dT. To analyze gene expression, first strand cDNA was amplified via PCR, using gene-specific primer sets (Table 1) and PrimeStar™ HS DNA Polymerase (Takara), followed by agarose gel electrophoresis.

Quantitative gene expression analysis was performed via real-time PCR, using TaqMan Universal Master Mix reagents with TaqMan Probes or SYBR Green PCR master mix reagents (Applied Biosystems) with gene-specific primer sets (Table 1) on an ABI PRISM® 7900HT thermal cycler (Applied Biosystems). *Col2a1*, *Runx2*, *Col10a1*, and *Gapdh* mRNAs were measured using mouse TaqMan probes Mm00491889_m1, Mm00501578_m1, Mm00487041_m1, and Mm99999915_g1, respectively (Applied Biosystems). In each experiment, expression data was normalized to *Gapdh* expression.

Lentivirus production and transduction

Oligonucleotide sequences for short hairpin RNA (shRNA) targeting Sox9, Bapx1, or LacZ as a control were cloned into the pLenti4/BLOCK-iT™-DEST vector (Invitrogen), and *Sox9* cDNA was cloned into the pLenti6 vector (Invitrogen). Each vector and ViraPower packaging vector (Invitrogen) were transfected to 293FT cells, and lentivirus was produced. Lentivirus was transduced into cells with 10µg/ml polybrene. The following oligonucleotide sequences were cloned into the pLenti4/BLOCK-iT™-DEST vector: LacZ (5'-

AAATCGCTGATTTGTGTAGTCGGAGACGACTACACAAATCAGCGA-3'), Sox9 (5'-GCGACAACCTTTACCAGTTTCACGAATGAACTGGTAAAGTTGTCGC-3'), Sox9-2 (5'-GCGACGTCATCTCCAACATTGCGAACAATGTTGGAGATGACGTCGC-3'), and Bapx1 (5'-GAGATGTCAGCCAGCGTTTCACGAATGAAACGCTGGCTGACATCTC-3')

Luciferase reporter assay and transfections

pGL4.12-Luc vector (Promega) including the indicated cloned genomic regions and thymidine kinase (TK) promoter was transfected into cultured cells. A Renilla luciferase reporter pRL-TK (Promega) was co-transfected as a control for evaluating transfection efficiency. Transfections were performed with Lipofectamine 2000 or LTX (Invitrogen) according to the manufacturer's instructions. After 24 hours, cells were washed before measuring firefly and Renilla luciferase activities using the Dual-Glo™ Luciferase Assay System (Promega). In each experiment, firefly luciferase activity was normalized to Renilla luciferase activity.

Mutagenesis

Mutations were introduced using QuikChange® Site-Directed Mutagenesis (Stratagene) according to the manufacturer's instruction.

Chromatin immunoprecipitation (ChIP)

Cells were cross-linked with 1% formaldehyde for 10 minutes at RT, followed by addition of glycine to a final concentration of 0.125 M. Next, cells were washed with PBS, collected, washed with cell lysis buffer (5mM PIPES pH 8.0, 85 mM KCl, 0.5% NP-40, and 10 µg of PMSF/ml) containing protease inhibitors, and resuspended in nuclear lysis buffer (50 mM Tris-HCl pH 8.1, 10 mM EDTA, 1% SDS, and 10 µg of PMSF/ml) containing protease inhibitors. Chromatin was sheared to approximately 200-1000 bp by sonication, prior to a 1/5 dilution in ChIP dilution buffer (16.7 mM Tris-HCl pH 8.1, 167 mM NaCl, 1.2 mM EDTA, 0.01% SDS, 1.1% Triton X-100, and 10 µg of PMSF/ml) containing protease inhibitors. Then, the chromatin solution was incubated with the indicated antibody and Dynabeads® M-280 sheep anti-rabbit IgG (Dyna Biotech) overnight at 4°C. Beads were washed three times with ChIP wash buffer (50 mM HEPES-KOH (pH 7.0), 0.5 M LiCl, 1 mM EDTA, 0.7% sodium deoxycholate, and 1% NP-40). Immunocomplexes were eluted from beads with ChIP elution buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, and 1% SDS) for 1 hour at 65°C. Eluates were then incubated overnight at 65°C to reverse cross-linking, prior to the addition of 0.5 mg of protease K/ml for 2 hours at 55°C. DNA was purified using the MinElute PCR purification kit (QIAGEN) and analyzed using the site-specific primer pair sets described in Table 1. Also, whole cell extract (WCE) was analyzed by PCR.

Electrophoretic mobility shift assay (EMSA)

DNA-protein binding was assayed with DNA probes that had been ³²P-labeled by end-filling with Klenow fragment and Sox9 in the presence or absence of anti-Sox9 antibody or rabbit IgG. Anti-Sox9 antibody or rabbit IgG was pre-incubated with Sox9 for 30 minutes at 25°C before addition of radiolabeled probes. Reactions were performed in binding buffer [20mM HEPES (pH 7.9), 10% glycerol, 50 mM KCl, 0.05% NP-40, 0.5 mM EDTA, 0.5 mM DTT, and 1 mM PMSF] in the presence of 0.5 µg of poly (dG-dI), a nonspecific competitor, for 30 minutes at 25°C. Products of the binding reactions were separated by polyacrylamide gel electrophoresis (PAGE) on a 4% gel for 3 hours at 100 V.

Results

Bapx1 is expressed in early chondrogenesis, and its expression is up-regulated by Sox9 in chondrocytes

The temporal sequence of Sox9 and Bapx1 expression in chondrogenesis was first examined by a well-established *in vitro* model, in which C3H10T1/2 cells are induced to undergo chondrogenesis by being plated as a high-density micromass culture in the presence of BMP-2 [24,25]. During the chondrogenesis assay, expression of Sox9 and Bapx1 were examined by western blot. Sox9 protein increased for 5 days during chondrogenesis induction. However, in the next 4 days, the expression of Sox9 protein slightly decreased (Fig. 1a). Bapx1 expression mirrored that of Sox9; it increased for 5 days and decreased by day 9 (Fig. 1a). This parallel expression pattern of Sox9 and Bapx1 was also observed by RT-PCR analysis (Fig. 1b). Correspondingly, RT-PCR revealed that the Sox9 target gene, *Col2a1*, a marker gene of immature chondroblasts [4], increased until day 5 after chondrogenesis induction, but slightly decreased by day 9 (Fig. 1b). As marker genes of late stage chondrogenesis, *Runx2* and its target, *Col10a1* [26], demonstrated significantly increased expression from days 5 to 9 (Fig. 1b). Thus, Bapx1 is expressed during early chondrogenesis, and its expression decreased in accordance with the incremental increase in expression of the mature chondrocyte markers *Runx2* and *Col10a1*.

To test the potential relationship between Sox9 and Bapx1, we examined the effect of gain- or loss-of-function of Sox9 on Bapx1 expression. Sox9 expression was efficiently silenced by transducing cultured mouse chondrocytes with two different shRNA-expressing lentiviruses (Fig. 2a). Under these conditions, *Col2a1* mRNA expression significantly decreased, confirming that Sox9 function is diminished in these chondrocytes (Fig. 2b).

Bapx1 expression in these cells was further assayed by western blot and RT-quantitative PCR. Bapx1 expression decreased in Sox9 knockdown chondrocytes (Fig. 2a and Fig. 2b). On the contrary, Sox9 over-expression through lentiviral transduction in cultured mouse chondrocytes (Fig. 2c) increased Bapx1 and *Col2a1* expression (Fig. 2c, d). These results indicate that Sox9 is a critical activator of Bapx1 expression during early chondrogenesis.

Sox9 directly binds the Bapx1 promoter and enhances its activity

To assess whether Bapx1 is directly regulated by Sox9, *Bapx1* promoter activity was examined by luciferase reporter assays. We cloned a promoter region of -4427 to +41 upstream of the *Bapx1* transcriptional start site (TSS) and ligated it into a pGL4.12 vector containing a *TK* promoter and a luciferase reporter gene (Fig. 3a). We found that Sox9 expression increased *Bapx1* promoter activity in a dose-dependent manner in C3H10T1/2 cells (Fig. 3b).

To identify the Sox9 responsive element, we further performed promoter analyses with serial deletions of the *Bapx1* promoter (Fig. 3a). Deletions up to position -995 did not alter the incremental increase in luciferase activity due to Sox9 co-transfection; however, deletion to the position of -424 diminished this incremental activation (Fig. 3c). These results suggest that the Sox9 responsive element is located between position -995 and -424 upstream of the *Bapx1* TSS.

In this Sox9 responsive region, we found multiple Sox9 binding motifs [(A/T)(A/T)CAA(T/A)G] (data not shown). Sox9 homodimers bind to enhancer regions of chondrocyte target genes containing inverted Sox9 binding sites separated by 3–4 bp that represent a Sox9 palindromic motif [27–30]. Based on this, we searched for a Sox9 palindromic motif in the Sox9 responsive element and found one at the position between -868 and -852 from the *Bapx1* TSS (Fig. 4a). We further examined promoter activity with a fragment containing the putative Sox9 binding site corresponding to the region between -1152 and -727 (Fig. 4b). Sox9 increased the

luciferase activity of this construct in a dose-dependent manner in C3H10T1/2 cells (Fig. 4c). Taken together, these results indicate that the Sox9 responsive element is located between –995 and –727 from the *Bapx1* TSS.

To further determine whether Sox9 binds to the endogenous *Bapx1* promoter, we performed ChIP using cultured mouse chondrocytes. Previous studies have detailed several Sox9 target genes and binding regions. For instance, Sox9 binds to the enhancer within the first intron of the chondrocyte-specific gene *Col2a1* [31]. Consistent with this, we found that Sox9 bound to this region in cultured mouse chondrocytes by ChIP (Fig. 4d). We further found that Sox9 bound to the *Bapx1* promoter containing putative Sox9 binding motifs in cultured mouse chondrocytes (Fig. 4d). Furthermore, our ChIP analysis did not detect Sox9 binding to an enhancer region of *anti-Mullerian hormone* (*Amh*), a well-known target in testis [32,33] (Fig. 4d). Moreover, our RT-PCR analysis did not detect *Amh* transcripts in cultured mouse chondrocytes (data not shown). These results suggest that *Bapx1* is a Sox9 target gene in chondrocytes.

Sox9 interacts with the *Bapx1* promoter

We prepared oligonucleotide probes for the putative Sox9 binding regions (Fig. 5a) and performed EMSAs. Upon incubating the *Bapx1*-WT oligonucleotide probes with recombinant Sox9 protein, we detected two different bands, presumably corresponding to the binding of Sox9 as a monomer to one site (lower band) or as a dimer to both sites (upper band) (Fig. 5b). Addition of anti-Sox9 antibody to this reaction reduced the intensity of both bands, with an upward supershift observed, although incubation with anti-rabbit IgG antibody did not alter either the putative monomer or putative dimer bands (Fig. 5b).

Oligonucleotide probes with point mutations in one or both of the Sox9 motif sites were synthesized and EMSAs were performed. A single Sox9 motif point mutation resulted in a reduction of both bands, especially diminishing the upper band, whereas mutation of both sites completely abolished these bands (Fig. 5c). The supershifted band produced by incubation with anti-Sox9 antibody was reduced by a single mutation and abolished by dual mutations of the Sox9 motifs (Fig. 5c). These results indicated that Sox9 could bind as a monomer and/or dimer to the *Bapx1* promoter site.

To further assess Sox9 binding and the contribution of this site to the *Bapx1* promoter, a luciferase reporter assay was performed using a vector containing point mutations in one or both of the Sox9 binding sites (Fig. 5d). A single mutation in the Sox9 motif reduced, whereas dual mutations almost completely abolished, the incremental increase in luciferase activity by Sox9 in C3H10T1/2 cells (Fig. 5e). Taken together, these results indicate that Sox9 directly regulates *Bapx1*, and Sox9 homodimer binding is essential for this regulation.

Sox9 represses *Runx2* by regulating *Bapx1* in chondrocytes

Based on our finding that *Bapx1* is a direct Sox9 target in chondrocytes, we speculated that Sox9 might repress *Runx2* expression by enhancing *Bapx1* expression, resulting in a suppression of terminal chondrocyte differentiation.

To test this hypothesis, *Runx2* mRNA expression was assessed by quantitative PCR in Sox9 knockdown chondrocytes. Indeed, expression of *Runx2* and its target, *Col10a1*, was increased after Sox9 knockdown in cultured mouse chondrocytes (Fig. 6a). Furthermore, over-expression of Sox9 inhibited the expression of *Runx2* and *Col10a1* mRNA (Fig. 6b).

We next assessed the effect of *Bapx1* knockdown on *Runx2* and *Col10a1* expression and found that while lentivirus-mediated knockdown of *Bapx1* did not change Sox9 and *Col2a1* expression, it increased *Runx2* and *Col10a1* expression (Figure 7a-b). We further found that

Bapx1 knockdown diminished the suppressive effect of *Runx2* and *Col10a1* expression by over-expression of Sox9 (Figure 7a-b). These results suggest that Sox9 negatively regulates Runx2 by enhancing Bapx1 expression, leading to the inhibition of terminal chondrocyte differentiation.

Discussion

Bapx1 is a direct Sox9 target gene

Various transcription factors are expressed and play essential roles during endochondral ossification. In immature chondrocytes, the transcription factors Sox9, Sox5, Sox6, and Bapx1, as well as several chondrocyte-specific ECM proteins, including Col2a1, Col11a2, and Agc1, are expressed. In contrast, these genes are down-regulated in differentiated mature chondrocytes; instead, Runx2 and the ECM protein, Col10a1, are expressed [2]. Previous studies have revealed the importance of each transcription factor in this process, but details including identification of target genes, downstream molecular mechanisms, and molecular crosstalk between these players were poorly understood.

Although past studies suggested that Sox9 increased Bapx1 expression [21], the mechanism behind this induction was unclear. In the present study, we identified Bapx1 as a direct Sox9 target gene in chondrocytes. Paired box gene 1 (Pax1), Pax9, and mesenchyme homeobox 1 (Meox1) proteins regulate Bapx1 expression by directly activating its promoter [34,35]. Interestingly, binding sites for Pax1/Pax9 (position between -873 and -853), Meox1 (position between -828 and -823), and Sox9 (position between -868 and -852) are in close proximity within the *Bapx1* promoter. These binding sites are concentrated between -880 and -820, with Pax1/Pax9 and Sox9 binding sites mostly overlapping. Based on these observations, it is possible that these factors cooperate to regulate Bapx1 expression.

However, Pax1/Pax9 and Meox1 expression is maintained in chondrogenic mesenchymal cells until they reach the pre-chondrocyte stage [36–40], whereas Sox9 expression is maintained throughout chondrocyte differentiation, except in hypertrophic chondrocytes [41–43]. Thus, Pax1/Pax9 and Meox1 expression occurs earlier than Sox9 expression, and these genes typically exhibit distinct expression patterns during chondrogenesis. Alternatively, Bapx1 is expressed in chondrogenic mesenchymal cells and subsequent chondroblast differentiation, so that its expression mirrors that of Sox9 [21,44]. These observations suggest that Pax1/9 and Meox1 may regulate initial and/or early induction of Bapx1, and Sox9 contributes to maintain Bapx1 expression during subsequent chondrogenic differentiation. Nevertheless, whether these events actually happen during embryogenesis remains to be determined.

Runx2 repression is critical for hypertrophic suppression by Sox9

Bapx1 involvement in endochondral ossification was previously suggested by its function as a transcriptional repressor [22]. More recently, the direct targets of Bapx1 have been clarified. Runx2, a critical transcription factor for osteoblast differentiation that also regulates hypertrophic chondrocytes during endochondral ossification, was identified as a direct Bapx1 target [19]. Moreover, Sox9 promotes early chondrogenesis and suppresses hypertrophic chondrocyte differentiation [9], although the mechanisms remain to be elucidated. Additionally, retrovirus-mediated over-expression of Bapx1 in chick wings resulted in repressed hypertrophic differentiation [20]. These observations suggest a possibility that Sox9 suppresses hypertrophic differentiation by repressing Runx2 through regulating Bapx1 expression.

Our results showed that Sox9 knockdown resulted in incremental changes in Runx2 expression and its target gene, and inverse effects resulted from Sox9 over-expression in cultured

chondrocytes. We further showed that *Bapx1* knockdown diminished the suppressive effect of *Runx2* expression and its target gene by over-expression of *Sox9* in cultured chondrocytes. These findings strongly suggest that *Sox9* suppresses *Runx2* function indirectly, at least in part, by regulating *Bapx1*, leading to repression of hypertrophic differentiation.

A recent study indicated that *Sox9* directly binds to *Runx2* and blocks its function [15]. Our findings and reports by other investigators suggest that *Runx2* repression is a critical event for *Sox9* suppression of hypertrophic chondrocyte differentiation. During endochondral ossification, *Sox9* and *Runx2* expression are distinct events. *Sox9* is expressed in immature chondroblasts, and *Runx2* is expressed in mature chondrocytes. These findings may indicate that *Sox9* sustains an immature state of chondroblasts by suppressing hypertrophic chondrocyte differentiation by repressing *Runx2*. In other words, *Runx2* expression during chondrogenesis may function as a proliferation/differentiation switch.

However, a recent report revealed that *S100A1* and *S100B* were direct *Sox9* targets, and that they suppress hypertrophic chondrocyte differentiation [45]. Although a detailed mechanism was not worked out, it suggests a possibility for another mechanism of hypertrophic suppression by *Sox9*. Further analysis is required to clarify the precise mechanism for hypertrophic chondrocyte suppression by *Sox9*.

Bapx1 is a critical mediator of endochondral bone formation

In cartilaginous development, *Sox9* enhances chondrogenesis by promoting the expression of chondrocyte-specific proteins, such as *Col2a1*, *Col11a2*, and *Agc1* [4–6]. In addition, *Sox9* plays a critical role in testicular development by regulating targets such as *Amh* and *Ptgds* in Sertoli cells [32,33,46]. However, the regulatory mechanism of tissue-specific target distribution is less well understood. Recent studies have shown that a missense mutation (A76E) in human *SOX9* in an XY patient resulted in skeletal abnormalities without gender reversal by perturbing *SOX9* dimerization, which results in dysregulation of chondrocyte-specific genes, but normal regulation of Sertoli cell-specific target [27,28]. Furthermore, *Sox9* homodimers may bind to enhancer regions of chondrocyte target genes containing inverted *Sox9* binding motifs that represent a *Sox9* palindromic motif [29,30]. These reports suggest that dimer formation is critical for chondrocyte-specific gene regulation. Given that our results indicate that *Bapx1* expression is regulated by *Sox9* homodimer binding to the *Bapx1* promoter, *Bapx1* may be a critical regulator of endochondral bone formation. Indeed, *Bapx1* homozygous mutant mice die perinatally and exhibit skeletal dysplasia, and transgenic mice over-expressing *Bapx1* in limb buds exhibit preaxial polydactyly [47]. However, the abnormal skeletal phenotype of *Bapx1* mutant mice was limited to the vertebral column and some craniofacial bones [16–18]. These findings may indicate that the necessity of *Bapx1* for endochondral bone formation is limited to specific tissues, and other factors with similar functions play the role in other tissues.

In this study, we focused on *Bapx1* function as a transcriptional repressor of *Runx2*, given that *Bapx1* targets, except for *Runx2*, were virtually unknown. Analysis of *Bapx1*-deficient mice by other researchers has revealed that *Bapx1* is also necessary for somite proliferation and differentiation into chondrocytes [16–18]. Moreover, an autoregulatory loop between *Bapx1* and *Sox9* was demonstrated in a chick somite culture system, although the mechanism of *Sox9* up-regulation by *Bapx1* was not clarified [21]. In the present study, our *Bapx1* knockdown chondrocytes did not exhibit altered expression of *Sox9* and *Col2a1*. The possibility exists that during embryogenesis, depending on the specific stage of development and tissues involved, *Bapx1* up-regulates *Sox9*, resulting in the promotion of proliferation and early chondrogenesis. Further analyses are needed to determine precise *Bapx1* functions during chondrogenesis.

In the present study, we revealed that Bapx1 is a direct Sox9 target gene. Our findings strongly suggest that Sox9 repressed Runx2 through Bapx1 regulation, resulting in suppressed hypertrophic chondrocyte differentiation, thereby promoting normal endochondral ossification.

Acknowledgments

We thank members of the Department of Regenerative Medicine for discussions and technical support. This study was supported by grants from JST SORST, Genome Network Project (MEXT), Grants-in Aid for Scientific Research (MEXT), Research on Child Health and Development, Research on Publicly Essential Drugs and Medical Devices (The Japan Health Sciences Foundation), and NIH (AR50631) (H. A.).

Abbreviations

Bapx1	vertebrate homolog of <i>Drosophila</i> bagpipe
Col10a1	type X collagen alpha 1
Col2a1	type II collagen alpha 1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
KD	knockdown
Runx2	runt-related transcription factor 2
Sox9	Sry-related High Mobility Group box containing gene 9

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Fig. 1. Bapx1 is expressed during early chondrogenesis in C3H10T1/2 cells

a. Western blot detection of Sox9 and Bapx1 proteins at the indicated time points. **b.** RT-PCR detection of mRNA of *Sox9*, *Bapx1*, *Col2a1*, *Bapx1*, *Runx2*, *Col10a1*, and *Gapdh* at several time points.

Fig. 2. Bapx1 expression is up-regulated by Sox9 in chondrocytes

a. LacZ as a negative control or two Sox9-targeting shRNA expressing lentiviruses were transduced into cultured mouse chondrocytes and evaluated by western blot for knockdown (KD) efficiency and Bapx1 expression. **b.** *Bapx1* and *Col2a1* mRNA expression after Sox9 knockdown (KD) was examined by RT-quantitative PCR. Mean values (n=3) $\pm$ standard deviation are shown. **c.** Over-expression (OE) of Sox9 in cultured mouse chondrocytes and detection of Sox9 and Bapx1 proteins by western blot. **d.** Transcript levels of *Bapx1* and *Col2a1* in Sox9-over-expressing (OE) chondrocytes were assayed by RT-quantitative PCR. Mean values (n=3) $\pm$ standard deviation are shown.

a **b** **c**

Fig. 3. Sox9-activated *Bapx1* proximal promoter activity

a. Luciferase reporter constructs containing the *Bapx1* promoter are shown. Numbers indicate the position from the transcription start site (TSS). **b.** C3H10T1/2 cells in 24-well plates were co-transfected with 200 ng of the indicated luciferase reporter plasmid and the specified amounts of the Sox9-encoded plasmid. Mean values (n=3) of luciferase reporter activity $\pm$ standard deviation are shown. **c.** Mean values (n=3) of luciferase reporter activity $\pm$ standard deviation in C3H10T1/2 cells transfected with 200 ng of the indicated luciferase reporter plasmid and either 200 ng of Sox9-expressing or empty pcDNA3 plasmid are shown.

a

-989 GAGACGAATACCCAGCTCCAAAAATTCCTACCCAAATGCTTCCAATTCCTCGCCCCACTTGGTGCTTAAC
-917 TAATATTTTCAACCTAGCTTTTAAAGTCGCTACAGAAATCCAATCCAATCAACGTAATATGATAGGAGTA
-846 CTTAGTAGAGTGATTTTAATTATCAGATTACTTCAGTAAAGAGCATTAGCAGAGAAACAGCTGAGAAACTTT

b

c

d

Fig. 4. Minimal Sox9 binding site in the *Bapx1* promoter in chondrocytes

a. Sequence of the *Bapx1* promoter between -989 and -775 from the TSS. A potential Sox9 binding site is indicated in red. **b.** A luciferase reporter construct containing the *Bapx1* minimum promoter is shown. Numbers indicate the position from the TSS. **c.** C3H10T1/2 cells were co-transfected with 200 ng of the indicated luciferase reporter plasmid and specified amounts of the plasmid-borne Sox9. Mean values (n= 3) of luciferase reporter activity $\pm$ standard deviation are shown. **d.** Chromatin-immunoprecipitated Sox9-binding non-enriched (rabbit IgG) DNA, enriched (Sox9) DNA, and 0.01% whole cell extract (WCE) DNA in chondrocytes was amplified by PCR using Sox9 site-specific primer sets for the Sox9 putative binding site, chondrocyte-specific target *Col2a1*, and Sertoli cell-specific target *Amh*.

Fig. 5. Induction of *Bapx1* promoter activity through the binding of a Sox9 homodimer
a. Oligonucleotide sequences corresponding to the *Bapx1* promoter are shown, with distance from the TSS indicated. Putative Sox9-binding consensus sequences are marked with a grey background; arrows denote site orientation. **b.** ³²P-labeled oligonucleotide probes corresponding to the *Bapx1* promoter complexed to Sox9 in the presence of anti-Sox9 antibody or control rabbit IgG were detected by electrophoretic mobility shift assays (EMSA). **c.** Binding of oligonucleotide probes with mutated putative Sox9 binding consensus sequences complexed to Sox9 protein (rSox9) was detected by electrophoretic mobility shift assays (EMSA). Mutations in one (mut1, 2) or both (mut3) Sox9-binding motifs were compared to wild-type (WT) in the presence or absence of anti-Sox9 antibody. **d.** Luciferase reporter constructs for *Bapx1* Sox9-binding sites containing mutations are shown. **e.** C3H10T1/2 cells were transfected with the indicated reporter plasmids and co-transfected with Sox9-expressing or empty pcDNA3 plasmid. Mean values (n=3) of luciferase reporter activity $\pm$ standard deviation are shown.

Fig. 6. Sox9 represses Runx2 expression and function

a. *Runx2* and *Col10a1* mRNA levels in Sox9 knockdown chondrocytes were assayed by RT-quantitative PCR. **b.** *Runx2* and *Col10a1* expression in Sox9 over-expressing chondrocytes were analyzed by quantitative RT-PCR.

Fig. 7. Bapx1 knockdown diminishes the suppression of Runx2 expression and function by Sox9 over-expression in chondrocytes

a. Over-expression of Sox9 and/or Bapx1 targeting shRNA in cultured mouse chondrocytes and detection of Sox9 and Bapx1 proteins by western blot. **b.** *Col2a1*, *Runx2* and *Col10a1* mRNA expression in chondrocytes with Sox9 over-expression (OE) and/or Bapx1 knockdown (KD) were assayed by quantitative RT-PCR. Mean values ($n=3$) $\pm$ standard deviation are shown.

Table 1

Primer sequences.

a) Sequence for each primer set used in qualitative and quantitative RT-PCR		
Gene Name	Forward primer sequence	Reverse primer sequence
<i>Sox9</i>	5'-TACGACTGGACGCTGGTGCC-3'	5'-CCGTTCTTCACCGACTTCCTCC-3'
<i>Col2a1</i>	5'-TCATCGAGTACCGATCACAGA-3'	5'-GTTTCGGGGGTTTACAAGAAG-3'
<i>Bapxl</i>	5'-CCACTGCAGCCCTCCTACTA-3'	5'-GTGTCAAGTCCCAGCAGGAT-3'
<i>Runx2</i>	5'-GGACGAGGCAAGAGTTTCAC-3'	5'-CCAGAGGCAGAAGTCAGAGG-3'
<i>Col10a1</i>	5'-GTCCAAGAGGTGAACCTGGA-3'	5'-TCTGTGAGCTCCATGATTGC-3'
<i>Gapdh</i>	5'-CCTGGTCACCAGGGCTGC-3'	5'-CGTCCTGGAAGATGGTGATG-3'
b) Sequence for each primer set used in chromatin immunoprecipitation (ChIP)-PCR		
Gene Name	Forward primer sequence	Reverse primer sequence
<i>Col2a1</i>	5'-GGGAGACCTCAGTCCTCCTT-3'	5'-GGAGGCTGTGCATTGTGG-3'
<i>Amh</i>	5'-GCTCAGGCCTCTGCAGTTAT-3'	5'-GGGTGGCCCTGCTTAT ATGT-3'
<i>Bapxl</i>	5'-GAGACGAATACCCAGCTCCA-3'	5'-GAGCCCAGAGTCGAAGAACA-3'