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% CO_2. To induce chondrogenesis, 10 μl aliquots of a suspension containing 10^7 cells/ml were plated into wells of a 24-well plate containing culture medium. After 2 h, 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% CO_2.

Western blot

Cells were lysed with NP-40 lysis buffer (20 mM Tris–HCl (pH 7.6), 3 mM EDTA, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM β-glycerophosphate, 5 mM $Na_4P_2O_7 \cdot 10\ H_2O$, 1 mM Na_3VO_4, 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,

Table 1 – Primer sequences.

Gene name	Forward primer sequence	Reverse primer sequence
a) Sequence for each primer set used in qualitative and quantitative RT-PCR		
Sox9	5′-TACGACTGGACGCTGGTGCC-3′	5′-CCGTTCTTCACCGACTTCCTCC-3′
Col2a1	5′-TCATCGAGTACCGATCACAGA-3′	5′-GTTCGGGGGTTTTACAAGAAG-3′
Bapx1	5′-CCACTGCAGCCCTCCTACTA-3′	5′-GTGTCAAGTCCCAGCAGGAT-3′
Runx2	5′-GGACGAGGCAAGAGTTTCAC-3′	5′-CCAGAGGCAGAAGTCAGAGG-3′
Col10a1	5′-GTCCAAGAGGTGAACCTGGA-3′	5′-TCTGTGAGCTCCATGATTGC-3′
Gapdh	5′-CCTGGTCACCAGGGCTGC-3′	5′-CGCTCCTGGAAGATGGTGATG-3′
b) Sequence for each primer set used in chromatin immunoprecipitation (ChIP)-PCR		
Col2a1	5′-GGGAGACCTCAGTCCTCCTT-3′	5′-GGAGGCTGTGCATTGTGG-3′
Amh	5′-GCTCAGGCCTCTGCAGTTAT-3′	5′-GGGTGGCCCTGCTTATATGT-3′
Bapx1	5′-GAGACGAATACCCAGCTCCA-3′	5′-GAGCCCAGAGTCGAAGAACA-3′

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 h 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 h 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 10 μg/ml polybrene. The following oligonucleotide sequences were cloned into the pLenti4/BLOCK-iT™-DEST vector:

LacZ (5′-AAATCGCTGATTTGTGTAGTCGGAGACGACTACACAAATCAGCGA-3′),
Sox9 (5′-GCGACAACTTTACCAGTTTCACGAATGAAACTGGTAAAGTTGTCGC-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 h, 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 min 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 (5 mM 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 (Dynal 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 h 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 h 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 ^{32}P-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 min at 25 °C before addition of radiolabeled probes. Reactions were performed in binding buffer [20 mM 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 min at 25 °C. Products of the binding reactions were separated by polyacrylamide gel electrophoresis (PAGE) on a 4% gel for 3 h 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*

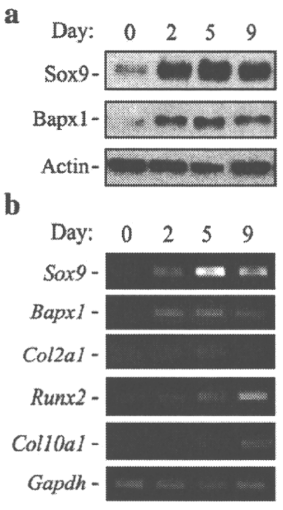

Fig. 1 – Bapx1 is expressed during early chondrogenesis in C3H10T1/2 cells. (a) Western blot detection of Sox9 and Bapx1 proteins at the indicated time points. (b) RT-PCR detection of mRNA of *Sox9*, *Bapx1*, *Col2a1*, *Bapx1*, *Runx2*, *Col10a1*, and *Gapdh* at several time points.

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 (Figs. 2a and b). On the contrary, Sox9 over-expression through lentiviral transduction in cultured mouse chondrocytes (Fig. 2c) increased Bapx1 and *Col2a1* expression (Figs. 2c, d). These results indicate that Sox9 is a critical activator of Bapx1 expression during early chondrogenesis.

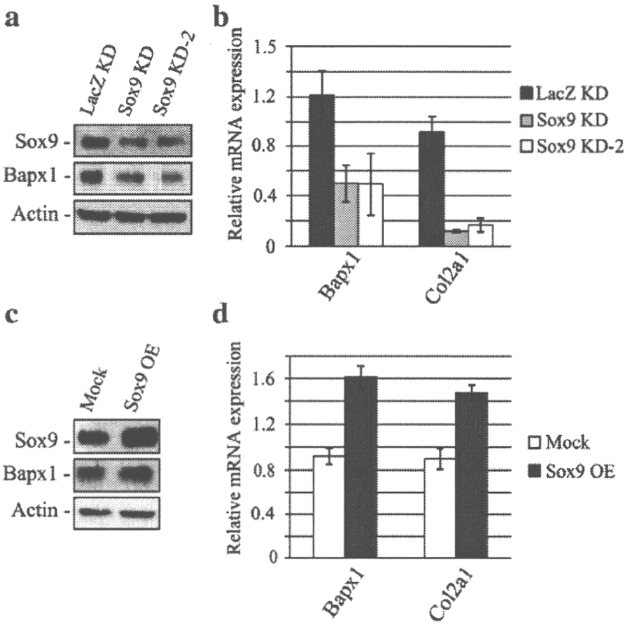

Fig. 2 – Bapx1 expression is up-regulated by Sox9 in chondrocytes. (a) LacZ as a negative control or two Sox9-targeting shRNA-expressing lentiviruses were transduced into cultured mouse chondrocytes and evaluated by western blot for knockdown (KD) efficiency and Bapx1 expression. (b) *Bapx1* and *Col2a1* mRNA expression after Sox9 knockdown (KD) was examined by RT-quantitative PCR. Mean values ($n = 3$) ± 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 ($n = 3$) ± standard deviation are shown.

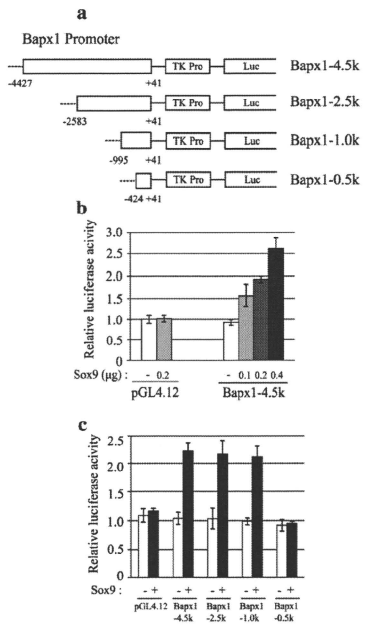

Fig. 3 – Sox9-activated *Bapx1* proximal promoter activity. (a) Luciferase reporter constructs containing the *Bapx1* promoter are shown. Numbers indicate the position from the transcription start site (TSS). (b) C3H10T1/2 cells in 24-well plates were co-transfected with 200 ng of the indicated luciferase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

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

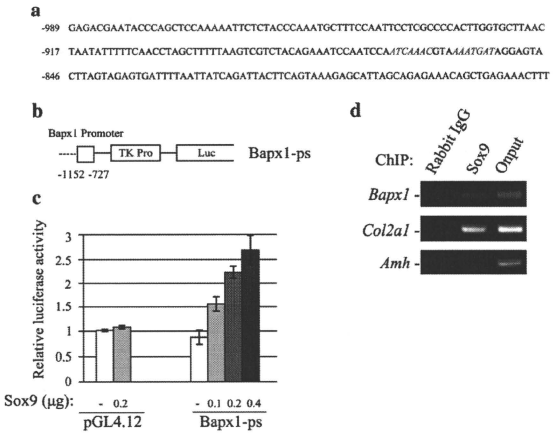

Fig. 4 – Minimal Sox9 binding site in the *Bapx1* promoter in chondrocytes. (a) Sequence of the *Bapx1* promoter between −989 and −775 from the TSS. A potential Sox9 binding site is indicated in red. (b) A luciferase reporter construct containing the *Bapx1* minimum promoter is shown. Numbers indicate 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 ± 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*.

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 (Figs. 7a, b). We further found that Bapx1 knockdown diminished the suppressive effect of *Runx2* and *Col10a1* expression by over-expression of Sox9 (Figs. 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

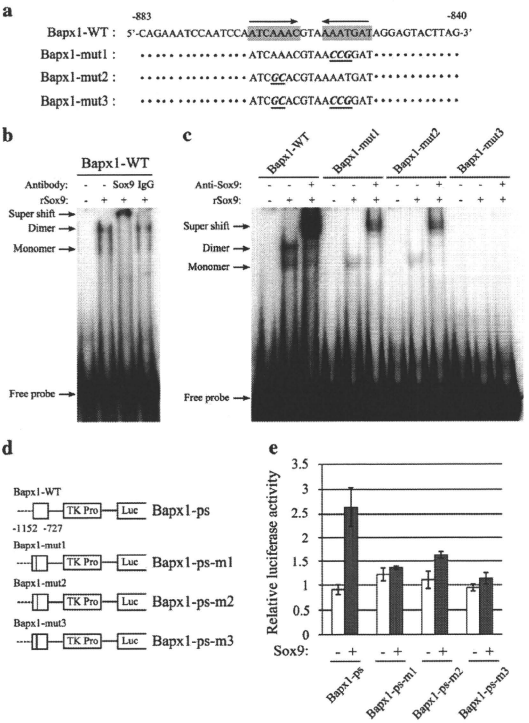

Fig. 5 – Induction of *Bapx1* promoter activity through the binding of a Sox9 homodimer. (a) Oligonucleotide sequences corresponding to the *Bapx1* promoter are shown, with distance from the TSS indicated. Putative Sox9-binding consensus sequences are marked with a grey background; arrows denote site orientation. (b) ^{32}P-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 ± standard deviation are shown.

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.

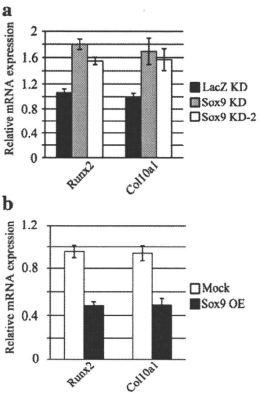

Fig. 6 – Sox9 represses Runx2 expression and function. (a) *Runx2* and *Col10a1* mRNA levels in Sox9 knockdown chondrocytes were assayed by RT-quantitative PCR. (b) *Runx2* and *Col10a1* expression in Sox9 over-expressing chondrocytes were analyzed by quantitative RT-PCR.

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

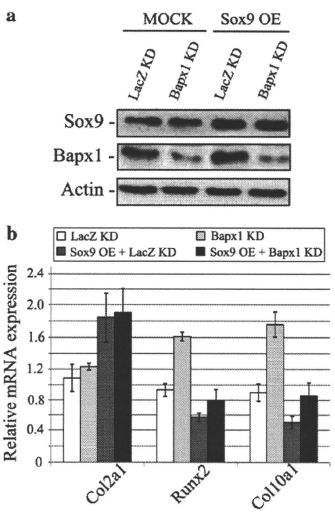

Fig. 7 – Bapx1 knockdown diminishes the suppression of Runx2 expression and function by Sox9 over-expression in chondrocytes. (a) Over-expression of Sox9 and/or Bapx1 targeting shRNA in cultured mouse chondrocytes and detection of Sox9 and Bapx1 proteins by western blot. (b) *Col2a1*, *Runx2* and *Col10a1* mRNA expression in chondrocytes with Sox9 over-expression (OE) and/or Bapx1 knockdown (KD) were assayed by quantitative RT-PCR. Mean values ($n = 3$) ± standard deviation are shown.

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.

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MicroRNA-140 Is Expressed in Differentiated Human Articular Chondrocytes and Modulates Interleukin-1 Responses

Shigeru Miyaki,[1] Tomoyuki Nakasa,[2] Shuhei Otsuki,[1] Shawn P. Grogan,[1] Reiji Higashiyama,[1] Atsushi Inoue,[2] Yoshio Kato,[3] Tempei Sato,[2] Martin K. Lotz,[1] and Hiroshi Asahara[4]

Objective. **MicroRNA (miRNA) are a class of noncoding small RNAs that act as negative regulators of gene expression. MiRNA exhibit tissue-specific expression patterns, and changes in their expression may contribute to pathogenesis. The objectives of this study were to identify miRNA expressed in articular chondrocytes, to determine changes in osteoarthritic (OA) cartilage, and to address the function of miRNA-140 (miR-140).**

Methods. **To identify miRNA specifically expressed in chondrocytes, we performed gene expression profiling using miRNA microarrays and quantitative polymerase chain reaction with human articular chondrocytes compared with human mesenchymal stem cells (MSCs). The expression pattern of miR-140 was monitored during chondrogenic differentiation of human MSCs in pellet cultures and in human articular cartilage from normal and OA knee joints. We tested the effects of interleukin-1β (IL-1β) on miR-140 expression. Double-stranded miR-140 (ds–miR-140) was transfected into chondrocytes to analyze changes in the expression of genes associated with OA.**

Results. **Microarray analysis showed that miR-140 had the largest difference in expression between chondrocytes and MSCs. During chondrogenesis, miR-140 expression in MSC cultures increased in parallel with the expression of *SOX9* and *COL2A1*. Normal human articular cartilage expressed miR-140, and this expression was significantly reduced in OA tissue. In vitro treatment of chondrocytes with IL-1β suppressed miR-140 expression. Transfection of chondrocytes with ds–miR-140 down-regulated IL-1β–induced *ADAMTS5* expression and rescued the IL-1β–dependent repression of *AGGRECAN* gene expression.**

Conclusion. **This study shows that miR-140 has a chondrocyte differentiation–related expression pattern. The reduction in miR-140 expression in OA cartilage and in response to IL-1β may contribute to the abnormal gene expression pattern characteristic of OA.**

Osteoarthritis (OA) is a chronic and highly prevalent degenerative joint disease. Approximately 40 million Americans are currently affected, and this number is predicted to increase to 60 million within the next 20 years as a result of population aging and an increase in life expectancy (1,2). Current treatment is limited to pain management, and disease-modifying therapies are not available in the late phase of the disease process, at which point joint replacement surgery is often indicated. OA has been associated with age-related loss of the homeostatic balance between degradation and repair mechanisms. Cartilage cellularity in OA is reduced by chondrocyte death, and remaining chondrocytes are activated by cytokines and growth factors to a catabolic and abnormal differentiation that leads to degradation

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[1]Shigeru Miyaki, PhD, Shuhei Otsuki, MD, PhD, Shawn P. Grogan, PhD, Reiji Higashiyama, MD, PhD, Martin K. Lotz, MD: Scripps Research Institute, La Jolla, California; [2]Tomoyuki Nakasa, MD, PhD, Atsushi Inoue, PhD, Tempei Sato, MS: National Research Institute for Child Health and Development, Tokyo, Japan; [3]Yoshio Kato, PhD: Research Institute for Cell Engineering, National Institute of Advanced Industrial Science and Technology, Osaka, Japan; [4]Hiroshi Asahara, MD, PhD: Scripps Research Institute, La Jolla, California, and National Research Institute for Child Health and Development, Tokyo, Japan.

Address correspondence and reprint requests to Hiroshi Asahara, MD, PhD, Department of Molecular and Experimental Medicine, Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037. E-mail: asahara@scripps.edu.

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of extracellular matrix (ECM) (3–6). Molecular mechanisms that govern articular chondrocyte differentiation during development and maintenance of articular cartilage are being characterized, and this has the potential to lead to new therapeutic interventions.

MicroRNA (miRNA) are a class of noncoding small RNAs that play roles in biologic processes as negative regulators of gene expression by promoting messenger RNA (mRNA) degradation and/or repressing translation through sequence-specific interactions with the 3′-untranslated regions of specific mRNA targets (7–10). Hundreds of miRNA have been found in various organisms, and many miRNA are evolutionarily conserved. Moreover, one-third of all mammalian mRNA seem to be under miRNA regulation, suggesting an essential role in regulating gene expression (11). Several miRNA exhibit a tissue- or developmental stage–specific expression pattern and have been associated with diseases such as cancer, heart disease, diabetes, and rheumatoid arthritis (12–16). Mice with limb- or cartilage-specific deletion of the miRNA processing enzyme Dicer showed a severe phenotype with reduced limb size but normal patterning (17,18). Because Dicer is indispensable for producing a functional, mature type of miRNA, this finding suggests that the presence of specific miRNA plays a critical role in skeletal development. Although Tuddenham et al showed cartilage-specific expression of miRNA-140 (miR-140) in mouse embryos (19), the role of tissue-specific miRNA in articular cartilage has not been reported.

We hypothesized that miRNA are novel regulators of cartilage homeostasis, and that changes in their expression and function play an important role in diseases affecting articular cartilage. The objectives of this study were to identify miRNA expressed in articular chondrocytes, to determine changes in OA cartilage, and to address the function of chondrocyte-specific miR-140.

MATERIALS AND METHODS

Human tissue samples, cell isolation, and culture. Human articular cartilage specimens were obtained from the knee joints of 8 normal donors (mean ± SD age 38.22 ± 5.31 years) and from 11 patients with OA (mean ± SD age 79.36 ± 9.72 years) who were undergoing total knee arthroplasty. Tissue collection was approved by the Scripps Human Subjects Committee. All samples were examined by Safranin O staining and graded according to a modified Mankin scale (20), with a score of <2 points being normal and a score of >5 representing OA. RNA was isolated from fresh-frozen cartilage by homogenizing the tissue in a freezer mill (Spex, Metuchen, NJ) and extracting the homogenate in TRIzol (Invitrogen, Carlsbad, CA). Human chondrocytes were isolated and cultured as described previously (21). Experiments with chondrocytes were performed in passages 1–2. Human bone marrow–derived mesenchymal stem cells (MSCs) were isolated from iliac crest bone marrow obtained from normal adult donors (with the approval of the Human Subjects Committee) and cultured as described previously (21,22). Experiments with MSCs were performed in passages 3–6.

Microarray analysis. Small RNAs of <200 nucleotides in length were extracted from MSCs and chondrocytes with the mirVana miRNA Isolation Kit (Ambion, Austin, TX) according to the manufacturer's protocol. Purified RNA was then labeled with Cy3 or Cy5, using the mirVana miRNA Labeling Kit. Briefly, RNA was subjected to a tailing reaction with amine-modified nucleotide triphosphates by poly(A) polymerase, followed by amide formation using Cy dye ester. Labeled RNAs were hybridized on slides, on which oligonucleotides against human miRNA had been arrayed (Hokkaido System Science, Hokkaido, Japan), and detected by a scanner (Agilent, Santa Clara, CA).

Chondrogenesis in human MSCs. Human bone marrow MSCs were used to prepare pellets (5.0×10^5 cells/pellet) by centrifuging the cells at 500*g* in 15-ml conical polypropylene tubes and culturing them in chondrogenic medium (Lonza, Walkersville, MD) supplemented with bone morphogeneic protein 2 (500 ng/ml) and transforming growth factor β3 (10 ng/ml). Medium was changed every 2–3 days. To monitor miR-140 throughout chondrogenesis, MSCs were processed for RNA isolation on day 7 and day 14. Chondrogenesis was monitored via *SOX9*, *AGGRECAN*, and *COL2A1* expression and Safranin O staining.

Treatment with interleukin-1β (IL-1β). Chondrocytes were maintained in 12-well plates containing Dulbecco's modified Eagle's medium plus 10% calf serum and 1% penicillin/streptomycin. Following treatment with recombinant human IL-1β (5 ng/ml; PeproTech, Rocky Hill, NJ), cells were washed with cold phosphate buffered saline, and total RNA was isolated with TRIzol reagent. Quantitative polymerase chain reaction (PCR) was performed with the TaqMan MicroRNA Assay Kit (Applied Biosystems, Foster City, CA) for mature

Table 1. MicroRNA expression in human articular chondrocytes and MSCs*

MicroRNA	Ratio, chondrocytes:MSCs	Chondrocytes	MSCs
miR-140	2.12	540.89	254.37
miR-197	1.88	5,395.03	2,869.38
miR-148	1.78	644.35	361.58
miR-328	1.70	2,768.41	1,632.95
miR-27b	1.63	6,092.21	3,746.00
miR-16	1.59	4,398.78	2,764.09
miR-222	1.55	6,349.65	4,087.27
miR-15b	1.55	668.29	430.56
miR-505	1.54	1,967.68	1,273.77
miR-23b	1.52	8,114.26	5,334.24

* RNA was isolated from articular chondrocytes and mesenchymal stem cells (MSCs) for microarray analysis of microRNA. Differentially expressed microRNA (at least 1.5-fold difference) are shown as the ratio of chondrocytes to MSCs. Values for chondrocytes and human MSCs are the raw signal intensities.

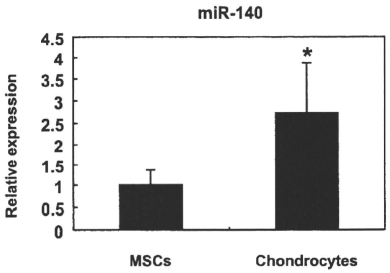

Figure 1. Expression of microRNA-140 (miR-140) in articular chondrocytes and human mesenchymal stem cells (MSCs). Array data for miR-140 were validated by quantitative polymerase chain reaction on MSCs (3 different preparations from 3 different donors) and articular chondrocytes (8 different preparations from 8 different donors). MiR-140 expression was significantly higher in chondrocytes compared with MSCs. Bars show the mean and SEM fold difference in relative expression. * = P = 0.015.

miR-140 or with the TaqMan Gene Expression Assay for chondrogenic markers *MMP13* and *ADAMTS5*.

Transfection of double-stranded miR-140 (ds–miR-140) into human articular chondrocytes. Double-stranded RNA (dsRNA) oligonucleotides representing mature sequences that mimic endogenous miR-140 were transfected into human chondrocytes at 80–90% confluence (4-n*M* concentration) with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Synthesized RNA oligonucleotides 5′-CAGUGGUUUUACCCUAUGGUAG-3′ and 5′-ACCACAGGGUAGAACCACGGAC-3′ were annealed to obtain ds–miR-140. Silencer Negative Control #1 small interfering RNA (Ambion) at the same concentration as the specific ds–miR-140 was used in each experiment.

Real-time quantitative PCR. Total RNA was isolated from cartilage tissues, monolayer cultures, or pellet cultures using TRIzol. Real-time quantitative PCR for miRNA was performed using the TaqMan MicroRNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol. Complementary DNA was produced using Ready-to-Go You-Prime First-Strand Beads (GE Healthcare, Chalfont, UK) with total RNA (1 μg) and oligo(dT)$_{18}$ primers. Real-time quantitative PCR was performed using TaqMan Gene Expression Assay probes for *COL2A1* (Hs00164004_m1),

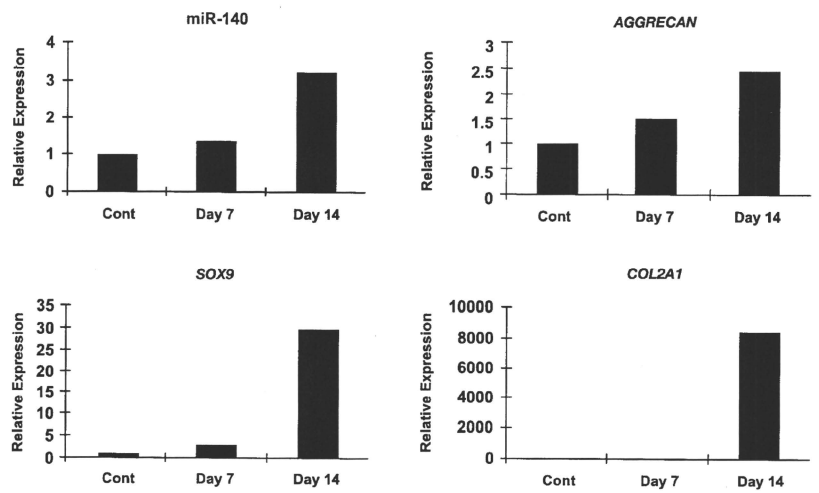

Figure 2. Changes in miR-140 expression during chondrogenesis. RNA was isolated from undifferentiated MSCs (controls [Cont]) and from MSC pellet cultures in chondrogenesis medium on day 7 and day 14. The expression of miR-140, *AGGRECAN*, *SOX9*, and *COL2A1* was analyzed by quantitative polymerase chain reaction. The expression of miR-140 increased during chondrogenesis, along with increased expression of *SOX9*, *AGGRECAN*, and *COL2A1*. Results are shown as the relative expression, where expression in undifferentiated MSCs is defined as 1. See Figure 1 for other definitions.

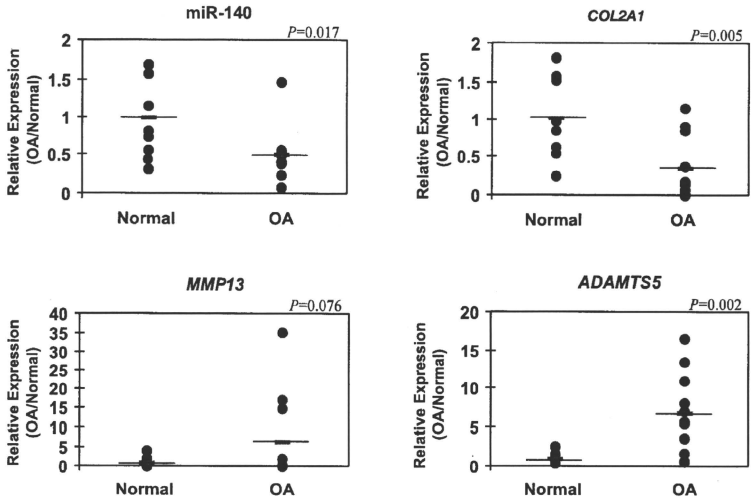

Figure 3. MicroRNA-140 (miR-140) expression in normal and osteoarthritis (OA) articular cartilage. Full-thickness cartilage specimens were collected from normal (n = 8) and OA (n = 11) knee joints for RNA isolation. The expression of miR-140, *COL2A1*, *ADAMTS5*, and *SOX9* was determined by quantitative polymerase chain reaction. The expression of miR-140 and *COL2A1* was significantly decreased, and the expression of *ADAMTS5* was significantly increased in OA cartilage compared with normal cartilage. Bars show the mean.

AGGRECAN (Hs00202971_m1), *ADAMTS5* (Hs00199841_m1), *MMP13* (Hs00233992_m1), and *GAPDH* (Hs99999905_m1) (Applied Biosystems). The U18 and GAPDH genes were used as an internal control to normalize differences in each sample. The expression levels for each gene were assessed relative to the expression of U18 or GAPDH.

Statistical analysis. Statistically significant differences between 2 groups were determined with *t*-tests. The results are reported as the mean ± SEM. *P* values less than 0.05 were considered significant.

RESULTS

MicroRNA-140 expression in articular chondrocytes and MSCs. Chondrogenic differentiation of MSCs involves dynamic changes in various gene expression patterns such as induced expression of chondrocyte-specific genes, including *SOX9* and *COL2A1*. In order to screen miRNA specifically expressed in chondrocytes, we performed gene expression profiling using miRNA microarrays comparing primary chondrocytes from articular cartilage with MSCs. Several miRNA were more abundant in primary articular chondrocytes than in undifferentiated MSCs. The largest difference was observed for miR-140 (Table 1). The high expression of miR-140 in chondrocytes compared with MSCs was confirmed by quantitative PCR (Figure 1).

Expression of miR-140 during chondrogenesis of MSCs. To examine the dynamic expression pattern of miR-140 during in vitro chondrogenesis, we performed a TaqMan quantitative PCR assay to analyze expression patterns of miR-140. Pellets of MSCs were strongly stained by Safranin O after chondrogenesis induction for 14 days (data not shown). In this model, miR-140 expression gradually increased during chondrogenesis in parallel with expression of *SOX9*, *AGGRECAN*, and *COL2A1* (Figure 2). These data indicate that the expres-

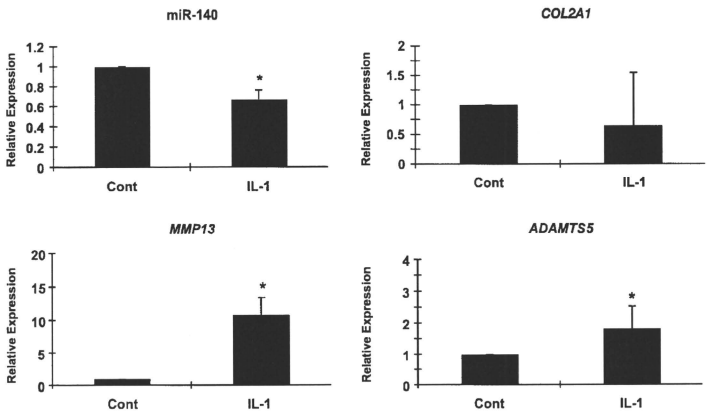

Figure 4. In vitro suppression of microRNA-140 (miR-140) by interleukin-1β (IL-1β). Articular chondrocytes (8 different preparations from 8 different donors) were treated with IL-1β (5 ng/ml) for 5 hours. The expression of miR-140, *COL2A1*, *MMP13*, and *ADAMTS5* was analyzed by quantitative polymerase chain reaction. IL-1β stimulation significantly decreased miR-140 expression and increased *MMP13* and *ADAMTS5* expression. *COL2A1* expression did not change significantly. Bars show the mean and SEM fold difference in relative expression. * = $P < 0.05$ versus control (Cont).

sion of miR-140 increases during chondrocytic differentiation of MSCs, and this is consistent with its high expression in chondrocytes.

Expression of miR-140 in normal and OA cartilage. In OA pathogenesis, several chondrocyte-specific genes, including *COL2A1* and *SOX9*, are down-regulated (23). In contrast, cartilage-degrading enzymes, including ADAMTS-5 and matrix metalloproteinase 13 (MMP-13), are up-regulated (24–26). To examine changes in miR-140 expression in OA articular cartilage, quantitative PCR of miR-140 together with OA-related marker genes was performed on 19 samples prepared from human knee articular cartilage (8 normal and 11 OA). As expected, the expression of *ADAMTS5* was significantly increased in OA cartilage, while the expression of *COL2A1* was significantly lower than that in normal cartilage (Figure 3). MiR-140 expression in articular cartilage from donors with OA (65–93 years old; Mankin score 5–10) was significantly lower than that in normal cartilage (obtained from individuals 30–44 years old; Mankin score 0–2) (Figure 3). These data demonstrate abnormally reduced miR-140 expression in OA cartilage and appear to correlate with increased *ADAMTS5* expression and reduced *COL2A1* expression in the same samples.

Effect of IL-1β on miR-140 expression in articular chondrocytes. IL-1β is one of the critical mediators of OA, and IL-1β stimulation of chondrocytes causes gene expression patterns similar to those of OA cartilage (27,28). To analyze the effects of IL-1β on the expression of miR-140 in articular chondrocytes, we performed quantitative PCR for miR-140 and *COL2A1*, *AGGRECAN*, *MMP13*, and *ADAMTS5*. In response to IL-1β stimulation, the expression of miR-140 was markedly decreased, while the expression of *MMP13* and *ADAMTS5* was significantly increased (Figure 4). Under the same experimental conditions, the expression of *COL2A1* did not change significantly. Taken together, these results show reduced miR-140 expression in the context of IL-1β–induced OA-like changes in chondrocyte gene expression.

Modulation of *ADAMTS5* and aggrecan expression in articular chondrocytes by miR-140. To investigate the function of miR-140 in chondrocytes, we examined whether expression of the OA-related genes *ADAMTS5*, *MMP13*, *COL2A1*, and *AGGRECAN* can be

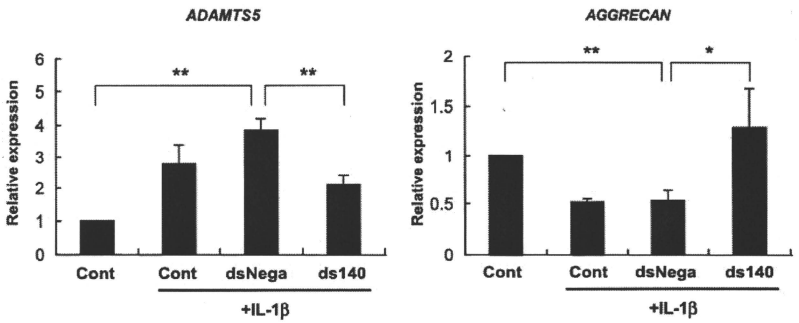

Figure 5. *ADAMTS5* and *AGGRECAN* expression by double-stranded microRNA-140 (ds–miR-140; ds140) in articular chondrocytes. Articular chondrocytes (n = 3) were transfected with ds–miR-140. The expression of *ADAMTS5* and *AGGRECAN* was analyzed by quantitative polymerase chain reaction. *ADAMTS5* expression was significantly reduced by ds–miR-140 with interleukin-1β (IL-1β) stimulation, and *AGGRECAN* expression was significantly increased by ds–miR-140 with IL-1β stimulation. Bars show the mean and SEM fold difference relative to control (Cont). dsNega = negative control of ds–miR-140 with nonspecific sequence. * = $P < 0.05$; ** = $P < 0.01$.

regulated by miR-140, when chondrocytes were stimulated with IL-1β with and without transfection of ds–miR-140. *ADAMTS5* expression with IL-1β stimulation was significantly reduced by ds–miR-140, and, conversely, *AGGRECAN* expression with IL-1β stimulation was significantly increased by ds–miR-140 (Figure 5). In the absence of IL-1β, the nonspecific dsRNA (dsNega) did not change the basal levels of *AGGRECAN*, and we observed an increase in *ADAMTS5* mRNA with ds–miR-140 as well as with dsNega. The expression of *MMP13* and *COL2A1* was not significantly changed by ds–miR-140 (data not shown). These results demonstrated that miR-140 regulates genes encoding ADAMTS-5 and aggrecan, suggesting that miR-140 plays an important role in regulating the balance between ECM formation and degradation.

DISCUSSION

This study is the first to identify miRNA that are expressed in a differentiation-dependent pattern in MSCs and articular chondrocytes. We also show changes in expression of the selected miR-140 in OA cartilage and in response to IL-1β stimulation. Moreover, we demonstrate that ADAMTS-5, a critical proteinase in OA pathogenesis (29–31), is regulated by miR-140.

Previous studies using systematic whole-mount in situ hybridization analysis for miRNA in zebrafish revealed that many miRNA are expressed in a tissue-specific pattern (32). From this database annotation, miR-140 was the only miRNA with a cartilage-specific expression pattern. Zebrafish embryos injected with ds–miR-140 had a profound facial phenotype, including cranial hemorrhaging and a hypoplastic roof of the mouth (33). Our miRNA array screen detected several miRNA that show large differences in expression in articular chondrocytes versus MSCs, including miR-140, which showed the largest expression difference between the 2 cell types. We also showed that during chondrogenesis, miR-140 expression increased in differentiated human MSCs compared with undifferentiated MSCs in parallel with expression of *SOX9*, *AGGRECAN*, and *COL2A1*. These findings suggest that miR-140 is a marker and possibly a regulator of chondrocyte differentiation.

The unique differentiation-related expression pattern of miR-140 is highlighted by our findings for miR-146, which is also expressed in chondrocytes. In contrast to miR-140, miR-146 has a broader tissue distribution, its expression is increased in response to IL-1 stimulation, it is up-regulated in OA (34), and it does not show changes related to chondrocyte differentiation (data not shown).

The ability of chondrocytes to remodel and repair cartilage ECM declines with aging, and in OA this is

related to a decline in the anabolic activity of chondrocytes (35,36). The expression of miR-140 was reduced in OA cartilage, and, in the same samples, expression of the proteinase *ADAMTS5* increased, and *COL2A1* expression decreased. Thus, the abnormal expression pattern of miR-140 correlates with the imbalance of anabolic–catabolic responses in OA. Our observations of abnormal miR-140 expression in OA are consistent with the findings of a recent study (37). IL-1β is one of the most prominent mediators of cartilage degradation and joint inflammation (38,39). IL-1β induces a cascade of inflammatory and catabolic events in chondrocytes. It also changes chondrocyte anabolism by suppressing the synthesis of proteoglycans and collagens and by enhancing the production of MMPs (27,28). The expression of miR-140 was down-regulated by IL-1β stimulation of chondrocytes in vitro. These data suggest that IL-1β may be a mediator that is involved in the suppression of miR-140 in OA.

Our studies using dsRNA mimicking miR-140 suggest that miR-140 suppresses *ADAMTS5* mRNA expression. This observation is supported by preliminary observations of increased *ADAMTS5* expression in miR-140–knockout mice (Miyaki S, et al: unpublished observations). The pathogenesis of OA is associated with abnormal activation and differentiation of chondrocytes that overexpress inflammation mediators and matrix-degrading enzymes (3–6). Previously examined mechanisms in these abnormal cellular responses include chondrocyte stimulation by extracellular stimuli such as cytokines, growth factors, mechanical stress, and matrix-degradation products. Intracellularly, these stimuli activate signaling cascades that lead to changes in gene expression (23,40). Alteration of miR-140 by IL-1β represents a novel mechanism to explain such aberrant changes in chondrocyte gene expression.

The present study was focused on miR-140, because it was shown to be the most cartilage-specific miRNA. We performed searches in 3 databases (TargetScan [http://www.targetscan.org/vert_50/], PicTar [http://pictar.mdc-berlin.de/], and miRanda [http://microrna.sanger.ac. uk/]), and this yielded 223–975 potential miR-140 targets. Only 9 potential targets were identified in all 3 databases, and, notably, this did not include *ADAMTS5*. Uncertainty remains regarding the rules for in silico miRNA target identification (41). At present, the most conclusive target validation is the demonstration of changes in protein expression, cell function, or phenotype in knockout or transgenic mice. Future studies are needed to determine the consequences of changes in the complete set of miR-140 targets for cartilage development and homeostasis. Currently, ongoing studies with miR-140–knockout mice and miR-140–transgenic mice will provide information in this regard.

In conclusion, the results of this study suggest that miR-140 is a chondrocyte differentiation–related miRNA. It may be a novel regulator of cartilage homeostasis, and changes in its expression and function play an important role in diseases affecting articular cartilage. Further studies of miR-140 have the potential to reveal important new regulatory pathways that control cartilage development and homeostasis and provide new insights into disease mechanisms and therapeutic interventions for OA.

AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Asahara had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Study conception and design. Miyaki, Asahara.

Acquisition of data. Miyaki, Nakasa, Otsuki, Grogan, Higashiyama, Inoue.

Analysis and interpretation of data. Miyaki, Nakasa, Otsuki, Grogan, Inoue, Kato, Sato, Lotz.

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Smad3 activates the Sox9-dependent transcription on chromatin

Takayuki Furumatsu [a,b,*], Toshifumi Ozaki [a], Hiroshi Asahara [b,c,**]

[a] Department of Orthopaedic Surgery, Okayama University Graduate School, 2-5-1 Shikatacho, Okayama 700-8558, Japan
[b] Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA
[c] National Center for Child Health and Development, 2-10-1 Okura, Setagaya, Tokyo 157-8535, Japan

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ABSTRACT

Transforming growth factor (TGF)-β has an essential role for the Sry-type high-mobility-group box (Sox)-regulated chondrogenesis. Chondrogenic differentiation is also controlled by chromatin-mediated transcription. We have previously reported that TGF-β-regulated Smad3 induces chondrogenesis through the activation of Sox9-dependent transcription. However, the cross-talk between TGF-β signal and Sox9 on chromatin-mediated transcription has not been elucidated. In the present study, we investigated the activity of Smad3, Sox9, and coactivator p300 using an in vitro chromatin assembly model. Luciferase reporter assays revealed that Smad3 stimulated the Sox9-mediated transcription in a TGF-β-dependent manner. Recombinant Sox9 associated with phosphorylated Smad3/4 and recognized the enhancer region of type II collagen gene. In vitro transcription and S1 nuclease assays showed that Smad3 and p300 cooperatively activated the Sox9-dependent transcription on chromatin template. The combination treatment of phosphorylated Smad3, Sox9, and p300 were necessary for the activation of chromatin-mediated transcription. These findings suggest that TGF-β signal Smad3 plays a key role for chromatin remodeling to induce chondrogenesis via its association with Sox9.

1. Introduction

Chondrogenesis is the fundamental process to form bones and articular surfaces. Mesenchymal condensation and the following chondrocyte differentiation are strictly regulated by several transcription factors and growth factors, such as Sry-type high-mobility-group box (Sox) genes and the transforming growth factor (TGF)-β superfamily, respectively. Sox5, 6, and 9 cooperatively regulate the sequential differentiation steps of chondrogenesis (Akiyama et al., 2002, 2004; Stricker et al., 2002). In these transcription factors, Sox9 has an essential role to initiate mesenchymal condensation and to maintain chondrogenic potential in early stages. The expression of α1 chain of type II collagen (Col2a1), a major component of cartilage extracellular matrix, is controlled by Sox9 through the Sox9-binding site on the Col2a1 enhancer region (Bell et al., 1997) and closely parallels that of Sox9 (Ng et al., 1997). The TGF-β superfamily including the two major families (TGF-β and bone morphogenetic protein) is a multifunctional growth factor for many cellular responses such as differentiation and proliferation (Heldin et al., 1997; Shi and Massagué, 2003). In chondrogenesis, TGF-β stimulation is necessary for primary chondrogenesis derived from mesenchymal stem cells (Pittenger et al., 1999). We previously described that TGF-β signal Smad3 promotes the early chondrogenesis through the activation of Sox9 (Furumatsu et al., 2005a). However, the precise mechanisms of Sox9 and TGF-β in the epigenetic regulation for initiating chondrogenesis are still unclear.

The epigenetic regulation is another dynamic system to control gene expression and other fundamental cellular processes, such as proliferation and differentiation (Li, 2002; Felsenfeld and Groudine, 2003; Jaenisch and Bird, 2003). Chromatin remodeling system including histone modification is the representative mechanism of epigenetics. The eukaryotic DNA and histones are packaged into chromatin as the nucleosome-repeated structure. Accesses of transcription factors and other regulators to DNA are highly restricted by chromatin structure. Many molecules have been revealed as important factors to form chromatin. Nucleosome assembly protein-1 (NAP-1) acts as a histone-shuttling protein (Ito

Abbreviations: AcCoA, acetyl-coenzyme A; ACF, ATP-utilizing chromatin assembly and remodeling factor; Col2a1, α1 chain of type II collagen; EMSA, electrophoretic mobility shift assay; MNase, micrococcal nuclease; MAPK, mitogen-activated protein kinase; NAP-1, nucleosome assembly protein-1; si-, small interfering; Sox, Sry-type high-mobility-group box; TβR-I(TD), constitutively active form of TβR-I; TGF, transforming growth factor.

* Corresponding author at: Department of Orthopaedic Surgery, Okayama University Graduate School, 2-5-1 Shikatacho, Okayama 700-8558, Japan.
Tel.: +81 86 235 7273; fax: +81 86 223 9727.
** Corresponding author at: Department of Molecular and Experimental Medicine, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, CA 92037, USA.
E-mail addresses: matino@md.okayama-u.ac.jp (T. Furumatsu), asahara@scripps.edu (H. Asahara).

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et al., 1996; Nakagawa et al., 2001). ACF (ATP-utilizing chromatin assembly and remodeling factor), consisting of Acf1 and ISWI subunits, assembles periodic nucleosome arrays on histone-attached DNA in an ATP-dependent process (Ito et al., 1999; Nakagawa et al., 2001). On the other hand, histone modification on chromatin, such as acetylation, enables transcription regulators to access to DNA sequences. DNA-binding transcription factors, such as CREB and MyoD, exert their transcriptional potential on histone-acetylated chromatin (Asahara et al., 2001; Dilworth et al., 2004). However, the relationship between chromatin-mediated transcription and signaling molecules is not elucidated. We previously reported that p300, which has an intrinsic histone acetyltransferase activity, directly associates with Sox9 (Tsuda et al., 2003) and activates the Sox9-dependent transcription on chromatin (Furumatsu et al., 2005b). In this study, we further analyzed the cross-talk between the Sox9-dependent transcription and TGF-β receptor-regulated Smad3 on chromatin using an in vitro chromatin assembly model.

The present study demonstrates that TGF-β-stimulated Smad3 activates the Sox9-dependent transcription on chromatin. This is the first report to explain the importance of TGF-β treatment in chromatin-mediated chondrogenesis.

2. Materials and methods

2.1. Cells, plasmids, si-RNA, and antibodies

A human chondrosarcoma cell line (SW1353) was used as an immature chondrogenic cell line. A plasmid encoding full-length of rat Sox9 and a small interfering (si-) RNA against Smad3 were used (Furumatsu et al., 2005a). p300 was a gift from Tso-Pang Yao. FLAG-tagged Smad3/4 and the constitutively active form of TβR-I [TβR-I(TD)] were generous gifts from Takeshi Imamura. pGL3-585E, which contains a mouse Col2a1 promoter and enhancer, was constructed with a pGL3-Basic (Promega) vector and used as a native Col2a1 reporter gene. 12 × 48-pGL3-P containing 12 sets of a 48-bp Col2a1 enhancer element was used as a reporter plasmid. PCR fragments of FLAG-tagged Sox9, FLAG-tagged Smad3, and Smad4 were subcloned into baculovirus expression vector pENTR3C (Invitrogen) as described (Furumatsu et al., 2005b). The following antibodies were used: FLAG M2, FLAG M2 affinity gel (Sigma), phospho-Smad2/3 (Santa Cruz), Smad2/3 (Upstate), Smad4 (Cell Signaling), and Sox9 (Chemicon).

2.2. Luciferase reporter assay

pGL3-585E and 12 × 48-pGL3-P were used as reporter genes for investigating the Sox9-dependent transcriptional activity. These reporter plasmids were different from our previous constructs (Furumatsu et al., 2005a). Appropriate plasmids (50 ng) and si-Smad3 (200 nM) were transiently transfected into SW1353 cells using FuGENE6 (Roche). pRL-CMV (10 ng, Promega) was used as an internal control. The cells were harvested for 24 h, and then the luciferase activities were analyzed using Dual-Luciferase Reporter Assay System (Promega). The assays were performed in triplicate.

2.3. Nuclear extract and immunoprecipitation

Nuclear extracts of SW1353 cells were prepared in 2× buffer D [20 mM HEPES (pH 7.9), 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT]. Protein concentrations were measured by BCA protein assay kit (Bio-Rad). Immunoprecipitation analyses using purified recombinant proteins were performed with anti-Sox9 or Smad2/3 antibody in 1× buffer D as described previously (Furumatsu et al., 2005b). Briefly, indicated amounts of recombinant proteins and/or nuclear extracts were incubated for 1 h at 25 °C. Ten percent volume of reaction mixture was loaded as an input fraction. Half of the mixture was incubated with each antibody and protein A beads (Sigma) for 1 h at 4 °C. Remaining mixture was incubated with rabbit IgG as a control.

2.4. Purification of histones and recombinant proteins

Core histones were purified from HeLa nuclear pellets and dialyzed in HEG buffer [10 mM HEPES (pH 7.6) 10% glycerol, 50 mM KCl, 0.1 mM EDTA]. Baculovirus of histidine-tagged NAP-1, FLAG-tagged ISWI, and Acf-1 were kindly gifts from Takashi Ito and used as chromatin assembling molecules (Ito et al., 1999, 2000). The baculovirus expression vectors carrying Sox9 and Smad3/4 were constructed using BaculoDirect Systems according to the manufacturer's protocol (Invitrogen). Recombinant NAP-1, recombinant ACF complex (FLAG-tagged ISWI and untagged Acf-1), FLAG-tagged p300, FLAG-tagged Sox9, and Smad3/4 complex (FLAG-tagged Smad3 and untagged Smad4) were produced in Sf9 cells (Invitrogen) and prepared as described previously (Furumatsu et al., 2005b). Recombinant Smad3/4 was purified after 30-min-treatments of TGF-β3 (R&D). Purified proteins were assessed by silver stain (BioRad) and Western blotting analyses.

2.5. Electrophoretic mobility shift assay (EMSA)

The Col2a1 enhancer probe containing the Sox9-binding site (in capital letters) was generated by annealing the following oligonucleotides: 5′-gcgcttgagaaaagcccCATTCATgagaggc-3′ and 5′-gcctctcATGAATGgggctttttctcaagcgc-3′. Probes were ^{32}P end-labeled using T4 polynucleotide kinase (Invitrogen). Purified Sox9 (30 ng) was incubated with the labeled probe (0.8 pmol). The unlabeled Col2a1 enhancer probe (16 pmol) was used as a competitor. In supershift analysis, 15 min treatment with anti-Sox9 antibody (0.2 μg) was performed before protein–DNA binding reaction.

2.6. Chromatin assembly and micrococcal nuclease (MNase) assay

Chromatin assembly and MNase digestion analyses were performed as described (Asahara et al., 2002) by using 12 × 48-pGL3-P. For chromatin reconstitution, standard reactions (20 μl) containing plasmid (150 ng), histones (100 ng), NAP-1 (500 ng), ISWI/Acf-1 (0.65 ng each), ATP (3 mM), and ATP regeneration systems (30 mM phosphocreatine and 20 ng creatine phosphokinase) were incubated at 30 °C for 4 h. In MNase assay, chromatinized plasmids (300 ng) were digested with MNase (0, 0.02, and 0.04 U/15 μl) for 5 min at 37 °C.

2.7. In vitro transcription and S1 nuclease assay

After chromatin assembly, standard reactions (12 × 48-pGL3-P, 150 ng) were incubated with Sox9 (10 ng), Smad3/4 (100 ng), p300 (40 ng), and acetyl-coenzyme A (AcCoA, 5 μM) for 30 min at 30 °C. For in vitro transcription, nuclear extracts from SW1353 cells (30 μg) were added and incubated with rNTPs at 30 °C for 40 min. In vitro-transcribed RNAs were recovered and subjected to S1 nuclease analyses using the specific primer (49 bp) against 12 × 48-pGL3-P luciferase gene as described (Furumatsu et al., 2005b). RNAs were annealed with ^{32}P end-labeled primers (0.2 pmol each) for 12 h, and then digested with 50 units of S1 nuclease (Invitrogen) for 30 min at 37 °C. The protected fragments were run on 8% denaturing polyacrylamide gels and visualized by autoradiography. Each experiment was performed at least three times.

3. Results

3.1. Smad3 stimulates the Sox9-mediated transcription in a TGF-β-dependent manner

To assess the fundamental role of Smad3 in chromatin remodeling during early chondrogenesis, we first analyzed the effect of Smad3 in the Sox9-regulated transcription using newly constructed reporter plasmids. Overexpressed Smad3 stimulated the transcriptional activity of Col2a1 reporter gene (Fig. 1A, pGL3-585E) in a Sox9-depedent manner (Fig. 1B). In addition, the effect of Smad3 was enhanced by the cotransfection of constitutively active form of TGF-β receptor I [TβR-I(TD)]. Twelve copies of the Sox9-binding fragment dramatically induced the Sox9-regulated transcription in reporter assays (Fig. 1C, 12 × 48-pGL3-P). Smad3 also activated the transcription of 12 × 48-pGL3-P in Sox9- and TGF-β-dependent manners. These findings suggest that Smad3 may act as a chromatin remodeling factor in chondrogenesis.

3.2. TGF-β and Smad3 are necessary for the activation of Sox9-dependent transcription

To investigate the effect of Smad3 itself in this reporter assay system, we used a si-RNA fragment against Smad3 as an inhibitor. The activities of Sox9-regulated transcription were stimulated by the addition of Smad3 in a dose-dependent fashion (Fig. 2A and B). si-Smad3 decreased the effect of activated TGF-β receptor and overexpressed Smad3 in Sox9-regulated reporters to the basal levels. However, si-Smad3 did not inhibit the Sox9-induced transactivation. These results prompted us to analyze the function of TGF-β-stimulated Smad3 and Sox9-related transcriptional apparatus on chromatin in chondrogenesis.

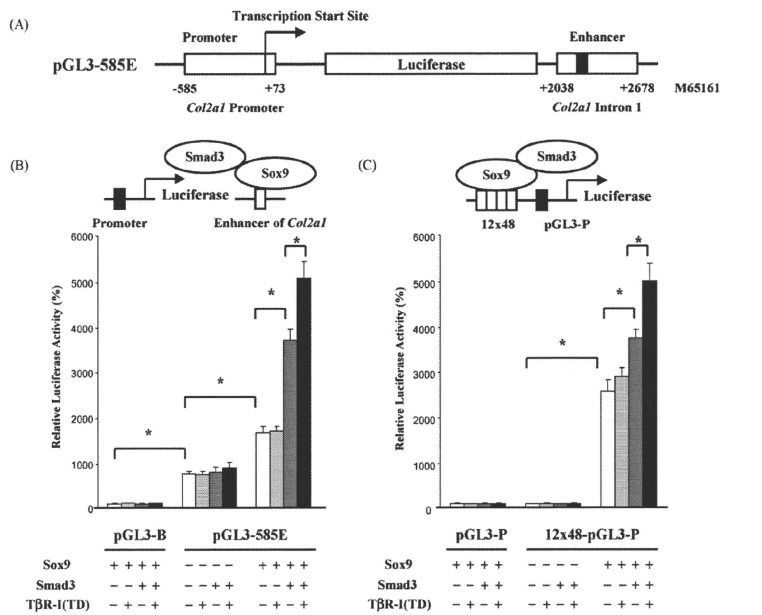

Fig. 1. Smad3 enhances the Sox9-mediated transcription in a TGF-β-dependent manner. (A) A schematic characterization of pGL3-585E which contains a native promoter and enhancer of mouse Col2a1 gene. Numbers indicate the distance from the transcription start site on mouse Col2a1 gene (National Center for Biotechnology Information, M65161). Black box denotes the SOX9-binding site on the enhancer region of Col2a1 intron 1. (B) Transient transfections of Sox9, Smad3, and TβR-I(TD) did not increase luciferase activities of pGL3-B plasmids in SW1353 cells (pGL3-B). In pGL3-585E systems, Sox9 enhanced a relative luciferase activity to a level as high as 2.2-fold over the control. Cotransfection of Smad3 augmented a luciferase activity up to 2.3-fold higher level of Sox9-transfected cells. The additional transfection of constitutively active form of TβR-I(TD) induced an approximately 36% increase of the activity in Sox9- and Smad3-transfected SW1353 cells. Luciferase activities of pGL3-585E were not increased in the absence of Sox9. Note that Smad3 and TβR-I(TD) synergistically activated the native Col2a1 reporter-mediated transcription in a Sox9-dependent manner. (C) The activity of 12 × 48 pGL3-P was enhanced by the addition of Sox9 up to 27.5-fold levels of the control. Smad3 increased the 12 × 48 pGL3-P-based luciferase activity up to 1.5-fold higher level in the presence of Sox9. TβR-I(TD) also induced 33% increase of the activity of Smad3-transfected cells in the presence of Sox9. However, the additional increase of luciferase activity was not observed in pGL3-P-transfected cells. Relative luciferase activities were calculated using the activity of pGL3-P as a control (100%). A schematic illustration of each reporter assay system is placed on the top of each figure (B and C). *Statistical significances ($p < 0.05$) were observed between the indicated bars with the Mann–Whitney *U*-test. Error bars, S.D.