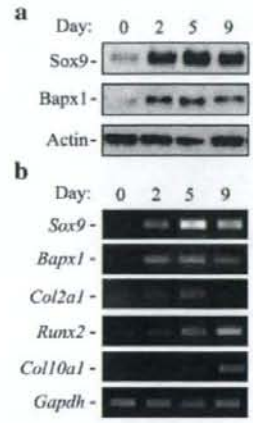

Fig. 1 – Bapx1 is expressed during early chondrogenesis in C3H10T1/2 cells. (a) Western blot detection of Sox9 and Bapx1 proteins at the indicated time points. (b) RT-PCR detection of mRNA of *Sox9*, *Bapx1*, *Col2a1*, *Bapx1*, *Runx2*, *Col10a1*, and *Gapdh* at several time points.

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.

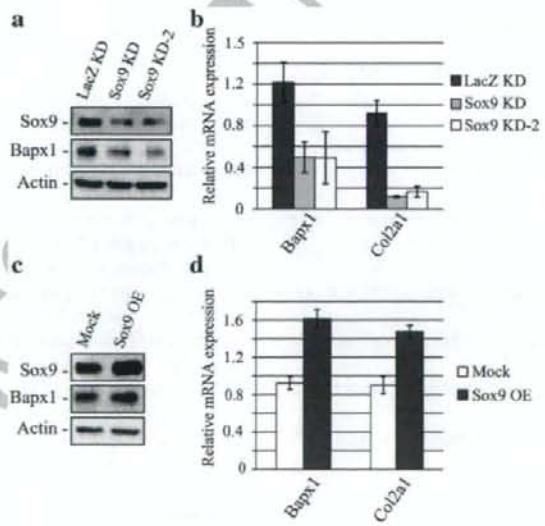

Fig. 2 – Bapx1 expression is up-regulated by Sox9 in chondrocytes. (a) LacZ as a negative control or two Sox9-targeting shRNA-expressing lentiviruses were transduced into cultured mouse chondrocytes and evaluated by western blot for knockdown (KD) efficiency and Bapx1 expression. (b) *Bapx1* and *Col2a1* 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.

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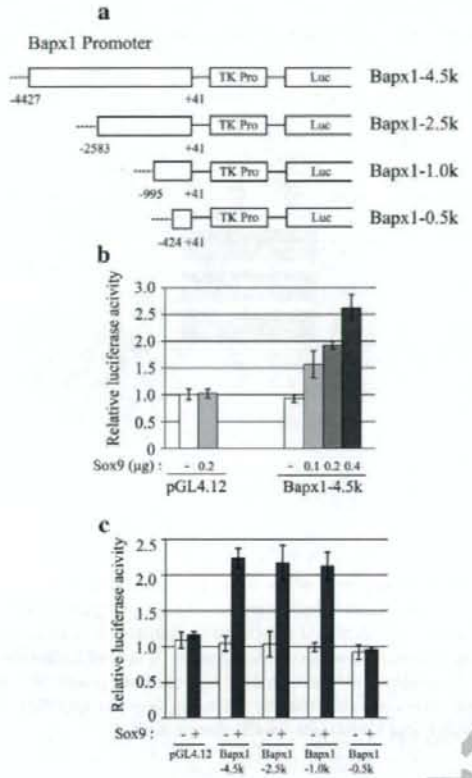

Fig. 3 – Sox9-activated *Bapx1* proximal promoter activity. (a) Luciferase reporter constructs containing the *Bapx1* promoter are shown. Numbers indicate the position from the transcription start site (TSS). (b) C3H10T1/2 cells in 24-well plates were co-transfected with 200 ng of the indicated luciferase 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

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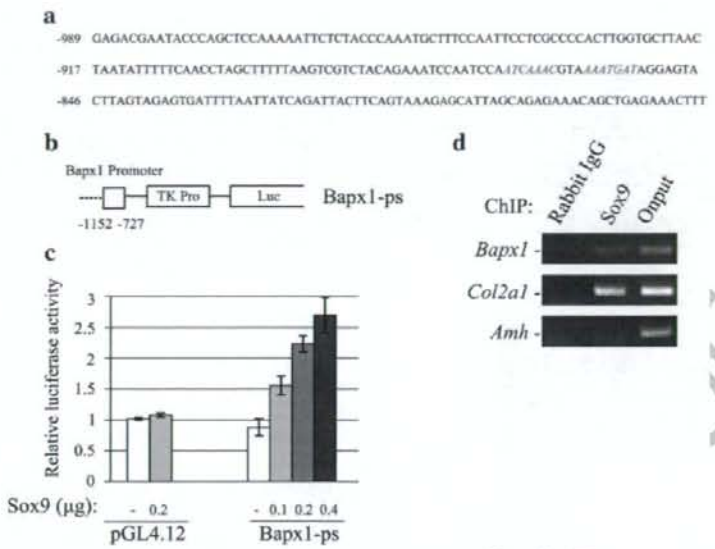

Fig. 4 – Minimal Sox9 binding site in the *Bapx1* promoter in chondrocytes. (a) Sequence of the *Bapx1* promoter between −989 and −775 from the TSS. A potential Sox9 binding site is indicated in red. (b) A luciferase reporter construct containing the *Bapx1* minimum promoter is shown. Numbers indicate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

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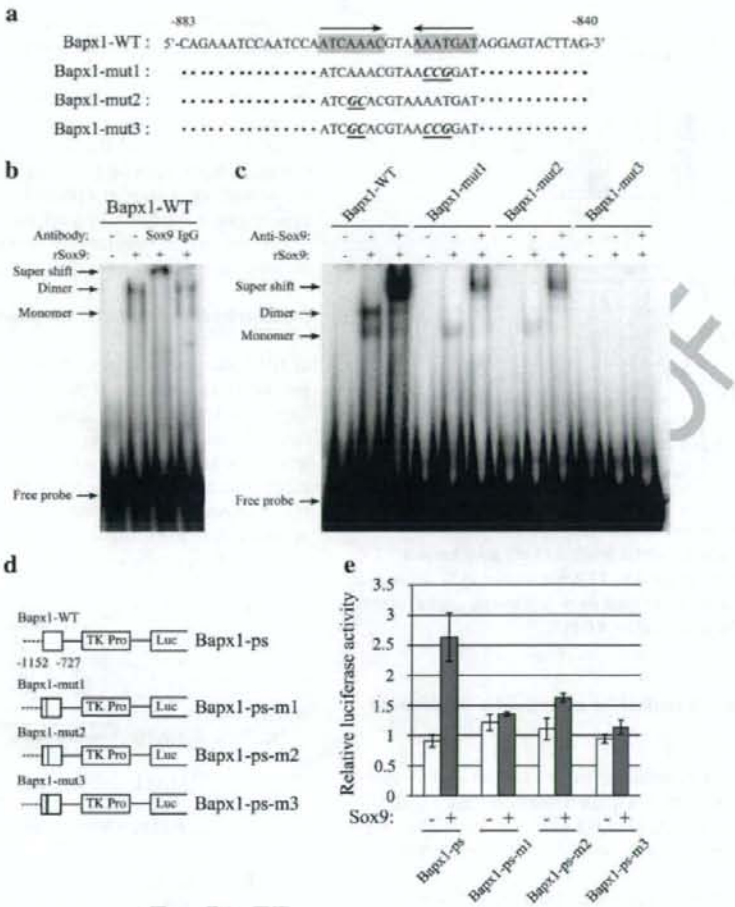

Fig. 5 – Induction of *Bapx1* promoter activity through the binding of a Sox9 homodimer. (a) Oligonucleotide sequences corresponding to the *Bapx1* promoter are shown, with distance from the TSS indicated. Putative Sox9-binding consensus sequences are marked with a grey background; arrows denote site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

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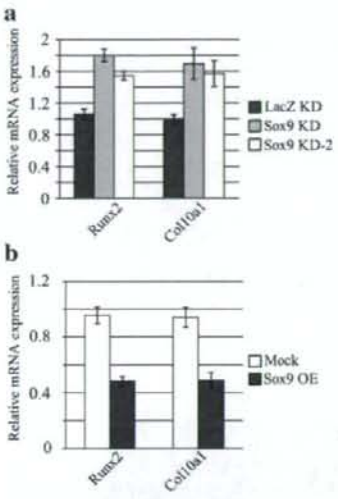

Fig. 6 – Sox9 represses Runx2 expression and function. (a) *Runx2* and *Col10a1* mRNA levels in Sox9 knockdown chondrocytes were assayed by RT-quantitative PCR. (b) *Runx2* and *Col10a1* expression in Sox9 over-expressing chondrocytes were analyzed by quantitative RT-PCR.

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

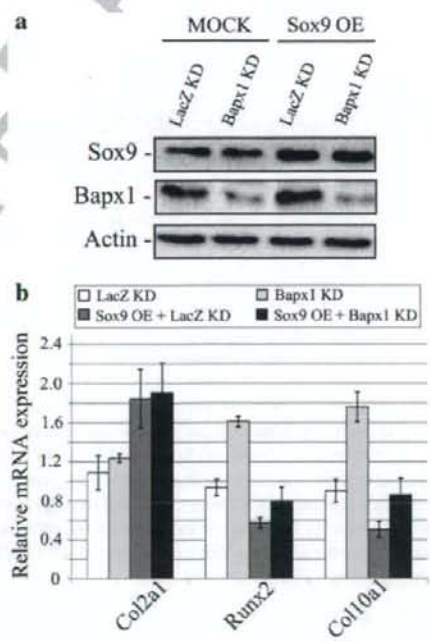

Fig. 7 – Bapx1 knockdown diminishes the suppression of Runx2 expression and function by Sox9 over-expression in chondrocytes. (a) Over-expression of Sox9 and/or Bapx1 targeting shRNA in cultured mouse chondrocytes and detection of Sox9 and Bapx1 proteins by western blot. (b) *Col2a1*, *Runx2* and *Col10a1* 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.

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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 IL-1 responses

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ABSTRACT

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

METHODS: To identify miRNAs specifically expressed in chondrocytes, we performed gene expression profiling using miRNA microarrays and quantitative PCR with human articular chondrocytes compared to human mesenchymal stem cells (MSC). The expression pattern of miR-140 was monitored during chondrogenic differentiation of hMSC in pellet cultures and in human articular cartilage from normal and osteoarthritic knee joints. We tested effects of IL-1ß on miR-140 expression. Double-strand (ds) miR-140 was transfected into chondrocytes to analyze changes in the expression of genes associated with osteoarthritis.

RESULTS: Microarray analysis showed that miR-140 has the largest difference in expression between chondrocytes and MSC. During chondrogenesis cultures of MSC miR-140 expression increased in parallel with *Sox9* and *Col2a1*. Normal human articular cartilage expressed miR-140 and this was significantly reduced in OA tissue. In vitro treatment of chondrocytes with IL-1ß suppressed miR-140 expression. Transfection of chondrocytes with ds-miR-140 downregulated IL-1ß-induced *ADAMTS-5* expression and rescued the IL-1ß –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-1ß may contribute to the abnormal gene expression pattern characteristic of OA.

INTRODUCTION

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 twenty 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 and in the late phase of the disease process where 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 of extracellular matrix (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 (miRNAs) are a class of non-coding small RNAs that play roles in biological processes as negative regulators of gene expression by promoting mRNA degradation and/or repressing translation through sequence-specific interactions with the 3' untranslated regions (UTRs) of specific mRNA targets (7-10). Hundreds of miRNAs have been found in various organisms, and many miRNAs are evolutionarily conserved. Moreover, one third of all mammalian mRNAs seem to be under miRNA regulation suggesting an essential role in regulating gene expression (11). Several miRNAs 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). As Dicer is indispensable for producing a functional, mature type of miRNA, this finding suggests that the presence of specific miRNAs plays a critical role in skeletal development. Although Tuddenham et al showed cartilage specific expression of miR-140 in mouse embryos (19), the role of tissue-specific miRNAs in articular cartilage has not been reported.

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

MATERIALS AND METHODS

Human tissue samples, cell isolation and culture

Human articular cartilages from knee joints were obtained from 8 normal donors (38.22 ± 5.31 years of age, mean ± SD) and from 11 OA patients (79.36 ± 9.72 years of age, mean ± SD) 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, with a score of less than 2 points being normal and a score of greater than 5 representing OA. RNA was isolated from fresh frozen cartilage by homogenizing the tissue in a freezer mill (Spex CertiPrep, Inc., Metuchen, NJ, USA) and extracting the homogenate in Trizol (Invitrogen Corporation, Carlsbad, CA, USA). Human chondrocytes were isolated and cultured as described previously (20). Experiments with chondrocytes were performed in passage 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 Human Subjects Committee) and cultured as described previously (20, 21). Experiments with MSCs were performed in passage 3-6.

Microarray analysis

Small RNAs of less than 200 nucleotides in length were extracted from MSCs and chondrocytes with mirVana miRNA isolation kit (Ambion, Austin, TX) according to the manufacturer's protocol. Purified RNA was then labeled with Cy3 or Cy5 by mirVana miRNA labeling kit (Ambion). In brief, 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

RNA was hybridized on slides, on which oligonucleotides against human miRNA had been arrayed (Hokkaido-System Science, Japan), and detected by a scanner (Agilent Technologies, Santa Clara, CA).

Chondrogenesis in human mesenchymal stem cells

Human bone marrow mesenchymal stem cells (MSC) were used to prepare pellets (5.0×10^5 cells/pellet) by centrifuging the cells at 500g in 15ml conical polypropylene tubes and cultured in chondrogenic medium (Lonza, Walkersville, MD) supplemented with BMP-2 (500ng/ml) and TGFß3 (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 IL-1ß

Chondrocytes were maintained in 12-well plates, containing DMEM plus 10% calf serum and 1% penicillin/streptomycin. Following treatment with recombinant human IL-1ß (5 ng/ml; PeproTech, Rocky Hill, NJ). Cells were washed with cold PBS, and total RNA was isolated with Trizol reagent. Quantitative PCR was performed with the TaqMan microRNA assay kit for mature miR-140 or Taqman Gene Expression Assay.

Transfection of double-stranded miR-140 into human articular chondrocytes

Double-strand (ds) RNA oligos representing mature sequences that mimic endogenous miR-140 were transfected into human chondrocytes at 80-90% confluence at 4nM concentration with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Synthesized RNA oligos 5'-CAGUGGUUUUACCCUAUGGUAG and 5'-

ACCACAGGGUAGAACCACGGAC were annealed to obtain ds-miR-140. Silencer Negative Control siRNA #1 (Ambion, CA) at the same concentration as the specific miR-140 ds RNA was included in each experiment.

Quantitative real-time polymerase chain reaction (PCR)

Total RNA was isolated from cartilage tissues, monolayer or pellet cultures using Trizol (Invitrogen).

Quantitative real time PCR (qPCR) for miRNAs was performed using the TaqMan MicroRNA reverse Transcription kit (Applied Biosystems, Foster City, CA) according to the manufacturer's protocol. Complementary DNA (cDNA) was produced using Ready To- GO You-Prime First-Strand Beads (GE Healthcare UK, UK) with total RNA 1μg and oligo (dT)18 primers.

Quantitative real time PCR was performed using TaqMan Gene Expression Assay probe for *Col2a1* (Hs00164004_m1), *Aggecan* Hs00202971_m1), *ADAMTS-5* (Hs00199841_m1), *MMP-13* (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. Expression levels for each gene were assessed relative to U18 or *GAPDH* expression.

Statistical analysis

Statistically significant differences between two groups were determined with *t* tests. The results are reported as mean ± SEM. *P* values of less than 0.05 were considered significant.

RESULTS

miR-140 expression in articular chondrocytes and mesenchymal stem cells

Chondrogenic differentiation of MSCs involves dynamic changes of various gene expression patterns including induced expression of chondrocytes specific genes, including *Sox9* and *Col2*. In order to screen miRNAs specifically expressed in chondrocytes, we performed gene expression profiling using miRNA microarrays comparing primary chondrocytes from articular cartilage to MSCs. In primary articular chondrocytes, several miRNAs were more abundant as compared with undifferentiated MSCs. The largest difference was observed for miR-140 (Table 1). The high expression miR-140 in chondrocytes compared to MSCs was confirmed by qPCR (Figure 1).

Expression of miR-140 during chondrogenesis of MSCs

In vitro chondrogenesis assay using MSCs reflects, in part, *in vivo* skeletal formation. To examine dynamic expression pattern of miR140 during *in vitro* chondrogenesis, we performed Taqman-qPCR assay to analyze expression patterns of miR-140. Pellets of MSCs were strongly stained by safranin O after chondrogenesis induction for 14 day (data not shown). In this model, miR-140 expression gradually increased during chondrogenesis in parallel with *Sox9*, *Aggrecan* and *Col2a1* expression (Figure 2). These data indicate that miR140 increases during chondrocytic differentiation of MSC 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

downregulated (22). On the other hand, cartilage degrading enzymes, including ADAMTS5 and MMP-13, are upregulated (23-25). To examine changes in miR-140 expression in OA articular cartilage, qPCR of miR-140 together with OA related marker genes was performed on 19 samples prepared from human knee articular cartilage (normal=8, OA=11). As expected, the expression of *ADAMTS-5* was significantly increased in OA cartilage while the expression of *Col2a1* was significantly lower than in normal cartilage (Figure 3). MiR-140 expression in articular cartilage from OA donors (65 to 93 years old; Mankin score: 5-10) was significantly lower than in normal cartilage (30 to 44 years old; Mankin score: 0-2) (Figure 3). These data demonstrate abnormally reduced miR-140 expression in OA cartilage, appears to correlate with increased *ADAMTS-5* expression and reduced *Col2a1* expression in the same samples.

Effect of IL-1ß on miR-140 expression in articular chondrocytes

IL-1ß is one of the critical mediators of osteoarthritis and IL-1ß stimulation on chondrocytes causes similar gene expression patterns with OA cartilage (26, 27). To analyze effects of IL-1ß on the expression of miR-140 in articular chondrocytes, we performed qPCR for miR-140 and *Col2a1*, *Aggrecan*, *MMP-13* and *ADAMTS-5*. In response to IL-1ß stimulation the expression of miR-140 was markedly decreased, while *MMP-13* and *ADAMTS-5* expression were significantly increased (Figure 4). Under the same experimental conditions expression of *Col2a1* did not significantly change. Taken together, these results show reduced mi-R140 expression in the context of IL-1ß induced OA-like changes in chondrocyte gene expression.

miR-140 modulates *ADAMTS-5* and *Aggrecan* expression in articular chondrocytes

To investigate miR-140 function in chondrocytes, we examined whether expression of the above osteoarthritis related genes; *ADAMTS-5*, *MMP-13*, *Col2a1* and *Aggrecan*, can be regulated by miR-140, when chondrocytes were stimulated with IL-1ß with and without transfection of ds-miR-140. The ds-miR-140 significantly reduced *ADAMTS-5* expression with IL-1ß stimulation and conversely, *Aggrecan* expression with IL-1ß stimulation was significantly increased by ds-miR-140 (Figure 5). In the absence of IL-1ß ds140 miRNA did not change the basal levels of aggrecan and we observed an increase in *ADAMTS-5* mRNA with the ds140 miRNA as well as with the non-specific dsRNA. The expression of *MMP-13* and *Col2a1* were 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 extracellular matrix formation and degradation.

DISCUSSION

This is the first study to identify miRs that are expressed in a differentiation-dependent pattern in mesenchymal stem cells and articular chondrocytes. We also show changes in expression of the selected miR-140 in OA cartilage and in response to IL-1ß. Moreover, we demonstrate that ADAMTS-5, a critical proteinase in OA pathogenesis, is regulated by miR-140.

Previous studies using systematic whole mount in situ hybridization analysis for miRs in zebrafish revealed that many miRs are expressed in a tissue-specific pattern (28). From this database annotation, miR-140 was the only miR with a cartilage specific expression pattern. Zebrafish embryos injected with miR-140 duplex RNA had a profound facial phenotype, including cranial hemorrhaging and a hypoplastic roof of the mouth (29). Our miR-array screen detected several miRs that show large difference in expression in articular chondrocytes versus

MSCs and this includes miR-140 with the largest expression difference between the two cell types. We also showed that during chondrogenesis, miR-140 expression increased in differentiated hMSC cells compared to undifferentiated MSC in parallel with *Sox9* and *Col2a1* expression. These findings suggest that miR-140 is a marker and possibly a regulator of chondrocytic differentiation.

The unique differentiation-related expression pattern of miR-140 is highlighted by our findings on miR-146 which is also expressed in chondrocytes. In contrast to miR-140, miR-146 has a broader tissue distribution, it is increased in response to IL-1, it is upregulated in OA and does not show changes related to chondrocyte differentiation (Arthritis Rheum, in press).

The ability of the chondrocytes to remodel and repair the cartilage ECM declines with aging and in OA and this is related to a decline in the anabolic activity of chondrocytes (30, 31). MiR-140 expression was reduced in OA cartilage and in the same samples expression of proteinases *ADAMTS-5* 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 one recent publication (32). IL-1ß is one of the most prominent mediators of cartilage degradation and joint inflammation (33, 34). IL-1ß 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 matrix metalloproteinases (MMPs) (26, 27). miR-140 expression was down-regulated by IL-1ß stimulation of chondrocytes *in vitro*. These data suggest that IL-1ß may be at least one mechanism that is involved in the suppression of miR-140 in OA.

Our studies using dsRNA mimicking miR-140 suggest that miR-140 suppresses