SOX9-dependent and -independent Transcriptional Regulation of Human Cartilage Link Protein*

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Cartilage link protein is a key component of the cartilage extracellular matrix. The transcriptional regulation of the gene encoding cartilage link protein (CRTL1) is largely unknown, however. Here, we investigated the regulation of CRTL1 by SOX9, a key regulator of cartilage matrix genes and chondrogenesis. Knockdown of SOX9 resulted in decreased CRTL1 expression. SOX9 induced CRTL1 expression effectively in human non-chondrocytic immortalized cell lines as well as in mesenchymal stem cell and adult dermal fibroblast. These results indicate that, like other cartilage matrix genes, SOX9 is a key regulator of CRTL1. Unlike other cartilage matrix genes, however, the activation of CRTL1 by SOX9 and its known transcriptional co-activators 1-SOX5 and SOX6 was cell-type dependent. Two cis-acting enhancer elements resided in the 5′-untranslated region of CRTL1. One contained a heptameric SOX binding sequence and showed SOX9-dependent enhancer activity in several cell lines. The other showed cell type-specific SOX9-independent enhancer activity. These findings suggest that the enhancer elements may mediate differential expression of CRTL1 during chondrocyte differentiation and maturation.

Cartilage link protein (CRTL-LP) is a glycoprotein that exists as a monomer of 339 amino acids (1). A major component of cartilage extracellular matrix, CRT-LP stabilizes aggregates of aggrecan and hyaluronic acid (HA). The three functional domains of CRT-LP include an Ig-fold, which interacts with the aggrecan G1 domain, and two proteoglycan tandem repeats that mediate binding to HA. CRT-LP binds aggrecan along the HA chain in a 1:1 stoichiometry (2–4). The resulting aggregates are entrapped within the mesh-like network of type II collagen fibrils, producing a large, stable macromolecular structure that contributes to compression resistance and shock absorption in the joint (2–4).

CRTL-LP is expressed in numerous non-cartilaginous tissues, such as mesonephros, brain, and sclera. However, the phenotype associated with loss of function of the CRT-LP gene, CRTL1, is restricted to the skeleton; CRTL1-null mice exhibit a perinatally lethal chondrodysplasia. The cartilage in these mice contains significantly reduced aggrecan deposits in the hypertrophic zone and decreased numbers of prehypertrophic and hypertrophic chondrocytes (5). Cartilage-specific transgene expression of CRTL1 can completely prevent perinatal mortality in CRTL1-null mice and rescue skeletal abnormalities at levels dependent upon the amount of CRTL-LP expression (6). In addition, CRT-LP may function as a growth factor to up-regulate the synthesis of aggrecan and type II collagen in cartilage (7). Thus, production of CRT-LP at appropriate levels is crucial to the formation of proteoglycan aggregates and the normal organization of hypertrophic chondrocytes. To date, however, the mechanisms that control CRTL1 expression remain largely unknown.

A key transcriptional regulator of chondrogenesis is SOX9, a member of the SOX (Sry-type HMG box) family, that is characterized by a reduced amino acid sequence within the HMG (high mobility group) domain (8, 9). Prechondrogenic mesenchymal cells with a homozygous deletion of SOX9 are unable to differentiate into chondrocytes and cannot express chondrocyte-specific matrix genes such as Col2a1, Col9a2, Col11a2, and Agc1 (10). In developing mouse embryos, SOX9 expression closely parallels that of Col2a1 (11, 12). SOX family proteins bind to DNA through a heptameric enhancer motif, (AT)7(TG)3 (13, 14). SOX9 has been demonstrated to regulate expression of Col2a1 via several of these heptameric motifs (15). Other cartilage matrix genes, including Col9a1, Col9a2, Col11a2, CD-Rap, and Agc1, have also been identified as direct targets of SOX9 (16–20). These findings indicate that cartilage genes might have common mechanisms for transcriptional regulation and suggest a role for SOX9 in regulating CRTL1 expression.

In this study, we have shown that the 5′-untranslated region (5′-UTR) of CRTL1 contains a cis-acting element that is directly regulated by SOX9. SOX9 can induce CRTL1 expression in human non-chondrocytic cell lines, mesenchymal stem cells, and adult dermal fibroblasts. We also show that the 5′-UTR of CRTL1 contains another cis-acting element that responds in a cell type-specific manner and is independent of SOX9.

MATERIALS AND METHODS

Cell Culture—HuH-7 cells were obtained from the Riken Cell Bank (Tsukuba, Japan), and HeLa cells were obtained from the Japanese Collection of Research Bioresource Cell Bank (Osaka, Japan). HEK293 cells were purchased from Clontech (Palo Alto, CA). All cell lines were cultured at 37 °C under 5% CO2 in Dulbecco’s modified Eagle’s medium (DMEM)-high glucose supplemented with penicillin (50 units/ml), streptomycin (50 μg/ml), and 10% fetal bovine serum. OUMS-27 cells were obtained from the Institute for Fermentation, Osaka Animal Cell Bank (Osaka, Japan) and cultured in DMEM-high glucose supplemented with kanamycin (50 μg/ml) and 10% fetal bovine serum. HuH

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1 The abbreviations used are: CRT-LP, cartilage link protein; UTR, untranslated region; DMEM, Dulbecco’s modified Eagle’s medium; hMSC, human mesenchymal stem cell; ADPB, human adult dermal fibroblast; RNAI, RNA interference; EMSA, electrophoretic mobility shift assay; WT, wild type; DIG, digoxigenin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
man mesenchymal stem cells (hMSC) and human adult dermal fibroblasts (hDFB) were purchased from Cambrex (East Rutherford, NJ). The hMSC line was cultured in MSC growing medium, and the hDFB line was cultured in DMEM-high glucose supplemented with penicillin (50 units/ml), streptomycin (50 µg/ml), and 10% fetal bovine serum. For all cultures, the growth medium was replaced every 3–4 days.

Virus titers were estimated using the AdenoX rapid titer assay (Clontech). AdenoX expression vectors containing L-SOX5, SOX6, and SOX9 were constructed using the AdenoX expression system (Clontech) according to the manufacturer's instructions. The adenoviruses were packed and amplified in HEK293 cells and then purified using the AdenoX virus purification kit (Clontech). Virus titers were estimated using the AdenoX rapid titer assay kit (Clontech).

Induction of Adenoviruses into hMSC and hDFB—Confluent hMSC and hDFB cultures were transduced with SOX-expressing adenoviruses at a multiplicity of infection of 50. SOX-induced hMSC and hDFB cells were collected 2 days after transduction and gently centrifuged into pellets (5 x 10^6 cells/pellet). Cell pellets were cultured in serum-free high-glucose DMEM. After 3, 7, 14, and 21 days of pellet culture, mRNA was harvested from cell pellets, and gene expression levels were measured using a real-time PCR assay. Three-dimensional cultures on collagen gel matrices at a density of 2.5 x 10^5 cells/pellet were transduced with SOX-expressing adenoviruses at a multiplicity of infection of 50 (50 units/ml), and SOX9, SOX5, and SOX6 were amplified by PCR. DNA sequences of PCR products were verified by automated DNA sequencing (model 3700; ABI, Foster City, CA) and then cloned into pEGFP-C1 or pShuttle mammalian expression vectors (Clontech). AdenoX expression vectors containing L-SOX5, SOX6, and SOX9 were constructed using the AdenoX expression system (Clontech) according to the manufacturer's instructions. The adenoviruses were packed and amplified in HEK293 cells and then purified using the AdenoX virus purification kit (Clontech). Virus titers were estimated using the AdenoX rapid titer assay kit (Clontech).

RNA Interference (RNAi)—The pSilencer2.1-U6 neo expression vector kit (Ambion, Austin, TX) was used according to the manufacturer's protocol. RNAi target sequences for human SOX9 mRNA were selected using a small interfering RNA target design online tool (www.takara-bio.co.jp/rna/target_design/index.htm) and were analyzed by a BLAST search to ensure the gene specificity. Several small interfering RNA constructs were tested, and the most effective one was used. The target sequence was 5'-AACUGUGGUUGUCGCAUGGU-3' (Sigma Genosis, Tokyo, Japan). These fragments were multimerized to 35-bp wild type and mutant fragments were synthesized as double-stranded oligonucleotides containing MluI-cleaved sites at both ends. The endogenous CRTL1 mRNA levels were quantified by SYBR Green real-time PCR and normalized to GAPDH mRNA. The values represent the means ± S.D. of quadruplicate measurements.

Real-time Quantitative PCR Assays—Total RNAs from cells were isolated using the RNaseasy mini kit (Qiagen, Hilden, Germany), and treated with DNaseI. Total RNAs (50 ng-1 µg) were reverse transcribed into cDNA using the Taqman core reagent kit (ABI) according to the manufacturer's protocol. Subsequently, 1 µl of each room temperature reaction was used as a template for the second step of SYBR Green reverse transcribed PCR (Qiagen). A partial cDNA of CRTL1 and SOX9 was amplified by PCR (using the primer sequences 5'-TACACAGAGGTGCACATGT-3', 5'-CAGCCAGAGGTGACACTCC-3', 5'-TCCCTTCCCTACAGAGATT-3', and 5'-CCCTCTACAGACCATCT-3', respectively) and cloned into the pCR-TOPOII vector (Invitrogen). SYBR Green PCR amplification and real-time fluorescence detection were performed using the ABI PRISM 7700 sequence detection system. PCR cycling conditions were as follows, 94 °C for 15 min followed by 40 cycles at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s.

Construction of CRTL1 Plasmids—CRTL1-luciferase fusion genes were constructed in the pGL3-Basic vector (Promega, Madison, WI). All CRTL1 mRNA levels were quantified by SYBR Green real-time PCR and normalized to GAPDH mRNA. The values represent the means ± S.D. of quadruplicate measurements.
create sequences of 2–4 tandem repeats (Fig. 6). All plasmid constructs were verified by DNA sequencing.

Luciferase Reporter Assays—Plasmid DNA transfections were performed using FuGENE 6 (Roche Applied Science). Cells were plated at a density of 5 \times 10^4 cells/well in 24-well tissue culture plates. Luciferase reporter plasmids were co-transfected with the pRL-TK plasmid as an internal control for transfection efficiency. Forty-eight hours after transfection, cells were harvested, and luciferase activity was measured using the PG-DUAL-SP reporter assay system (TOYO Ink, Tokyo, Japan). Relative transcriptional activity was expressed as a ratio of luciferase reporter gene activity from the experimental vector to that from the internal control vector.

Electrophoretic Mobility Shift Assays (EMSAs)—Wild type (WT) and mutant (m-1) probes were prepared by annealing 35-bp complementary oligonucleotides labeled with digoxigenin (DIG)-11-ddUTP. DNA-protein binding reactions were performed using in vitro translated SOX9 or nuclear extract with excess unlabeled oligonucleotides prior to adding labeled oligonucleotides. Reaction mixtures were incubated for 20 min at room temperature. DNA-protein complexes were resolved on 6% Tris-boric acid-EDTA gels (Invitrogen), and signal was detected using a chemiluminescent detection system (Roche Applied Science).

RESULTS

CRTL1 Induction by SOX9 in Human Non-chondrocytic immortalized Cell Lines—We examined the effect of SOX9 on CRTL1 expression in the human non-chondrocytic cell line HuH-7. Endogenous CRTL1 expression is undetectable in this cell line.\(^2\) Transient transfection of plasmids expressing green fluorescent protein-tagged SOX genes into HuH-7 cells produced easily detectable levels of SOX protein expression, with peak expression observed 48–72 h following transfection (data not shown). In SOX9-transfected cells, endogenous CRTL1 expression increased gradually beginning at 48 h after transfection and was remarkably elevated from 72 until 96 h after transfection (Fig. 1).

Previous studies of other cartilage matrix genes, including Col2a1, Col11a2, and Agc1, have shown that the transcriptional activity of SOX9 is enhanced by the co-activators L-2 I. Kou and S. Ikegawa, unpublished data.

\(^2\) I. Kou and S. Ikegawa, unpublished data.
SOX5 and SOX6 (23–25). Therefore, we investigated whether L-SOX5 and SOX6 could enhance the effect of SOX9 on CRTL1 expression. In cells co-expressing SOX9, L-SOX5, and SOX6, CRTL1 expression increased sooner, beginning at 24 h after transfection (Fig. 1). However, both L-SOX5 and SOX6 appear to have no direct effect on SOX9. These results suggest that SOX9 can regulate endogenous CRTL1 in addition to other major cartilage matrix genes and that L-SOX5 and SOX6 may enhance the transcriptional activity of SOX9.

CRTL1 Induction by SOX9 in hMSC and hDFB Cells—To confirm SOX9-mediated regulation of CRTL1 expression in other cell lines, we evaluated the effect of SOX9 on CRTL1 expression in hMSC cells. Two days after adenovirus transduction of SOX9, hMSCs were collected into small pellets and cultured in serum-free growth medium. We detected an increase in CRTL1 mRNA expression 3 days after SOX9 transduction, and expression levels remained elevated for 3 weeks following transduction. We observed similar patterns of CRTL1 mRNA expression in cells co-transduced with SOX9, L-SOX5, and SOX6 (Fig. 2A).

In addition, we examined whether CRTL1 could be induced by SOX9 in well differentiated mesenchymal cells such as hDFB. In hDFB cells, CRTL1 expression was first induced at Day 3 of pellet culture, and expression levels remained increased for 3 weeks (Fig. 2B). We further examined CRTL1 induction in hMSC and hDFB cells using a three-dimensional culture system. Interestingly, SOX9 with L-SOX5 and SOX6 could co-activate CRTL1 expression more strongly than SOX9 alone (Fig. 3). These results indicate that SOX9 is a key regulator of CRTL1 and that CRTL1 can be induced by SOX9 in both differentiated and undifferentiated human mesenchymal cells.

Reduction of CRTL1 Expression by RNAi for SOX9—To test whether SOX9 is necessary for CRTL1 expression, we inhibited SOX9 expression using small interfering RNA in a human chondrosarcoma cell line, OUMS-27, which normally expresses high levels of SOX9 and CRTL1 (data not shown). OUMS-27 cells were transfected with an RNAi expression plasmid or a control vector. GAPDH mRNA levels were similar in test cells and mock controls (data not shown). The SOX9 RNAi reduced SOX9 mRNA levels to ~50% of the original level and resulted in inhibition of CRTL1 expression (Fig. 4). Thus, SOX9 is necessary to maintain expression of CRTL1.

Transcriptional Activity of the 5′-Flanking Sequence of CRTL1 in Non-chondrocytic Cells—To examine whether CRTL1 transcription is directly regulated by SOX9, we measured the effect of SOX9 expression on transcriptional activity from the 5′-flanking sequence of the CRTL1 gene. We performed transient transfection assays in several non-chondrocytic cell lines (HuH-7, HeLa, and HEK293) using the construct pCRT1, which contains ~1.2 kb of the 5′-flanking sequence (~924 to +318) of the CRTL1 gene (NM_001884.1). In cells co-transfected with the SOX9 plasmid, transcriptional activity from pCRT1 plasmid was greatly increased relative to control experiments (Fig. 5). This observation suggests that the transcriptional activity of CRTL1 is directly regulated by SOX9 and that a regulatory region resides within the 1.2-kb region. Next, we tested the effect of SOX9 on pCRT2, which contains a 3′-deletion of the flanking sequence (~924 to –3). Transcriptional activity from pCRT2 was considerably lower than that from pCRT1 in cells co-transfected with SOX9, further indicating that a SOX9-responsive element resides within the 3′-segment of the 1.2-kb enhancer fragment (~2 to +318), the 5′-UTR of the CRTL1 gene (Fig. 6). To localize the cis-element within the 5′-UTR, we constructed plasmids containing various 5′-deletions of the ~400-bp enhancer fragment (~69 to +318). A 5′-deletion to +117 retained almost complete enhancer activities, whereas a 5′-deletion to +177 showed remarkably decreased activity (Fig. 6). These experiments localized the minimal enhancer element to the sequence between nucleotides +117 and +177. Sequence analysis of this region identified a motif that is identical to consensus SOX binding sequences, with the exception of a 1-bp mismatch (Fig. 7A, CACAAAG, +120 to +126). Next, we tested the SOX9-dependent enhancer activity of multiple, tandemly arranged copies of the 35-bp enhancer element containing the putative SOX binding motif. In all cell lines co-transfected with the SOX9 plasmid, SOX9-dependent activity increased in correlation with the number of enhancer element repeats (Fig. 7B). Substitution of a 3-bp sequence within the motif abolished enhancer activity, indicating that this sequence is critical to SOX activity (Fig. 7A and C).

Binding of SOX9 to the CRTL1 Enhancer Sequence—We used EMSA to determine whether SOX9 binds the 35-bp 5′-UTR cis-element directly. Incubation of a DIG-labeled WT probe with in vitro translated SOX9 protein resulted in the
SOX9-dependent and -independent Enhancer Elements in CRTL1

Fig. 7. Transcriptional activity of the 35-bp enhancer fragment depends on the consensus SOX-binding site. A, nucleotide sequences of the 35-bp enhancer element (WT) and the mutated element (m-1). Only mutated nucleotides are indicated; unchanged nucleotides are represented by dashes. The putative SOX-binding site is in bold. B, enhancer activities are increased by multimerization of the 35-bp element (shaded box) in three non-chondrocytic cell lines. Luciferase activities are presented as means ± S.D. and are normalized to 1 for the activity obtained with the pGL3 vector. Results are shown for four independent cultures tested in two representative experiments. C, mutation in the SOX-binding site abolishes transcriptional activity of the 35-bp enhancer fragments. Four copies of the wild type (WT) and mutated (m-1) fragments were cloned upstream of the luciferase reporter gene. Transcriptional activities of both fragments were examined by transient transfection in three non-chondrocytic cell lines, HuH-7 (white), HeLa (black), and HEK293 (gray). Luciferase activities are presented as means ± S.D. and are normalized to 1 for the activity obtained with four repeats of the wild type 35-bp element. Results are shown for four independent cultures tested in two representative experiments.

Fig. 8. Binding of SOX9 protein to the CRTL1 enhancer sequence. A, EMSA using the 35-bp enhancer oligonucleotide (probe) and in vitro translated SOX9. A DIG-labeled probe was incubated with SOX9 (lane 1), SOX9 and anti-SOX9 antibody (lane 2), and SOX9 and a 125-fold excess of unlabeled probe (lane 3). Arrow indicates super-shifted SOX9 enhancer complex. B, EMSA using in vitro translated SOX9 with the wild type (WT, lane 1) and mutated (m-1, lane 2) probes. Competition analyses were performed using 125-fold excess of the unlabeled 35-bp WT (lane 3) and m-1 (lane 4) probe as competitor.

We have demonstrated that SOX9 can regulate the transcriptional activity of CRTL1 through a heptamer SOX-binding site within the 5′-UTR. Other major cartilage matrix genes, including Col2a1, Col11a2, and Age1, also are regulated by SOX9 through heptamer SOX binding elements (16, 18, 26).
Thus, SOX9-dependent regulation is considered to be a common mechanism for transcriptional regulation of cartilage matrix genes. Most SOX9 binding elements in the Col2a1, Col11a2, and Agc1 genes are localized to the first intron within each gene. In contrast, the intron 1 sequence of CRTL1 contains no similar regulatory regions and is not highly conserved between species. Comparison of the human and mouse genomic sequences revealed that the SOX9-dependent cis-element within 5'-UTR is highly conserved (more than 90% identity), suggesting that transcriptional regulation of CRTL1 by SOX9 is a common mechanism between species.

SOX9 appears to function as the master regulator of CRTL1. Our RNAi analysis shows that SOX9 is necessary for CRTL1 expression. We also found that SOX9 effectively induces CRTL1 in human cells, even in non-chondrogenic cell lines and in differentiated and undifferentiated mesenchymal cells. However, we observed a delay between the time of SOX9 transduction and induction of CRTL1 mRNA expression. This finding suggests the presence of additional factor(s) that are regulated by SOX9 and mediate SOX9 function. Likely candidates for this role include the SOX9 transcriptional co-activators L-SOX5 and SOX6, which are known to be induced by SOX9 and cooperate with SOX9 to induce COL2A1 expression (11, 27, 28). Our present experiments using HuH-7 cells support this suggestion, as CRTL1 induction in cells transduced with SOX9, L-SOX5, and SOX6 began earlier than that in cells expressing SOX9 alone. By Day 4, however, CRTL1 induction levels were equivalent under both experimental conditions.

This effect of L-SOX5 and SOX6 to SOX9 is not consistent among all cartilage matrix genes, however. For example, SOX9-mediated transcription from the COL9A1 promoter is not stimulated by L-SOX5 and SOX6 (19). In the pellet culture of hMSC and hDFB cells, we observed no additive effect of L-SOX5 and SOX6 to SOX9 activity, whereas SOX9 expression itself was sufficient for CRTL1 induction. In contrast, L-SOX5 and SOX6 have a marked additive effect on SOX9 activity in the three-dimensional culture of hDFB and MSC cells. Our findings indicate that the regulatory mechanism of SOX9 will differ between genes and is likely specific to cell type and/or stages of differentiation.

We have also demonstrated the presence of another sequence element within the 5'-UTR that shows strong, SOX9-independent enhancer activity. In contrast to the SOX9-dependent element, the activity of the element is cell type-specific. Binding experiments using nuclear extracts from chondrocytic and non-chondrocytic cell lines showed a cell type-specific binding pattern with a DNA-protein complex that was specific to the cells where the SOX9-independent element is active. These obser-
vations suggest that the difference in enhancer activity between the cell lines results from the difference in the trans-factor(s) in the cells. In this context, original tissues of OUMS-27 and HEK293 cells are chondrocyte and kidney, respectively, where the CRTL1 expression is strong (27). Therefore, we speculate that one or more factors in addition to SOX9 contribute to transcriptional regulation of CRTL1. The expression profile of CRTL1 and SOX9 during chondrocyte proliferation and differentiation is consistent with this idea; CRTL1 is expressed in hypertrophic chondrocytes, where SOX9 expression is no longer detectable (12, 28–30). In addition, the SOX9 expression is strong (27). There-

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