Original article

Scleraxis and E47 cooperatively regulate the Sox9-dependent transcription

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Abstract

During musculoskeletal development, Sry-type HMG box 9 (Sox9) has a crucial role in mesenchymal condensation and chondrogenesis. On the other hand, a tissue-specific basic helix-loop-helix (bHLH) transcription factor Scleraxis (Scx) regulates the differentiation of tendon and ligament progenitors. Whereas these two transcription factors cooperatively participate in the determination of cellular lineages, the precise interaction between Sox9 and Scx remains unclear. We have previously demonstrated that the Sox9-dependent transcription is synergistically activated by several Sox9-associating molecules, such as p300 and Smad3, on chromatin. In this study, we investigated the function of Scx in the Sox9-dependent transcription. The expression of α1(II) collagen (Col2a1) gene was stimulated by an appropriate transduction of Sox9 and Scx. Scx and its partner E47, which dimerizes with other bHLH proteins, cooperatively enhanced the Sox9-dependent transcription in luciferase reporter assays. Coactivator p300 synergistically increased the activity of Sox9-regulated reporter gene, which contains promoter and enhancer regions of Col2a1, in the presence of Scx and E47. Immunoprecipitation analyses revealed that Scx and E47 formed a transcriptional complex with Sox9 and p300. Scx/E47 heterodimer also associated with a conserved E-box sequence (CAGGTG) in the Col2a1 promoter on chromatin. These findings suggest that Scx and E47 might modulate the primary chondrogenesis by associating with the Sox9-related transcriptional complex, and by binding to the conserved E-box on Col2a1 promoter.

Keywords: E47; p300; Scleraxis; Sox9; Transcription

The abbreviations used are: AD, activation domain; bHLH, basic helix-loop-helix; BMP, bone morphogenetic protein; Col2a1, α1(II) collagen; EMSA, electrophoretic mobility shift assay; FGF, fibroblast growth factor; G3PDH, glyceraldehyde-3-phosphate dehydrogenase; bHLH, basic helix-

loop-helix; IP, immunoprecipitation; Scx, Scleraxis; siRNA, small interfering RNA; Sox9, Sry-type HMG box 9; TGF, transforming growth factor; WB, Western blot.

1. Introduction

A functional musculoskeletal system depends on the coordinated development of bone, cartilage, tendon, and muscle. During somite development, the interactions between myotome and sclerotome have crucial roles in the formation of syndetome, a somitic compartment of Scleraxis (Scx)-expressing tendon progenitors (Brent et al., 2003). Fibroblast growth factor (FGF) signals from muscle progenitors (myotome) induce syndetome. On the other hand, cartilage progenitors (sclerotome) indirectly suppress the Scx expression in tendon progenitors (Brent et al., 2003). In embryonic development, a class II basic helix-loop-helix (bHLH) transcription factor Scx and a Sry-type HMG box 9 (Sox9) coordinately regulate the process of cellular differentiation in mesenchymal progenitors (Asou et al., 2002). However, the precise interaction between these two transcription factors in the determination of different cellular lineages, such as tendon and chondrocyte precursor cells, has not been fully elucidated.

Sox9, which encodes a HMG DNA-binding domain, has been identified as a master transcription factor in chondrogenesis (Kamachi et al., 2000). Heterozygous mutations in Sox9 cause the skeletal malformation syndrome, campomelic dysplasia (Foster et al., 1994; Wagner et al., 1994). Sox9 regulates the expression of its target genes by through the association with the consensus DNA sequences (WWCAAWG) on promoters or enhancers of cartilage-specific genes, such as α1(II) collagen (Col2a1), α1(IX), α2(XI) collagen, aggrecan, cartilage link protein, and Cd-rap (Bell et al., 1997; Bridgewater et al., 1998; Xie et al., 1999; Sekiya et al., 2000; Zhang et al., 2003; Kou and Ikegawa, 2004). We have previously reported that the Sox9-dependent transcription is synergistically activated by cofactor p300, Smad3, and PGC-1α in chondrogenesis (Tsuda et al., 2003; Furumatsu et

al., 2005a; Kawakami et al., 2005). In addition, the Sox9-related transcriptional complex has an important role in the epigenetic regulation during chondrogenesis. Transforming growth factor (TGF)-β receptor-regulated Smad3 and p300, which has an intrinsic histone acetyltransferase activity, cooperatively enhance the Sox9-dependent transcription on chromatin through the acetylation of histones (Furumatsu et al., 2005b, 2009). These findings suggest that Sox9-associating molecules should modulate the Sox9-regulated chondrogenesis on chromatin.

Scx is a distinct marker for tendon and ligament progenitors (Schweitzer et al., 2001). Scx homozygous mutant embryos are unable to form mesoderm and to gastrulate (Brown et al., 1999). Conditional knockout mice of Scx gene show a failure in the condensation of tendon progenitors and a severe disruption of force-transmitting and intermuscular tendons (Murchison et al., 2007). We have previously demonstrated that Scx positively regulates the expression of tenomodulin, a marker of tendon formation (Shukunami et al., 2006). Several authors have also indicated that Scx is a key transcription factor to regulate the transcription of aggrecan, α1(I), and α2(I) collagen genes (Liu et al., 1997; Terraz et al., 2002; Lejard et al., 2007; Espira et al., 2009). The tissue-specific bHLH transcription factor Scx interacts with cis-acting elements (CANNTG), called E-boxes, in the promoter regions of target genes as a Scx/E47 heterodimer (Liu et al., 1997; Carlberg et al., 2000; Terraz et al., 2002; Lejard et al., 2007; Espira et al., 2009). E47 is an alternative splice variant of the E2A gene, a class I ubiquitous bHLH protein. E47 dimerizes with a class II bHLH protein, such as the myogenic bHLH MyoD, by through each bHLH domain (Lu and Sloan, 2002). Class I/II bHLH heterodimers strongly activate the transcription of their target genes by binding to specific E-box sequences, rather than bHLH homodimers (Massari and Murre, 2000). In our previous study, we described that the recruitment of p300 may have a critical role for the synergistic activation of insulin promoter by the BETA2/E47 heterodimer (Amemiya-Kudo et al., 2005). Several reports have shown that the associations between E2A and p300 coactivators are important in many cellular responses,

such as proliferation, differentiation, and transcriptional activation (Qiu et al., 1998; Bradney et al., 2003; Bayly et al., 2004). However, the crosstalk among Scx, E47, and p300 is still unclear.

Here, we hypothesized that Scx and E47 might modulate the Sox9-dependent transcription through the association with Sox9-associating cofactor p300. In the present study, we investigated the interaction among Sox9, Scx, E47, and p300. Our study demonstrates that Scx and E47 cooperatively activate the Sox9-dependent Col2a1 expression by associating with the Sox9-p300 transcriptional complex, and by binding to a conserved E-box sequence in the Col2a1 promoter on chromatin.

2. Materials and methods

2.1. Cells, plasmids, and antibodies

A human chondrosarcoma cell line (SW1353) was used as an immature chondrogenic cell line. Plasmids encoding full-length (1-507 amino acids) and partial fragments (1-327, 1-423, 182-507 amino acids) of rat Sox9 were used (Furumatsu et al., 2005a). Expression plasmids of mouse Scx and rat E47 (full length, dAD1/2, dHLH) have been described previously (Amemiya-Kudo et al., 2005; Shukunami et al., 2006). p300 was a gift from Tso-Pang Yao. pGL3-585, which contains a mouse Col2a1 promoter extending from -585 to +73 bp of the transcription start site, was constructed with a pGL3-Basic vector (Promega). The site-directed mutation (-caggtg- to -caggAA-, in capital letters) on a conserved E-box (from -577 to -572 bp) was inserted (pGL3-585M). pGL3-569, which lacks the conserved E-box, was also constructed. The reporter plasmid containing Sox9-binding enhancer, pGL3-585E, was used as described (Furumatsu et al., 2009). The following antibodies were used: E2A Yae (Santa Cruz), β-actin, HA, HA affinity gel, FLAG M2, and FLAG M2 affinity gel (Sigma).

2.2. Transfection, small interfering RNA (siRNA), RT-PCR, and quantitative real-time PCR

Plasmids were transiently transfected using FuGENE6 (Roche) according to the manufacturer's protocol. Transfected cells were cultured for 24 h. A common siRNA against human/mouse Scx (si-Scx, 5'- CGCAC CAACA GCGUG AACAT T -3' and 5'- UGUUC ACGCU GUUGG UGCGT T -3') and Silencer select negative control siRNA (Ambion) were occasionally transfected using Oligofectamine (Invitrogen) after plasmid transfections. siRNA-transfected cells were harvested for 48 h. Total cellular RNAs were extracted using QuickGene SP kits (Fujifilm). Reverse transcribed cDNAs were subjected to PCR amplification (Date et al., 2009). Quantitative real-time PCR analyses were also performed using FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's protocol. The primer sets for human SOX9, rat Sox9, human SCX, mouse Scx, human E47, rat E47, human COL2A1, and human glyceraldehyde-3-phosphate dehydrogenase (G3PDH) are shown in Table 1. PCR reactions were allowed to proceed for 27-33 cycles. These assays were run in triplicate and relative mRNA expressions were calculated as described (Tetsunaga et al., 2009).

2.3. Luciferase reporter assay

To assess the Sox9-dependent transcriptional activity, Gal4-TK Luc reporter and Gal4-Sox9 constructs were used (Furumatsu et al., 2005a). pGL3-585, 585M, 569, and 585E were used as native Col2a1 reporter genes. Appropriate plasmids (50 ng) and/or si-Scx were transiently transfected into SW1353 cells using FuGENE6. pRL-CMV (10 ng, Promega) was used as an internal control. The cells were harvested for 24 hr, and then the luciferase activities were analyzed using Dual-Luciferase Reporter Assay System (Promega). The assays were performed in triplicate.

2.4. Immunoprecipitation (IP), nuclear extraction, and Western blot (WB)

Transiently transfected SW1353 cells (500 ng of each plasmid/3.5-cm dish) were harvested for 24 h. Cell extracts were prepared with IP buffer 0 (Furumatsu et al., 2005a). Ten percent volume of supernatant was loaded as an input fraction. Forty-five percent volume of extract was incubated with FLAG M2 or HA affinity gel for 1 hr at 4°C. The remaining supernatant was incubated with mouse

IgG and protein G beads (Sigma) as a control. Cytoplasmic fractions and nuclear extracts of SW1353 cells were prepared in buffer A and B, respectively, as described (Furumatsu et al., 2005a). Protein concentrations were measured by BCA protein assay kit (Bio-Rad). Western blot analyses were performed as described (Tsuda et al., 2003).

2.5. Electrophoretic mobility shift assay (EMSA)

The conserved E-box (in capital letters)-containing probe, -577 E-box probe, was generated by annealing the following oligonucleotides: 5'-cgaga ctcCA GGTGg gagct cac-3' and 5'-gtgag ctccC ACCTG gagtc tcg-3'. Probes were end-labeled using Biotin 3' end DNA labeling kit (Pierce). Binding reactions and detections were performed with 5 μg of SW1353 nuclear extracts and 20 fmol of end-labeled probe using LightShift chemiluminescent EMSA kit (Pierce) according to instructions. The unlabeled probe (400 fmol) was used as a competitor.

2.6. Chromatin immunoprecipitation

Chromatin IP assays were performed as described previously (Furumatsu et al., 2005a). In brief, SW1353 cells were transfected with indicated plasmids (2 μg/10-cm dish). Cell lysates were suspended in nuclear lysis buffer. Ten percent volume of each supernatant was stocked as an input sample. Half of each remaining sample was incubated with indicated antibodies. Immunoprecipitated DNAs were purified, and then PCR reactions were allowed to proceed for 27 cycles. The following primers were used to amplify the conserved E-box in COL2A1 promoter: 5'-CAGCT CTTTT CCTGG CTGTC-3' and 5'-CCCCG ACCTA TCTTT TAGCC-3'.

2.7. Statistical analysis

All experiments were repeated at least three times and similar results were obtained. Data were expressed as means. Differences among groups were compared by using the Mann-Whitney U-test. Statistical significance was established at $p < 0.05$.

3. Results

3.1. Sox9, Scx, and E47 cooperatively stimulate the expression of COL2A1 gene.

We firstly assessed the expression pattern of COL2A1 gene, a chondrogenic marker, under different balances between Sox9 and Scx. An individual transfection of Sox9 or Scx did not stimulate COL2A1 expression in quantitative real-time PCR analyses. However, a simultaneous transfection of Sox9 and Scx increased COL2A1 expression up to a 2.9-fold level of control in an immature chondrogenic cell line, SW1353 cells (Fig. 1, A and C). The increase of COL2A1 expression was enhanced up to a 5.5-fold level of control by the cotransfection of E47 (Fig. 1, B and D). In addition, the interfering analyses using si-Scx revealed that excessively high and low transduction of Scx did not increase the expression of endogenous SOX9 and COL2A1 (Fig. 2). COL2A1 expression was increased up to a 2.1-fold level of control in an appropriate balance between endogenous SOX9 (higher than Scx) and overexpressed Scx (lower than SOX9, 40 pmol of si-Scx). Endogenous gene expressions of SOX9, SCX, and E47 in human chondrocytic cells, such as SW1353 cells, a differentiated chondrosarcoma cell line OUMS-27 (Furumatsu et al., 2002; Kunisada et al., 1998), and human chondrocytes, were also shown in Supplemental Fig. 1.

3.2. Scx and E47 regulate the Sox9-dependent transcription.

To elucidate the transcriptional cross-talk among Sox9, Scx, and E47, we investigated the Sox9-dependent transcription using Gal4-TK luciferase reporter assays. Scx and E47 did not show any effects in the absence of Gal4-Sox9 (Fig. 3A, Gal4). However, a simultaneous transfection of Scx and E47 increased a relative luciferase activity up to a 7.3-fold level of Gal4 control in the presence of Gal4-Sox9 encoding the full length of Sox9 (Fig. 3A, Gal4-Sox9 1-507). We also examined the effects of coactivator p300 in this system. The activity of mutant Gal4-Sox9 1-327, which lacks the binding region to p300, was not influenced by the addition of Scx, E47, and p300 (Fig. 3B). On the

other hand, Scx, E47, and p300 synergistically enhanced the transcriptional activity of Gal4-Sox9 1-507 to a level as high as 29-fold over the Gal4 control (Fig. 3B). This synergistic effect was not observed by E47 deletion mutants. E47 dAD1/2, lacking the binding region to the KIX domain of p300, did not induce the additional enhancement of Scx and p300 (Fig. 3C, E47 dAD1/2). Deletion mutant lacking bHLH domain of E47 also showed no synergistic effect in the presence of Scx and p300 (Fig. 3C, E47 dHLH). These findings suggest that Scx and E47 might regulate the Sox9-dependent transcription through the association with Sox9 and p300.

3.3. E47 associates with Sox9, Scx, and p300.

E47 directly interacts with CREB-binding protein (a close paralog of p300) in hematopoietic cells (Bayly et al., 2004; Bradney et al., 2003). E47 also forms a heterodimer with Scx in tendon fibroblasts (Carlberg et al., 2000; Lejard et al., 2007). In our previous studies, Sox9 directly associates with p300 (Furumatsu et al., 2009; Tsuda et al., 2003). Here, we investigated the protein interactions among Sox9, p300, E47, and Scx to understand the cooperative effects of Scx and E47 in the Sox9-dependent transcription. Coactivator p300 associated with Sox9 and E47 in SW1353 cells (Fig. 4A). We also observed that E47 had an ability to interact with Sox9 in the presence or absence of p300 (Fig. 4B). IP analyses using truncated constructs revealed that the P/Q rich domain of Sox9 (encoding 328-423 amino acids) was necessary for the association between Sox9 and E47 (Fig. 4C). The complex formation between Scx and E47 predictably depended on the bHLH domain of E47 (Fig. 4D). Whereas the direct interaction between Sox9 and Scx was not detected, Scx was partly coimmunoprecipitated with Sox9 in the presence of transfected E47 (Fig. 4E).

3.4. Scx and E47 modulate the Sox9-dependent transcription by a putative E-box on Col2a1 promoter.

We searched putative E-box sequences on Col2a1 promoters in several species. Conserved putative E-boxes were found at approximately 580-bp and 150-bp upstream from the transcription start site of Col2a1 genes (Fig. 5A). To investigate the function of Scx and E47 on Col2a1 promoter, we

constructed the indicated reporter plasmids (Fig. 5B). Scx and E47 cooperatively stimulated the relative luciferase activity of pGL3-585 up to a 2-fold level of control (Fig. 5C). However, the cooperative effect of Scx and E47 was not observed in pGL3-585M and pGL3-569 (Fig. 5C). Scx, E47, and p300 synergistically increased the transcriptional activity of pGL3-585E, which encodes the Sox9-binding enhancer, up to an 11.3-fold level of control in the presence of Sox9 1-507 (Fig. 5D). These findings prompted us to investigate the interaction between Scx, E47, and the conserved E-box sequence on Col2a1 promoter (-577 to -572 bp).

3.5. Scx-E47 transcriptional complex associates with the -577 E-box on Col2a1 promoter.

Overexpressed Scx was abundantly detected in the nuclear fractions of SW1353 cells (Fig. 6A). Protein-DNA complexes were not detected by a single transfection of Scx or E47 in EMSA (Fig. 6B, Scx, E47). On the other hand, the -577 E-box containing probes formed a complex with the nuclear extract purified from Scx and E47-cotransfected cells (Fig. 6B, Scx + E47). Unlabeled competitors decreased the bindings between proteins and biotin end-labeled probes (Fig. 6B, Scx + E47, Compete). Chromatin IP analyses revealed that Scx associated with the conserved E-box region of COL2A1 promoter on chromatin in the presence of E47 (Fig. 6C). Our results indicated that Scx-E47 transcriptional complex might have a crucial role to regulate the Sox9-dependent transcription through the interaction with Sox9, p300, and the E-box sequence on Col2a1 promoter.

4. Discussion

The present study indicated that Scx, E47, and p300 cooperatively activated the Sox9-dependent transcription. Scx-E47 complex might have dual effects to modulate the Sox9-dependent Col2a1 expression: (i) by associating with Sox9 and p300, (ii) and by recognizing the conserved E-box sequence on Col2a1 promoter (Fig. 7). We revealed that E47 mediated the formation of Sox9-related transcriptional complex through the direct association with Scx, p300, and Sox9 (Fig. 4). In addition,

p300 associated with E47 and Sox9 (Fig. 4A) as described previously (Bayly et al., 2004; Tsuda et al., 2003). From these results, the Sox9-dependent Col2a1 expression might be affected by the balance among Sox9-p300, Scx-E47-p300, and Sox9-Scx-E47-p300 complexes. Coactivator p300 acts as a bridging factor for connecting DNA-binding transcription factors to the transcriptional apparatus, and as a protein scaffold to form multicomponent transcriptional complexes (Utley et al., 1998; Chan et al., 2001). In addition, we have reported that the histone acetyltransferase activity of p300 facilitates the Sox9-dependent transcription on chromatin by histone modification (Tsuda et al., 2003; Furumatsu et al., 2005b, 2009). These findings suggest that the Col2a1 expression might be epigenetically regulated by Sox9-p300 and/or Sox9-Scx-E47-p300 transcriptional complexes on chromatin.

The discrepancy between SOX9 and COL2A1 expression was observed in the presence of si-Scx (Fig. 2). Overexpressed Sox9 actually increases the transcriptional activity of Col2a1 reporter gene in luciferase assays as described previously. However, the Col2a1 mRNA expression does not seem to be sufficiently induced by a single transduction of Sox9 (Li et al., 2004; Hardingham et al., 2006). The combination of Sox genes, coactivators, and intracellular signals are necessary for chondrogenesis including Col2a1 expression in the presence of Sox9 (Ikeda et al., 2004; Furumatsu et al., 2005a, 2005b, 2009; Suzuki et al., 2006). In addition, cellular lineages and differentiation stages influence the Sox9-dependent transcription (Kypriotou et al., 2003; Hardingham et al., 2006). In our study, excessively high and low transduction of Scx did not increase the expression of endogenous SOX9 and COL2A1 (Fig. 2). We consider that the Col2a1 expression is enhanced by an optimum balance between Sox9 and Scx in early chondrocytic SW1353 cells. An appropriate amount of Scx, lower than that of Sox9, might stimulate Col2a1 expression by modulating the Sox9 expression itself in an early chondrogenesis.

Several transcription partners such as Sox5/6 (Ikeda et al., 2004; Lefebvre et al., 2001), Barx2 (Meech et al., 2005), β-catenin (Akiyama et al., 2004; Bernard et al., 2008), c-Maf (Huang et al., 2002), PIAS

(Hattori et al., 2006), and TRAP230 (Zhou et al., 2002) can modify the Sox9-dependent transcription in chondrogenesis and sex determination. Sox9 is necessary for mesenchymal condensations, and considered to be a key transcription factor for the following expression of Sox5/6 genes (Akiyama et al., 2002). However, Sox9-/- mouse embryonic stem cells successfully form early condensations expressing chondrogenic marker genes such as Sox5/6 and Scx (Hargus et al., 2008). On the other hand, the single gene transduction of Sox9 fails to induce a sufficient chondrogenesis (Ikeda et al., 2004). These reports suggest that the Sox9-regulated coordinated chondrogenesis largely depends on cooperative actions of Sox9-associating molecules. Brent et al. (2005) has described that Sox9-induced Sox5/6 inhibit the expression of Scx to block the differentiation towards tendon and ligament cell lineages. In our study, Sox9 transduction did not inhibit the effect of Scx in luciferase reporter assays (Fig. 3 and 5D). We also demonstrated that a simultaneous transfection of Sox9 and Scx enhanced the COL2A1 expression and the Col2a1 reporter activity, especially in the presence of E47 and p300 (Fig. 1, 3, and 5). Several authors have indicated that common mesenchymal precursor cells expressing both Sox9 and Scx are involved in the coordinated development of cartilages, tendons, and ligaments (Asou et al., 2002; Cserjesi et al., 1995). In the limb buds, diversification of cartilage and tendon cell lineages is regulated by antagonism between bone morphogenetic protein (BMP) and FGF signaling pathways (Brent and Tabin, 2002). BMP-2 not only promotes chondrogenesis, but also inhibits tendon development, while FGF-4 has the opposite effect (Edom-Vovard et al., 2002). The BMP inhibitor Noggin represses the expression of Sox9 while inducing the Scx expression (Schweitzer et al., 2001). In valvulogenesis, similar antagonistic relationships between BMP and FGF are also observed (Lincoln et al., 2006a, 2006b). In addition, increased or decreased levels of phosphorylated ERK, a MAPK signal transduction cascade of FGF, result in the loss of Scx transcripts and rib development (Smith et al., 2005). These findings suggest that the balance between Sox9 and Scx should play an important role to determine the lineages of mesenchymal and valve progenitor cells. In the early phase of embryonic development, the expressions of Scx and Sox9 are overlapped in sclerotome. However, Scx is reduced in the center of Sox9-expresssed sclerotome

during the following development (Asou et al., 2002). On the other hand, Scx expression is transiently increased in the early stage of embryonic stem cell-derived chondrogenesis (zur Nieden et al., 2005). In $Scx^{-/-}$ mutant mice, the deltoid tuberosities that act as the tendon-to-bone interfaces are not observed (Murchison et al., 2007). We consider that Scx and Sox9 could be involved in an early switch for the initiation of chondrogenic differentiation. The interaction between Scx and Sox9 might be not only synergistic in the early chondrogenesis but also antagonistic in the following development toward distinct cellular lineages. Further investigations will be required to understand the precise mechanism of chondrogenesis regulated by the interaction between Scx and Sox9.

In conclusion, the present study demonstrates that Scx/E47 modulates the Sox9-dependent Col2a1 expression by associating with Sox9-p300 transcriptional complex, and by binding to the conserved E-box on Col2a1 promoter. Our results suggest that the chondrogenic differentiation is delicately regulated by the balance between Sox9 and Scx.

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References

Akiyama, H., Chaboissier, M. C., Martin, J. F., Schedl, A., de Crombrugghe, B. The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 2002;16:2813-28.

Akiyama, H., Lyons, J. P., Mori-Akiyama, Y., Yang, X., Zhang, R., Zhang, Z., et al. Interactions between Sox9 and β-catenin control chondrocyte differentiation. Genes Dev 2004;18:1072-87.

Amemiya-Kudo M, Oka J, Ide T, Matsuzaka T, Sono H, Yoshikawa T, et al. Sterol regulatory element-binding proteins activate insulin gene promoter directly and indirectly through synergy with BETA2/E47. J Biol Chem 2005;280:34577-89.

Asou Y, Nifuji A, Tsuji K, Shinoyama K, Olson EN, Koopman P, et al. Coordinated expression of Scleraxis and Sox9 genes during embryonic development of tendons and cartilage. J Orthop Res 2002;20:827-33.

Bayly R, Chuen L, Currie RA, Hyndman BD, Casselman R, Blobel GA, et al. E2A-PBX1 interacts directly with the KIX domain of CBP/p300 in the induction of proliferation in primary hematopoietic cells. J Biol Chem 2004;279:55362-71.

Bell DM, Leung KK, Wheatley SC, Ng LJ, Zhou S, Ling KW, et al. SOX9 directly regulates the type-II collagen gene. Nat Genet 1997;16:174-8.

Bernard P, Tang P, Liu S, Dewing P, Harley V, Vilain E. Dimerization of SOX9 is required for chondrogenesis, but not for sex determination. Hum Mol Genet 2003;12:1755-65.

Bernard P, Sim H, Knower K, Vilain E, Harley V. Human SRY inhibits β-catenin-mediated transcription. Int J Biochem Cell Biol 2008;40:2889-900.

Bradney C, Hjelmeland M, Komatsu Y, Yoshida M, Yao TP, Zhuang Y. Regulation of E2A activities by histone acetyltransferases in B lymphocyte development. J Biol Chem 2003;278:2370-6.

Brent AE, Tabin CJ. Developmental regulation of somite derivatives: muscle, cartilage and tendon. Curr Opin Genet Dev 2002;12:548-57.

Brent AE, Schweitzer R, Tabin CJ. A Somitic Compartment of Tendon Progenitors. Cell 2003;113:235-48.

Brent AE, Braun T, Tabin CJ. Genetic analysis of interactions between the somitic muscle, cartilage and tendon cell lineages during mouse development. Development 2005;132:515-28.

Bridgewater LC, Lefebvre V, de Crombrugghe B. Chondrocyte-specific enhancer elements in the Col11a2 gene resemble the Col2a1 tissue-specific enhancer. J Biol Chem 1998;273:14998-5006.

Brown D, Wagner D, Li X, Richardson JA, Olson EN. Dual role of the basic helix-loop-helix transcription factor Scleraxis in mesoderm formation and chondrogenesis during mouse embryogenesis. Development 1999;126:4317-29.

Carlberg AL, Tuan RS, Hall DJ. Regulation of scleraxis function by interaction with the bHLH protein E47. Mol Cell Biol Res Commun 2000;3:82-6.

Chan HM, La Thangue NB. p300/CBP proteins: HATs for transcriptional bridges and scaffolds. J Cell Sci 2001;114:2363-73.

Cserjesi P, Brown D, Ligon KL, Lyons GE, Copeland NG, Gilbert DJ, et al. Scleraxis: a basic helix-loop-helix protein that prefigures skeletal formation during mouse embryogenesis. Development 1995;121:1099-110.

Date H, Furumatsu T, Sakoma Y, Yoshida A, Hayashi Y, Abe N, et al. GDF-5/7 and bFGF activate integrin α2-mediated cellular migration in rabbit ligament fibroblasts. J Orthop Res 2009;Epub.

Edom-Vovard F, SchulerB, Bonnin MA, Teillet MA, Duprez D. Fgf4 positively regulates Scleraxis and tenascin expression in chick limb tendons. Dev Biol 2002;247:351-66.

Espira L, Lamoureux L, Jones SC, Gerard RD, Dixon IM, Czubryt MP. The basic helix-loop-helix transcription factor scleraxis regulates fibroblast collagen synthesis. J Mol Cell Cardiol 2009;47:188-95.

Foster JW, Dominguez-Steglich MA, Guioli S, Kwok C, Weller PA, Stevanovic M, et al. Campomelic dysplasia and autosomal sex reversal caused by mutations in an SRY-related gene. Nature 1994;372:525-30.

Furumatsu T, Nishida K, Kawai A, Namba M, Inoue H, Ninomiya Y. Human chondrosarcoma secretes vascular endothelial growth factor to induce tumor angiogenesis and stores basic fibroblast growth factor for regulation of its own growth. Int J Cancer 2002;97:313-22.

Furumatsu T, Tsuda M, Taniguchi N, Tajima Y, Asahara H. Smad3 induces chondrogenesis through the activation of SOX9 via CREB-binding protein/p300 recruitment. J Biol Chem 2005a;280:8843-50.

Furumatsu T, Tsuda M, Yoshida K, Taniguchi N, Ito T, Hashimoto M, et al. Sox9 and p300 cooperatively regulate chromatin-mediated transcription. J Biol Chem 2005b;280:35203-8.

Furumatsu T, Ozaki T, Asahara H. Smad3 activates the Sox9-dependent transcription on chromatin. Int J Biochem Cell Biol 2009;41:1198-204.

Hardingham TE, Oldershaw RA, Tew SR. Cartilage, SOX9 and Notch signals in chondrogenesis. J Anat 2006;209:469-80.

Hargus G, Kist R, Kramer J, Gerstel D, Neitz A, Scherer G, et al. Loss of Sox9 function results in defective chondrocyte differentiation of mouse embryonic stem cells in vitro. Int J Dev Biol 2008;52:323-32.

Hattori T, Eberspaecher H, Lu J, Zhang R, Nishida T, Kahyo T, et al. Interactions between PIAS proteins and SOX9 result in an increase in the cellular concentrations of SOX9. J Biol Chem 2006;281:14417-28.

Huang W, Lu N, Eberspaecher H, de Crombrugghe B. A new long form of c-Maf cooperates with Sox9 to activate the type II collagen gene. J Biol Chem 2002;277:50668-75.

Ikeda, T., Kamekura, S., Mabuchi, A., Kou, I., Seki, S., Takato, T., et al. The combination of SOX5, SOX6, and SOX9 (the SOX trio) provides signals sufficient for induction of permanent cartilage. Arthritis Rheum 2004;50:3561-73.

Kamachi Y, Uchikawa M, Kondoh H. Pairing SOX off: with partners in the regulation of embryonic development. Trends Genet 2000;16:182-7.

Kawakami Y, Tsuda M, Takahashi S, Taniguchi N, Esteban CR, Zemmyo M, et al. Transcriptional coactivator PGC-1α regulates chondrogenesis via association with Sox9. Proc Natl Acad Sci USA 2005;102:2414-19.

Kou I, Ikegawa S. SOX9-dependent and -independent transcriptional regulation of human cartilage link protein. J Biol Chem 2004;279:50942-48.

Kypriotou M, Fossard-Demoor M, Chadjichristos C, Ghayor C, de Crombrugghe B, Pujol JP, et al. SOX9 exerts a bifunctional effect on type II collagen gene (COL2A1) expression in chondrocytes depending on the differentiation state. DNA Cell Biol 2003;22:119-29.

Kunisada T, Miyazaki M, Mihara K, Gao C, Kawai A, Inoue H, et al. A new human chondrosarcoma cell line (OUMS-27) that maintains chondrocytic differentiation. Int J Cancer 1998;77:854-9.

Lefebvre, V., Behringer, R. R., & de Crombrugghe, B. L-Sox5, Sox6 and Sox9 control essential steps of the chondrocyte differentiation pathway. Osteoarthritis Cartilage 2001;9:S69-75.

Lejard V, Brideau G, Blais F, Salingcarnboriboon R, Wagner G, Roehrl MH, et al. Scleraxis and NFATc regulate the expression of the pro-α1(I) collagen gene in tendon fibroblasts. J Biol Chem 2007;282:17665-75.

Li Y, Tew SR, Russell AM, Gonzalez K, Hardingham TE, Hawkins RE. Transduction of passaged human articular chondrocytes with adenoviral, retroviral, and lentiviral vectors and the effects of enhanced expression of SOX9. Tissue Eng 2004;10:575-84.

Lincoln J, Alfieri CM, Yutzey KE. BMP and FGF regulatory pathways control cell lineage diversification of heart valve precursor cells. Dev Biol 2006a;292:290-302.

Lincoln J, Lange AW, Yutzey KE. Hearts and bones: Shared regulatory mechanisms in heart valve, cartilage, tendon, and bone development. Dev Biol 2006b;294:292-302.

Liu Y, Watanabe H, Nifuji A, Yamada Y, Olson EN, Noda M. Overexpression of a single helix-loop-helix-type transcription factor, Scleraxis, enhances aggrecan gene expression in osteoblastic osteosarcoma ROS17/2.8 cells. J Biol Chem 1997;272:29880-5.

Lu J, Sloan SR. The basic helix-loop-helix domain of the E47 transcription factor requires other protein regions for full DNA binding activity. Biochem Biophys Res Commun 2002;290:1521-8.

Massari ME, Murre C. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. Mol Cell Biol 2000;20:429-40.

Meech, R., Edelman, D. B., Jones, F. S., Makarenkova, H. P. The homeobox transcription factor Barx2 regulates chondrogenesis during limb development. Development 2005;132:2135-46.

Murchison ND, Price BA, Conner DA, Keene DR, Olson EN, Tabin CJ, et al. Regulation of tendon differentiation by Scleraxis distinguishes force-transmitting tendons from muscle-anchoring tendons. Development 2007;134:2697-708.

Qiu Y, Sharma A, Stein R. p300 mediates transcriptional stimulation by the basic helix-loop-helix activators of the insulin gene. Mol Cell Biol 1998;18:2957-64.

Schweitzer R, Chyung JH, Murtaugh LC, Brent AE, Rosen V, Olson EN, et al. Analysis of the tendon cell fate using Scleraxis, a specific marker for tendons and ligaments. Development 2001;128:3855-66.

Sekiya I, Tsuji K, Koopman P, Watanabe H, Yamada Y, Shinomiya K, et al. SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line, TC6. J Biol Chem 2000;275:10738-44.

Shukunami C, Takimoto A, Oro M, Hiraki Y. Scleraxis positively regulates the expression of tenomodulin, a differentiation marker of tenocytes. Dev Biol 2006;298;234-47.

Smith TG, Sweetman D, Patterson M, Keyse SM, Munsterberg A. Feedback interactions between MKP3 and ERK MAP kinase control scleraxis expression and the specification of rib progenitors in the developing chick somite. Development 2005;132:1305-14.

Suzuki T, Sakai D, Osumi N, Wada H, Wakamatsu Y. Sox genes regulate type 2 collagen expression in avian neural crest cells. Develop, Growth Differ 2006;48:477-86.

Terraz C, Brideau G, Ronco P, Rossert J. A combination of cis-acting elements is required to activate the pro-α1(I) collagen promoter in tendon fibroblasts of transgenic mice. J Biol Chem 2002;277:19019-26.

Tetsunaga T, Furumatsu T, Abe N, Nishida K, Naruse K, Ozaki T. Mechanical stretch stimulates integrin αVβ3-mediated collagen expression in human anterior cruciate ligament cells. J Biomech 2009;42:2097-103.

Tsuda M, Takahashi S, Takahashi Y, Asahara H. Transcriptional co-activators CREB-binding protein and p300 regulate chondrocyte-specific gene expression via association with Sox9. J Biol Chem 2003;278:27224-9.

Utley RT, Ikeda K, Grant PA, Cote J, Steger DJ, et al. Eberharter A,Transcriptional activators direct histone acetyltransferase complexes to nucleosomes. Nature 1998;394:498-502.

Wagner T, Wirth J, Meyer J, Zabel B, Held M, Zimmer J, et al. Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene SOX9. Cell 1994;79:1111-20.

Xie WF, Zhang X, Sakano S, Lefebvre V, Sandell LJ. Trans-activation of the mouse cartilage-derived retinoic acid-sensitive protein gene by Sox9. J Bone Miner Res 1999;14:757-63.

Zhang P, Jimenez SA, Stokes DG. Regulation of human COL9A1 gene expression. Activation of the proximal promoter region by SOX9. J Biol Chem 2003;278:117-23.

Zhou, R., Bonneaud, N., Yuan, C. X., de Santa Barbara, P., Boizet, B., Schomber, T., et al. SOX9 interacts with a component of the human thyroid hormone receptor-associated protein complex. Nucleic Acids Res 2002;30:3245-52.

zur Nieden NI, Kempka G, Rancourt DE, Ahr HJ. Induction of chondro-, osteo- and adipogenesis in embryonic stem cells by bone morphogenetic protein-2: effect of cofactors on differentiating lineages. BMC Dev Biol 2005;5:1.

Figure legends

Fig. 1. Sox9, Scx, and E47 cooperatively activate the COL2A1 expression.

(A) Different balances of rat Sox9 and mouse Scx transductions were indicated as triangular boxes (0, 250, and 500 ng/3.5-cm dish). Transient transfection using both Sox9 and Scx (250 ng each) increased the COL2A1 expression in SW1353 cells.

(B) Rat E47 (250 ng) were cotransfected with different volumes of Sox9 and Scx. The COL2A1 expression was stimulated by cotransfection of Sox9, Scx, and rE47. Endogenous gene expressions (human SOX9, SCX, and E47) were also included using universal primer sets (Table 1) in SW1353 cells.

(C and D) Relative COL2A1 expressions were determined by quantitative real-time PCR analyses as described in Materials and methods. Cotransfection of Sox9 and Scx increased the COL2A1 expression to a 2.9-fold level of untransfected cells in the absence of rE47 transduction (C). Sox9, Scx, and rE47 cooperatively enhanced the COL2A1 expression up to a 5.5-fold level of control (D). * Statistical significances ($p < 0.05$) were observed in the Sox9/Scx-cotransfected cells using Mann-Whitney U test.

Fig. 2. The expression balance between SOX9 and Scx is important for the COL2A1 transactivation.

Mouse Scx (500 ng) and/or si-Scx (0, 20, 40, and 80 pmol/3.5-cm dish, indicated as triangular boxes) were transfected in SW1353 cells. Scx transduction was inhibited by si-Scx in a dose-dependent manner. Quantitative real-time PCR analyses demonstrated that approximately 20, 30, and 70% of interferences in mScx and SCX expressions were observed by 20, 40, and 80 pmol of si-Scx, respectively (C). On the other hand, the expression of endogenous SOX9 was stimulated to a 6.2-fold level of control by 40 pmol of si-Scx (A and B). Relative COL2A1 expression was increased up to a

2.1-fold level of control in an appropriate balance between SOX9 and Scx (40 pmol of si-Scx, A and D). * Statistical significances ($p < 0.05$) compared with each 0 pmol control.

Fig. 3. Scx, E47, and p300 synergistically enhance the Sox9-dependent transcription.

(A) Scx and E47 did not increase the luciferase activity of Gal4-TK Luc in the absence of Gal4-Sox9. The Gal4-TK Luc activity was stimulated up to a 3.2-fold level of control by Gal4-Sox9 1-507. No additional increase was observed by the single transfection of Scx or E47. However, a simultaneous transfection of Scx and E47 enhanced the Gal4-Sox9-dependent transcription to a 2.3-fold level of control.

(B) Scx, E47, and p300 did not influence the activity of Gal4-Sox9 1-327 lacking the C-terminal transactivation domain. In the presence of Gal4-Sox9 1-507 (full length), Scx, E47, and p300 synergistically stimulated the Sox9-dependent transcription to a 29-fold level of Gal4 control.

(C) Activation domain (AD) 1/2 and bHLH domain of E47 were necessary for the synergistic increase of Sox9-dependent transcription in the presence of Scx and p300. No significant differences were observed between two hatched bars. * Statistical significances ($p < 0.05$) between the indicated bars.

Fig. 4. E47 contributes to the indirect association between Scx and Sox9.

(A) p300 associated with E47 and Sox9 in SW1353 cells.

(B) E47 directly interacted with Sox9 even in the absence of p300.

(C) FLAG-tagged Sox9 corresponding to 1-327 amino acids (Sox9 1-327) was not coimmunoprecipitated with E47. However, Sox9 1-423 and 182-507 directly associated with E47.

(D) E47 formed a complex with Scx by its bHLH domain.

(E) Cotransfection of E47 triggered the indirect association between Sox9 and Scx.

* denotes a nonspecific band derived from IgG.

Fig. 5. Sox9, Scx, E47, and p300 cooperatively enhance the activity of native Col2a1 reporter genes.

(A) Putative E-box sequences on Col2a1 promoters are shown as letters. Numbers indicate the distance from the transcription start site of each Col2a1 gene (National Center for Biotechnology Information: human, AC004801; rat, M10613; mouse, M65161).

(B) Schematic illustrations of mouse Col2a1 reporter constructs. The site-directed mutant (capital letters) was used as pGL3-585M. The native Col2a1 enhancer, which contains the Sox9-binding sequence, was inserted into pGL3-585 as pGL3-585E (Furumatsu et al., 2009).

(C) A simultaneous transfection of Scx and E47 increased the relative luciferase activity of pGL3-585 up to a 2-fold level of untransfected control. However, Scx and E47 did not affect the activity of pGL3-585M and pGL3-569, which was inactivated or lacked the E-box sequence (-577 to -572 bp) of mouse Col2a1 promoter.

(D) Scx and E47 cooperatively stimulated the activity of pGL3-585E to approximately 1.8-fold levels of each control. However, the synergistic effect of p300 was not observed in Sox9 1-327-transfected cells. Scx, E47, and p300 synergistically increased the activity of pGL3-585E up to a 4.3-fold level of control in the presence of Sox9 1-507. * Statistical significances ($p < 0.05$) between the indicated bars.

Fig. 6. Scx-E47 associates with the conserved E-box of COL2A1 promoter on chromatin.

(A) Transiently transfected Scx was detected in the nuclear fractions of SW1353 cells (Nuc). Small amounts of transfected Scx remained in the cytoplasmic fractions (Cyto).

(B) Nuclear extracts as shown in (A) were used for EMSA. The conserved -577 E-box probes did not form protein-DNA complexes with the nuclear extracts of Scx or E47-single-transfected cells (Scx, E47). On the other hand, protein-DNA complexes were observed by using Scx and E47-cotransfected nuclear extracts (Scx + E47). Unlabeled -577 E-box probes (a 20-fold volume of labeled probes) decreased the complex formation between the Scx-E47-cotransfected nuclear extracts and labeled probes (Compete). Arrowhead and * denote the protein-DNA complex and free probes, respectively.

(C) The fragments, which include the conserved E-box, were coimmunoprecipitated with FLAG-tagged Scx in chromatin IP analyses. The amplified fragments were detected in Scx and E47-cotransfected cells, rather than in Scx-transfected cells (FLAG). Mouse IgG was used as a control for chromatin IP (IgG). A schema of human COL2A1 promoter and the conserved E-box are shown. Numbers indicate the distance from the transcription start site of COL2A1 gene (AC004801). Arrows denote the primer set for chromatin IP.

Fig. 7. Sox9, Scx, E47, and p300 cooperatively regulate the Col2a1 gene expression.

The schematic illustration of Col2a1 transactivation. Sox9 homodimer recognizes the Sox9-binding sequence (CATTCAT) on the Col2a1 enhancer in intron 1 (Bernard et al., 2003; Kamachi et al., 2000). Scx-E47 complex binds to the conserved E-box (CAGGTG) in the Col2a1 promoter. p300 acts as an important protein scaffold to connect Scx-E47 heterodimer with Sox9, and enhances the chromatin-mediated transcription by its histone acetyltransferase activity (HAT). E47 also directly associates with Sox9 (Fig. 4C). The crosstalk among transcription factors (Sox9, Scx) and coactivators (E47, p300) has a critical role to induce the Col2a1 expression and chondrogenesis. Arrows denote the interactions between indicated molecules. Important domains for bindings and activations are shown. N, N-terminal. C, C-terminal.

Table 1. Primer information

Gene	Species	Sequences (5'-3')	Basepairs	Source, Accession
SOX9	Human, Rat	CTGAACGAGAGCGAGAAG TTCTTCACCGACTTCCTCC	116	* NM_000346
SCX	Human, Mouse	TCTCCAAGATTGAGACGCTG TCTGTTTGGGCTGGGTGTTC	201	* XM_926116
E47	Human, Rat	ATGGGCTACCAGCTGCATGG GAGTGATCCGGGGAGTAGAT	215	* NM_003200
COL2A1	Human	AATTCCTGGAGCCAAAGGAT AGGACCAGTTGCACCTTGAG	103	Kawakami et al.
G3PDH	Human	CATCAAGAAGGTGGTGAAGCAG CGTCAAAGGTGGAGGAGTGG	119	Tetsunaga et al.

* GenBank accession numbers.

Table 1

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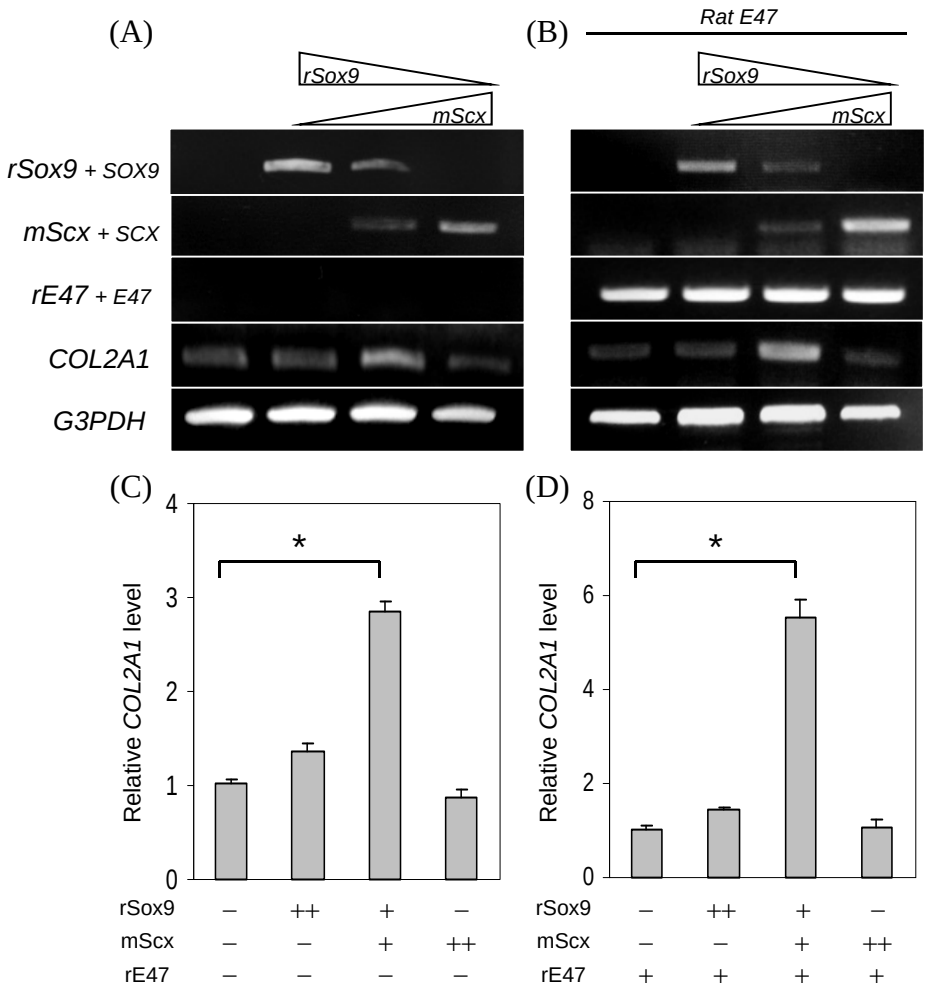

Figure 1

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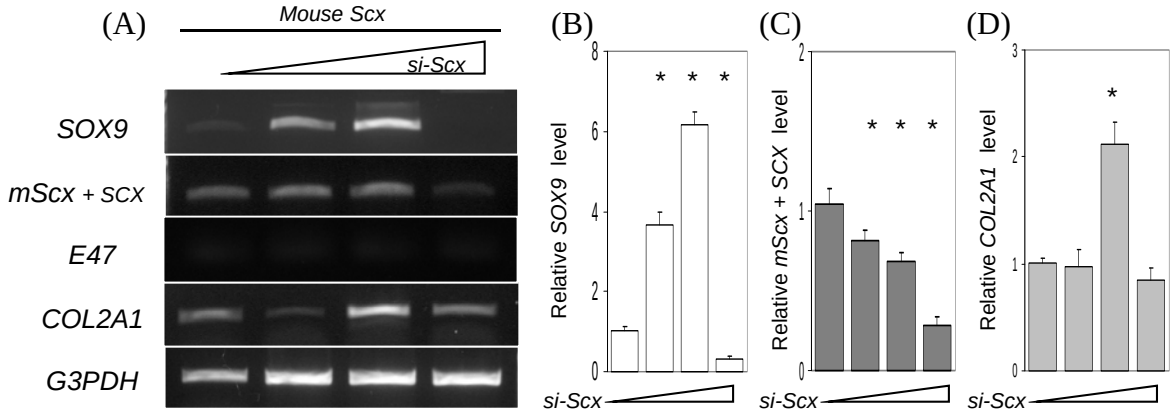

Figure 2

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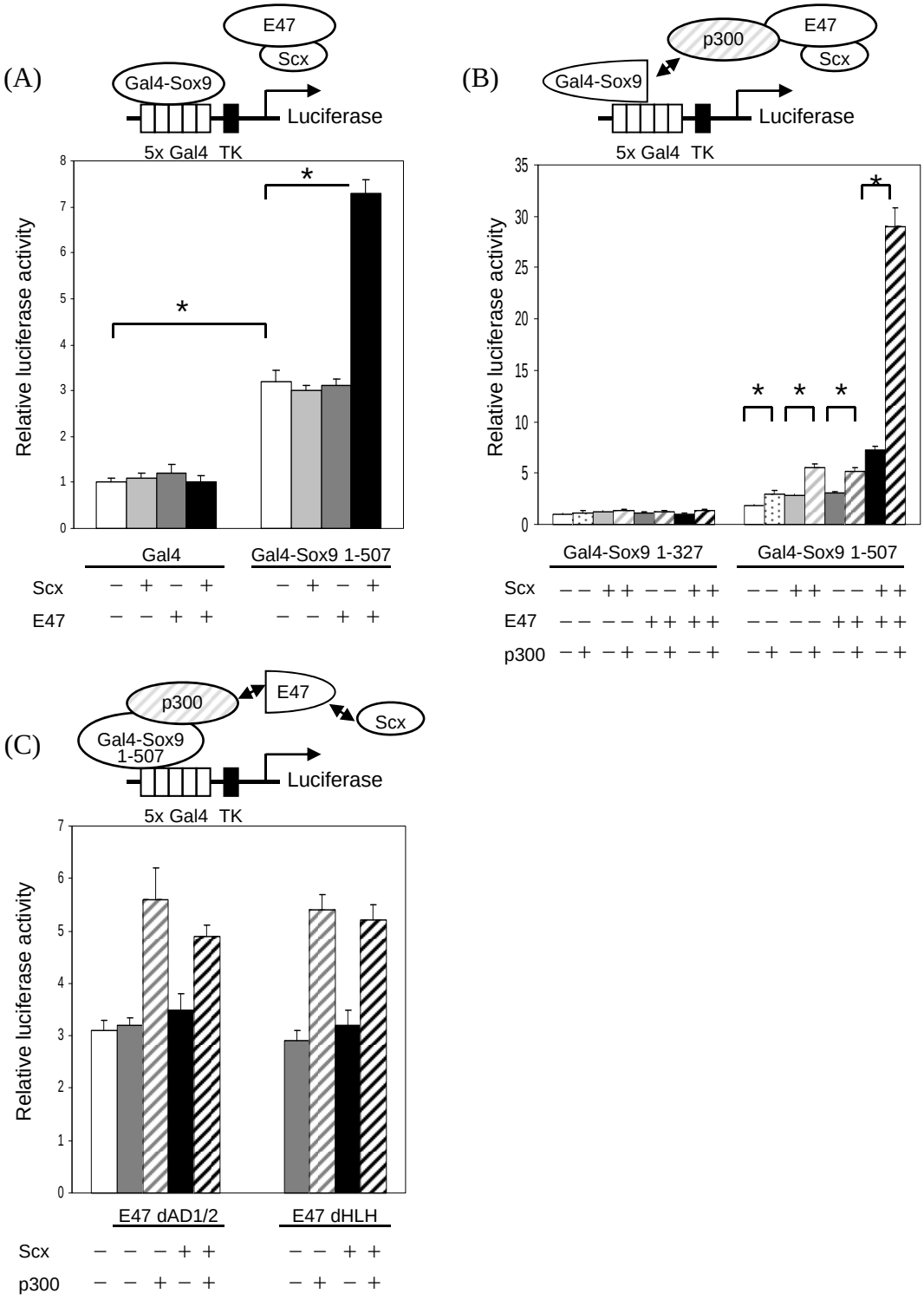

Figure 3

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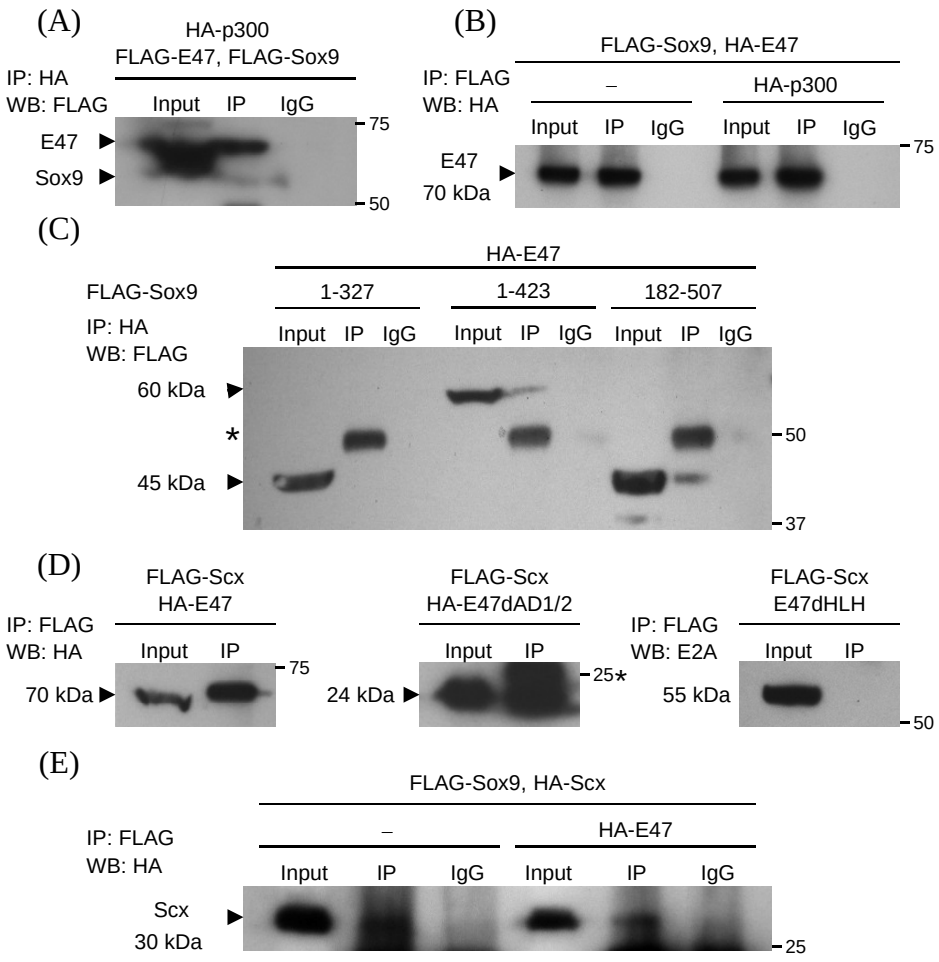

Figure 4

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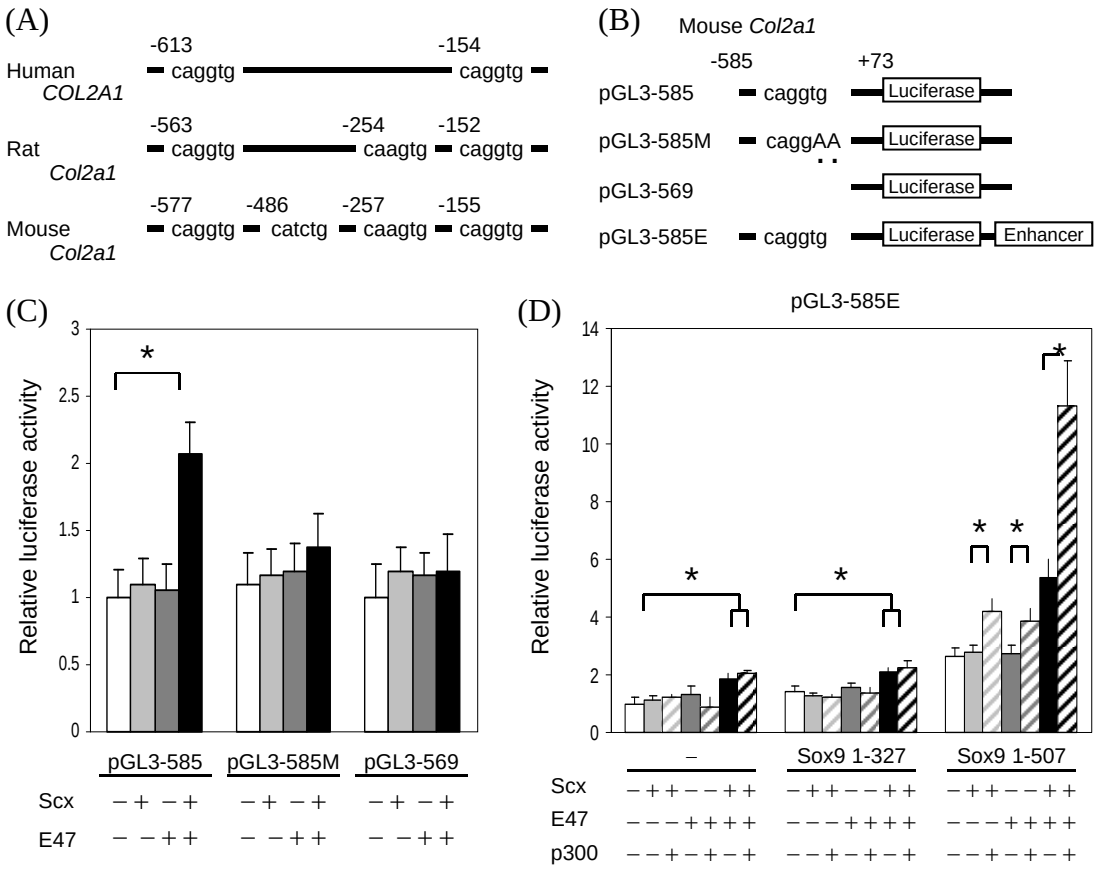

Figure 5

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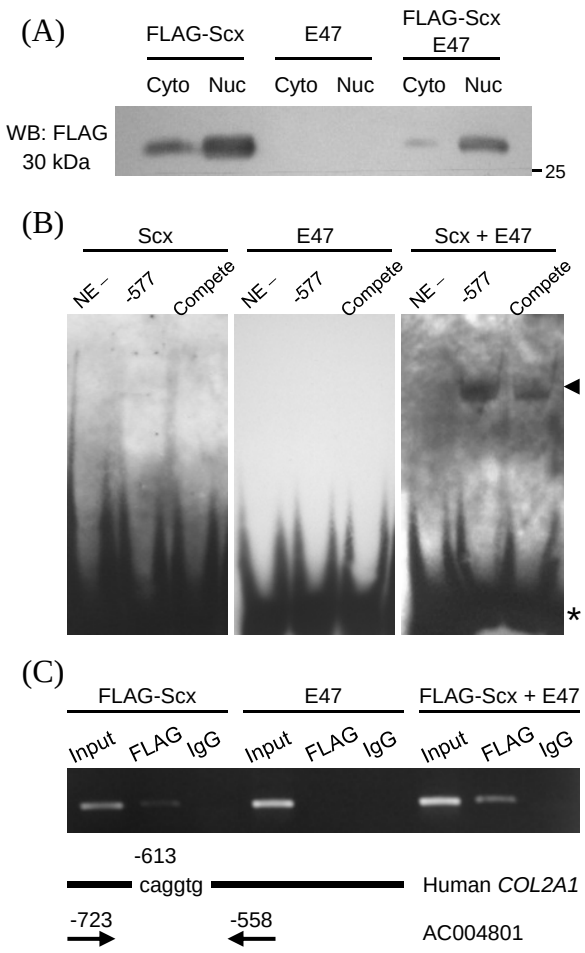

Figure 6

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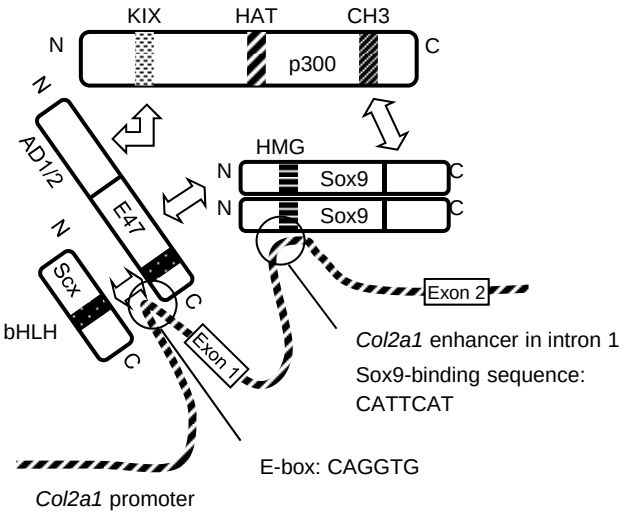

Figure 7

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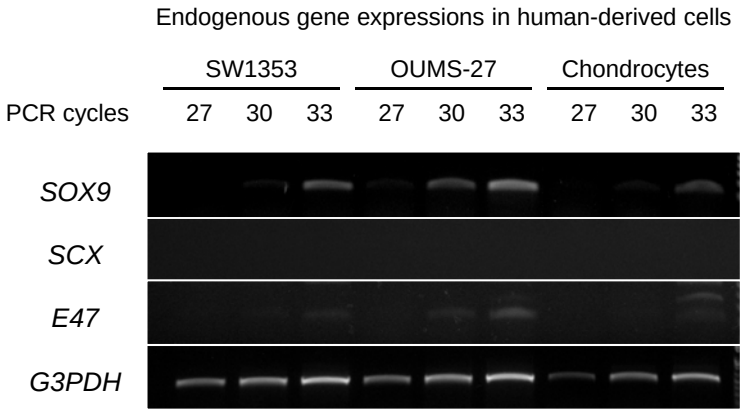

Supplemental Figure 1

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