Original article

Smad3 activates the Sox9-dependent transcription on chromatin

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Abstract

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

Keywords: Chondrogenesis; Chromatin; p300; Smad3; Sox9

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

1. Introduction

Chondrogenesis is the fundamental process to form bones and articular surfaces. Mesenchymal condensation and the following chondrocyte differentiation are strictly regulated by several transcription factors and growth factors, such as Sry-type high-mobility-group box (Sox) genes and the transforming growth factor (TGF)-β superfamily, respectively. Sox5, 6, and 9 cooperatively regulate the sequential differentiation steps of chondrogenesis (Akiyama et al., 2002, 2004; Stricker et al., 2002). In these

transcription factors, Sox9 has an essential role to initiate mesenchymal condensation and to maintain chondrogenic potential in early stages. The expression of α1 chain of type II collagen (Col2a1), a major component of cartilage extracellular matrix, is controlled by Sox9 through the Sox9-binding site on the Col2a1 enhancer region (Bell et al., 1997) and closely parallels that of Sox9 (Ng et al., 1997). The TGF-β superfamily including the two major families (TGF-β and bone morphogenetic protein) is a multifunctional growth factor for many cellular responses such as differentiation and proliferation (Heldin et al., 1997; Shi, & Massagué, 2003). In chondrogenesis, TGF-β stimulation is necessary for primary chondrogenesis derived from mesenchymal stem cells (Pittenger et al., 1999). We previously described that TGF-β signal Smad3 promotes the early chondrogenesis through the activation of Sox9 (Furumatsu et al., 2005a). However, the precise mechanisms of Sox9 and TGF-β in the epigenetic regulation for initiating chondrogenesis are still unclear.

The epigenetic regulation is another dynamic system to control gene expression and other fundamental cellular processes, such as proliferation and differentiation (Li, 2002; Felsenfeld, & Groudine, 2003; Jaenisch, & Bird, 2003). Chromatin remodeling system including histone modification is the representative mechanism of epigenetics. The eukaryotic DNA and histones are packaged into chromatin as the nucleosome-repeated structure. Accesses of transcription factors and other regulators to DNA are highly restricted by chromatin structure. Many molecules have been revealed as important factors to form chromatin. Nucleosome assembly protein-1 (NAP-1) acts as a histone-shuttling protein (Ito et al., 1996; Nakagawa et al., 2001). ACF (ATP-utilizing chromatin assembly and remodeling factor), consisting of Acf1 and ISWI subunits, assembles periodic nucleosome arrays on histone-attached DNA in an ATP-dependent process (Ito et al., 1999; Nakagawa et al., 2001). On the other hand, histone modification on chromatin, such as acetylation, enables transcription regulators to access to DNA sequences. DNA-binding transcription factors, such as CREB and MyoD, exert their transcriptional potential on histone-acetylated chromatin (Asahara et al., 2001; Dilworth et al., 2004). However, the relationship between chromatin-mediated transcription and signaling molecules is not elucidated. We previously reported that p300, which has an intrinsic histone acetyltransferase activity, directly associates with Sox9 (Tsuda et al., 2003) and activates the Sox9-dependent transcription on chromatin (Furumatsu et al., 2005b). In this study, we further analyzed the cross-talk between the Sox9-dependent transcription and TGF-β receptor-regulated Smad3 on chromatin using an in vitro chromatin assembly model.

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

2. Materials and methods

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

A human chondrosarcoma cell line (SW1353) was used as an immature chondrogenic cell line. A plasmid encoding full-length of rat Sox9 and a small interfering (si-) RNA against Smad3 were used (Furumatsu et al., 2005a). p300 was a gift from Tso-Pang Yao. FLAG-tagged Smad3/4 and the constitutively active form of TβR-I [TβR-I(TD)] were generous gifts from Takeshi Imamura. pGL3-585E, which contains a mouse Col2a1 promoter and enhancer, was constructed with a pGL3-Basic (Promega) vector and used as a native Col2a1 reporter gene. 12x48-pGL3-P containing 12 sets of a 48-bp Col2a1 enhancer element was used as a reporter plasmid. PCR fragments of FLAG-tagged Sox9, FLAG-tagged Smad3, and Smad4 were subcloned into baculovirus expression vector pENTR3C (Invitrogen) as described (Furumatsu et al., 2005b). The following antibodies were used: FLAG M2, FLAG M2 affinity gel (Sigma), phospho-Smad2/3 (Santa Cruz), Smad2/3 (Upstate), Smad4 (Cell Signaling), and Sox9 (Chemicon).

2.2. Luciferase reporter assay

pGL3-585E and 12x48-pGL3-P were used as reporter genes for investigating the Sox9-dependent transcriptional activity. These reporter plasmids were different from our previous constructs (Furumatsu et al., 2005a). Appropriate plasmids (50 ng) and si-Smad3 (200 nM) were transiently transfected into SW1353 cells using FuGENE6 (Roche). pRL-CMV (10 ng, Promega) was used as an internal control. The cells were harvested for 24 hr, and then the luciferase activities were analyzed using Dual-Luciferase Reporter Assay System (Promega). The assays were performed in triplicate.

2.3. Nuclear extract and immunoprecipitation

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

2.4. Purification of histones and recombinant proteins

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

2.5. Electrophoretic mobility shift assay (EMSA)

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

2.6. Chromatin assembly and micrococcal nuclease (MNase) assay

Chromatin assembly and MNase digestion analyses were performed as described (Asahara et al., 2002) by using 12x48-pGL3-P. For chromatin reconstitution, standard reactions (20 μl) containing plasmid (150 ng), histones (100 ng), NAP-1 (500 ng), ISWI/Acf-1 (0.65 ng each), ATP (3 mM), and ATP regeneration systems (30 mM phosphocreatine and 20 ng creatine phosphokinase) were incubated at 30°C for 4 hr. In MNase assay, chromatinized plasmids (300 ng) were digested with MNase (0, 0.02, and 0.04 U/15 μl) for 5 min at 37°C.

2.7. In vitro transcription and S1 nuclease assay

After chromatin assembly, standard reactions (12x48-pGL3-P, 150 ng) were incubated with Sox9 (10 ng), Smad3/4 (100 ng), p300 (40 ng), and acetyl-coenzyme A (AcCoA, 5 μM) for 30min at 30°C. For in vitro transcription, nuclear extracts from SW1353 cells (30 μg) were added and incubated with rNTPs at 30°C for 40 min. In-vitro-transcribed RNAs were recovered and subjected to S1 nuclease analyses using the

specific primer (49 bp) against 12x48-pGL3-P luciferase gene as described (Furumatsu et al., 2005b). RNAs were annealed with ^{32}P end-labeled primers (0.2 pmol each) for 12 hr, and then digested with 50 units of S1 nuclease (Invitrogen) for 30 min at 37°C. The protected fragments were run on 8% denaturing polyacrylamide gels and visualized by autoradiography. Each experiment was performed at least three times.

3. Results

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

To assess the fundamental role of Smad3 in chromatin remodeling during early chondrogenesis, we first analyzed the effect of Smad3 in the Sox9-regulated transcription using newly constructed reporter plasmids. Overexpressed Smad3 stimulated the transcriptional activity of Col2a1 reporter gene (Fig. 1A, pGL3-585E) in a Sox9-depedent manner (Fig. 1B). In addition, the effect of Smad3 was enhanced by the cotransfection of constitutively active form of TGF-β receptor I [TβR-I(TD)]. Twelve copies of the Sox9-binding fragment dramatically induced the Sox9-regulated transcription in reporter assays (Fig. 1C, 12x48-pGL3-P). Smad3 also activated the transcription of 12x48-pGL3-P in Sox9- and TGF-β-dependent manners. These findings suggest that Smad3 may act as a chromatin remodeling factor in chondrogenesis.

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

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

3.3. Recombinant Sox9 associates with p300 and binds to the Col2a1 enhancer in vitro

To examine the role of Sox9-associated transcriptional complex (Sox9, p300, and Smad3) on chromatin, we purified histones from HeLa cells, chromatin assembly-related molecules (NAP-1 and ACF complex), Sox9, p300, and Smad3 as described in materials and methods. Purified NAP-1 and ACF sufficiently assembled chromatin under histone-containing conditions. Chromatin assembling abilities of these molecules were estimated by MNase digestion assays (Fig. 3A). Recombinant Sox9 purified from Sf9 cells associated with recombinant p300 in vitro (Fig. 3B). Recombinant Sox9 also bound with high affinity to the Col2a1 enhancer probe, which contains the Sox9-binding sequence, in EMSA (Fig. 3C).

3.4. TGF-β-stimulated Smad3 and p300 cooperatively activate the Sox9-dependent transcription on chromatin

For in vitro transcription analyses after chromatin assembly (Fig. 4A), we assessed the complex formation of Smad3 and Smad4. Smad3 purified from the nuclear fraction of TGF-β-treated Sf9 cells was a phosphorylated form of Smad3 (Fig.4B). Smad4 was also detected in the same coimmunoprecipitated fraction using anti-FLAG M2 affinity gel (Fig. 4B). This result demonstrated that phosphorylated Smad3 was transferred into the nucleus with Smad4 by TGF-β treatment. In addition, purified Smad3/4 associated with recombinant Sox9 and p300 in vitro (Fig. 4C). Here we investigated the effect of phosphorylated Smad3 in the Sox9-dependent transcription on chromatin. In vitro transcription analyses on chromatinized templates revealed that the combination of Sox9, Smad3/4, and p300 were necessary for the activation of chromatin-mediated transcription (Fig. 4D). These findings suggest that the Sox9-

dependent chondrogenesis might be strictly controlled by TGF-β signal Smad3 and chromatin remodeling factor p300.

4. Discussion

The present study indicates that TGF-β receptor-regulated Smad3 and p300 cooperatively activate the Sox9-dependent transcription on chromatin. The TGF-β signal plays an essential role to induce primary chondrogenesis (Pittenger et al., 1999; Heng et al., 2004). However, the differentiation of chondrocyte is regulated by the conflictive effects of TGF-β. TGF-β3 enhances the early chondrogenesis derived from mesenchymal stem cells (Fan et al., 2008). The short-term treatment with TGF-β3 has been reported to maintain a chondrogenic phenotype (Mehlhorn et al., 2006). On the other hand, TGF-β inhibits chondrocyte maturation at the late stage (Ballock et al., 1993; Ferguson et al., 2000). We previously described that TGF-β signal Smad3 promotes the early chondrogenesis through the activation of Sox9 (Furumatsu et al., 2005a). However, the cross-talk between TGF-β signal and Sox9 in the epigenetic regulation for initiating chondrogenesis is still unclear. Here, we further analyzed a crucial role of Smad3 in the Sox9-dependent chondrogenesis on chromatin. In this study, Smad3 enhanced the Sox9-mediated transcription in luciferase reporter assay systems (Fig. 1B and C). The increase of relative luciferase activity with Smad3 was higher in pGL3-585E, which contains a native set of Col2a1 promoter and enhancer, than in 12x48-pGL3-P systems. These findings might be caused by the binding affinity of Sox9 against each reporter plasmid. The activity of 12x48-pGL3-P containing high copies of Sox9-binding site might be already excited by the cotransfection of Sox9. A Dose-dependent transactivation by Smad3 was observed in both systems, and was totally suppressed by the cotransfection of si-RNA against Smad3 itself (Fig. 2). We previously demonstrated that si-Smad3 completely decreased the Col2a1 expression in a mesenchymal stem cell-derived chondrogenic model (Furumatsu et al, 2005a). These results suggest that Smad3 is the major transducer of TGF-β signal in the Sox9-regulated early chondrogenesis.

The Sox9-dependent transcription is synergistically activated by p300 on chromatin (Furumatsu et al., 2005b). Transcriptional coactivator p300 has an important role for gene expression and cellular differentiation (Dilworth et al., 2004; Espinosa, & Emerson, 2001; Kitagawa et al., 2003). The effect of p300 is exerted through several mechanisms. p300 acts as a protein scaffold and a bridging factor for forming transcriptional complexes. In addition, the intrinsic histone acetyltransferase activity of p300 has a potential to facilitate the transcriptional activity by modulating chromatin structure (Chan, & La Thangue, 2001; Korzus et al., 1998; Utley et al., 1998). Several authors have reported that p300 plays a critical role for the activation of cAMP response element-binding protein-, MyoD-, p53-, or vitamin D receptor-dependent transcription on reconstituted chromatin (Asahara et al., 2001; Dilworth et al., 2004; Espinosa, & Emerson, 2001; Kitagawa et al., 2003). In previous studies, we described that p300 and Smad3 enhanced the Sox9-dependent transcription by associating with Sox9 (Tsuda et al., 2003; Furumatsu et al., 2005a). However, the precise effect of the third associating factor, such as Smad3, on chromatin is still unclear. To analyze the additional effect of the third factor in a chromatin assembly model is considered to be hard. This study revealed the additional effect of phosphorylated Smad3 in the Sox9- and p300-mediated transcription using 12x48-pGL3-P-based chromatin assembly model (Fig. 4D). However, the synergistic effect of Smad3 was not observed in a different balance of Sox9-associating molecules (data not shown). In pGL3-585E systems, we could not detect a significant effect of Smad3 on chromatin-derived transcription, either (data not shown). These findings suggest that the balance of Sox9-associating factors and the accessibility to chromatinized promoter might be important for the epigenetic regulation of chondrogenesis. In addition, the discrepancy of Smad3-induced transactivation between reporter assays (Fig. 1 and 2) and chromatin-derived transcription (Fig. 4D) might be caused by the following reasons: i) the chromatinized status of Sox9-reactive plasmid was different in each analysis, ii) the influence of Sox9 and p300 was more critical on chromatin-assembled plasmid, and iii) unknown factors in SW1353 nuclear extracts might have important roles in the Sox9-dependent transcription on

chromatin. Several transcription partners such as Sox-5/6, PGC-1α, Barx2, and TRAP230 can modify the Sox9-dependent transcription during chondrogenesis (Ikeda et al., 2004; Kawakami et al., 2005; Lefebvre et al., 2001; Meech et al., 2005; Zhou et al., 2002). Further analyses to identify the other unknown partners of Sox9-based transcriptional complex will be required.

Animal models for a loss of Smad3 function have revealed the importance of Smad3 in physiological systems. Smad3 null mice show skeletal defects including osteoarthritis (Datto et al., 1999). Haploinsufficiency of Smad2 and Smad3 causes an embryonic lethality due to endodermal defects and exhibits craniofacial defects (Liu et al., 2004). We previously reported that Smad3 had an important role for primary chondrogenesis (Furumatsu et al., 2005a). In addition to the Smad3 pathway, TGF-β activates mitogen-activated protein kinase (MAPK) pathway during chondrogenic differentiation (Stanton et al., 2003). Several authors have shown that MAPK pathway modulates Col2a1 and Sox9 expression in chondrogenesis (Murakami et al., 2000; Nakamura et al., 1999; Tuli et al., 2003). These reports suggest that TGF-β-stimulated MAPK pathway would also be involved in chondrogenesis with modifying the Sox9-dependent transcription. Further studies to analyze the relationships between MAPK pathway and the Sox9-mediated transcription on chromatin are required.

In conclusion, the present study demonstrates that Smad3 enhances the Sox9-dependent transcription on chromatin. Our findings suggest the potential molecular mechanism how TGF-β signals induce early chondrogenesis via chromatin regulation.

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Figure legends

Fig. 1. Smad3 enhances the Sox9-mediated transcription in a TGF-β-dependent manner.
(A) A schematic characterization of pGL3-585E which contains a native promoter and enhancer of mouse Col2a1 gene. Numbers indicate the distance from the transcription start site on mouse Col2a1 gene (National Center for Biotechnology Information, M65161). Black box denotes the SOX9-binding site on the enhancer region of Col2a1 intron 1.
(B) Transient transfections of Sox9, Smad3, and TβR-I(TD) did not increase luciferase activities of pGL3-B plasmids in SW1353 cells (pGL3-B). In pGL3-585E systems, Sox9 enhanced a relative luciferase activity to a level as high as 2.2-fold over the control. Cotransfection of Smad3 augmented a luciferase activity up to 2.3-fold higher level of Sox9-transfected cells. The additional transfection of constitutively active form of TβR-I(TD) induced an approximately 36% increase of the activity in Sox9- and Smad3-transfected SW1353 cells. Luciferase activities of pGL3-585E were not increased in the absence of Sox9. Note that Smad3 and TβR-I(TD) synergistically activated the native Col2a1 reporter-mediated transcription in a Sox9-dependent manner.
(C) The activity of 12x48 pGL3-P was enhanced by the addition of Sox9 up to 27.5-fold levels of the control. Smad3 increased the 12x48 pGL3-P-based luciferase activity up to 1.5-fold higher level in the presence of Sox9. TβR-I(TD) also induced 33% increase of the activity of Smad3-transfected cells in the presence of Sox9. However, the additional increase of luciferase activity was not observed in pGL3-P-transfected cells. Relative luciferase activities were calculated using the activity of pGL3-P as a control (100%).
A schematic illustration of each reporter assay system is placed on the top of each figure (B and C). * Statistical significances ($p < 0.05$) were observed between the indicated bars with the Mann-Whitney *U*-test. Error bars, SD.

Fig .2. Smad3 has an essential role for TGF-β-stimulated transactivation in the Sox9-regulated gene expression.
(A) Smad3 enhanced the Sox9-dependent transcription in a dose-dependent manner. Smad3 and TβR-I(TD) synergistically increased the luciferase activity in pGL3-585E reporter systems. si-RNA against Smad3 (si-Smad3) totally inhibited the synergistic effects of Smad3 and TβR-I(TD). Note that si-Smad3 did not inhibit the Sox9-induced transactivation of reporter genes.
(B) In 12x48 pGL3-P reporter systems, Smad3 and TβR-I(TD) cooperatively stimulated the relative luciferase activity up to 1.7-fold higher level in the presence of Sox9. A dose-dependent effect of Smad3 was observed. However, the increase of luciferase activity was suppressed by si-Smad3 in Smad3-transfected cells.
Relative luciferase activities were calculated using the activity of pGL3-B (A) or pGL3-P (B) as a control (100%). Triangular boxes denote the transfection volume of Smad3 expression plasmid (0, 25, and 50 ng). * Statistical significances ($p < 0.05$) were observed between the indicated bars with the Mann-Whitney *U*-test. Error bars, SD.

Fig. 3. MNase digestion analyses after chromatin assembly. Purified Sox9 form a complex with p300 or DNA probe containing the Sox9-binding site.
(A) Closed circular 12x48-pGL3-P (300 ng) was used as a template. Chromatin assembling steps were performed as shown in Fig. 4A. Plasmid DNAs were completely digested by MNase (0.02 and 0.04 U/15 μl) in the absence of histones, NAP-1, and ACF (Histone-free DNA). Chromatinized plasmids were protected from complete digestion (Chromatin). Nucleosome-repeated pattern (approximately 165 bp) was observed in chromatin template after MNase treatment (0.04 U/15 μl). M, 123-bp ladder (Invitrogen).
(B) Purified p300 was coimmunoprecipitated with recombinant Sox9 using anti-Sox9 antibody. Western blotting was performed with anti-FLAG M2 antibody. Sox9 (30 ng) was incubated with p300 (30 ng), and then the 10% of reaction was loaded as an input. Immunoprecipitation using rabbit IgG was performed as

a control. Numbers indicate molecular weight (kD).
(C) Purified Sox9 associated with the Col2a1 enhancer probe in EMSA. The unlabeled competitor decreased the signal of Sox9-DNA complex. Supershifted band was observed in the presence of anti-Sox9 antibody.

Fig. 4. Phosphorylated Smad3 and p300 cooperatively activate the Sox9-dependent transcription on chromatin.
(A) The sequential steps for chromatin assembly and in vitro transcription are illustrated. MNase assays were performed after chromatin assembly (Fig. 3A). To estimate the amounts of RNAs transcribed from chromatinized plasmid, S1 nuclease assays were performed as described in materials and methods. S1 nuclease digests a single-stranded part of RNA and excessive primers. Remaining double-stranded fragments (49-bp), which are annealed with ^{32}P end-labeled specific primers, represent transcriptional activities on chromatin.
(B) Recombinant Smad3/4 were prepared using baculovirus expression systems. The details are described in materials and methods. Smad3/4 complex were visualized with silver staining (left lane). Phosphorylated Smad3 were obtained after TGF-β treatments (middle lane). Smad4 was coimmunoprecipitated with FLAG-tagged Smad3 (right lane).
(C) Protein-protein interactions among recombinant proteins. Purified Sox9 (50 ng), p300 (50 ng), and Smad3/4 (50/15 ng) were incubated, and then immunoprecipitated with anti-Smad2/3 antibodies. Sox9 and p300 were coimmunoprecipitated with Smad3 (IP). Western blotting analyses were performed with anti-FLAG M2 antibodies.
(D) Sox9, Smad3/4, and p300 cooperatively enhanced the transcriptional activities of chromatinized 12x48-pGL3-P (12x48, upper bands). Chromatin-mediated transcription was not fully activated by the combined treatment with Sox9 and Smad3/4 (or p300). Note that the synergistic effect of triple combination with Sox9, Smad3/4, and p300 was observed (right lane). Digested denotes non-annealed probes, which were digested by S1 nuclease treatments (lower bands).

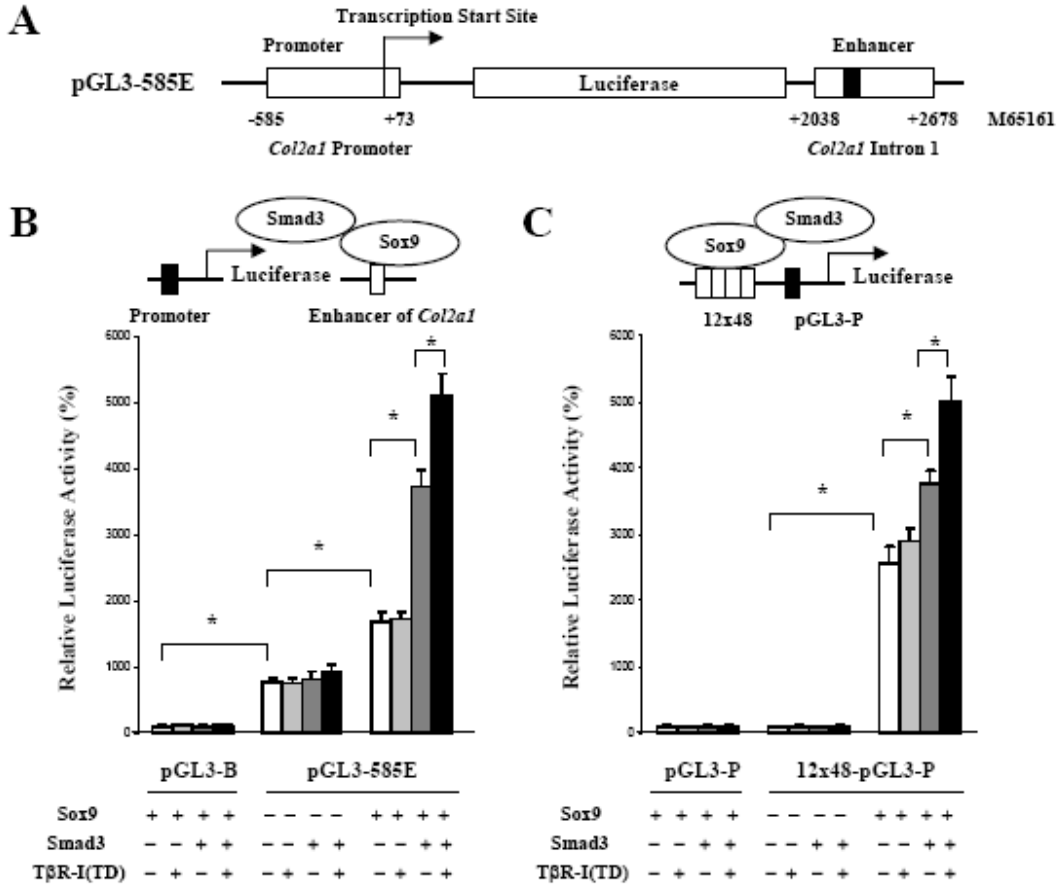

Figure 1
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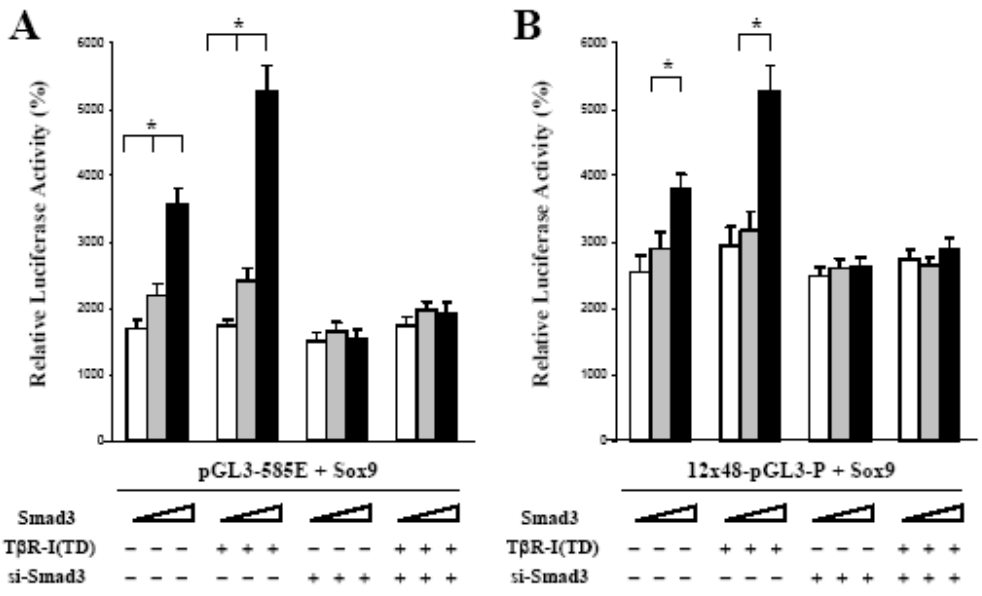

Figure 2
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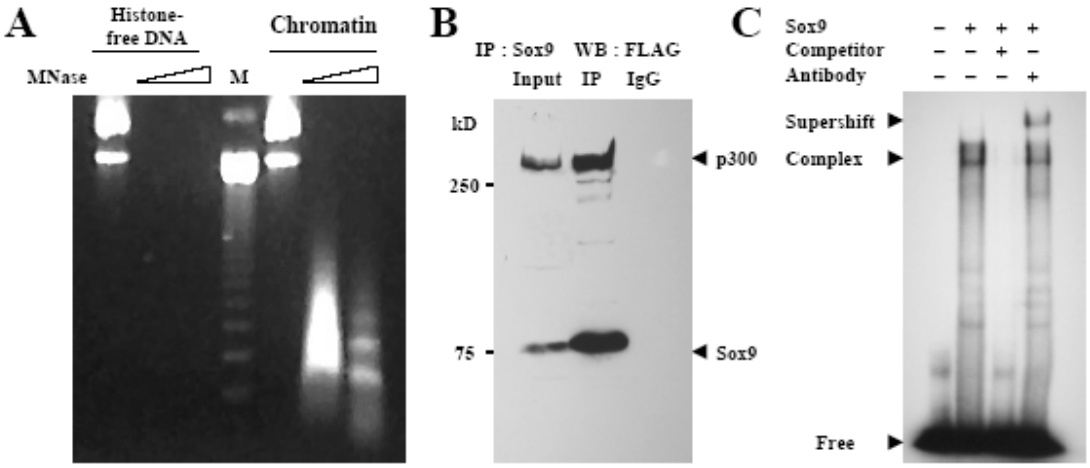

Figure 3
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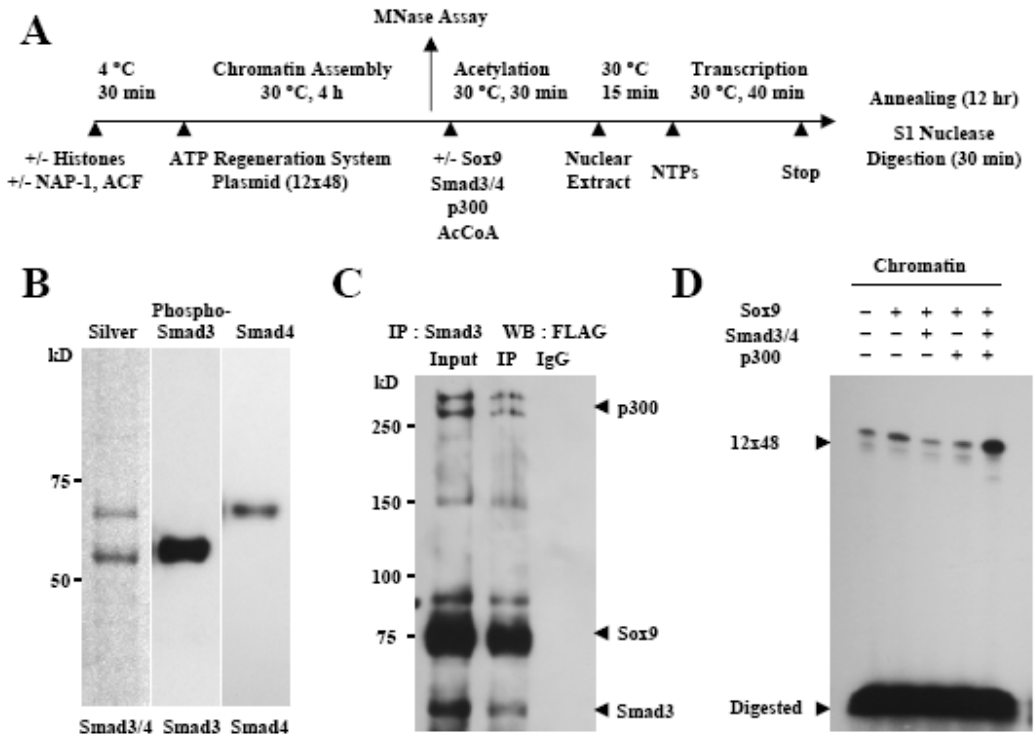

Figure 4
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