Smad3 Induces Chondrogenesis through the Activation of SOX9 via CREB-binding Protein/p300 Recruitment*§

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The transcriptional activation by SRY-type high mobility group box 9 (SOX9) and the transforming growth factor β (TGF-β) signals are necessary for chondrogenic differentiation. We have previously shown that CREB-binding protein (CBP/p300) act as an important SOX9 co-activator during chondrogenesis. In the present study, we investigated the relationship between TGF-β-dependent Smad2/3 signaling pathways and the SOX9-CBP/p300 transcriptional complex at the early stage of chondrogenesis. Overexpressed Smad3 strongly induced the primary chondrogenesis of human mesenchymal stem cells. In addition, Smad3 enhanced the transcriptional activity of SOX9 and the expression of α1(II) collagen gene (COL2A1), and small interference RNA against Smad3 (si-Smad3) inhibited them. We observed that Smad2/3 associated with Sox9 in a TGF-β-dependent manner and formed the transcriptional complexes with SOX9 on the enhancer region of COL2A1. Interestingly, the association between Sox9 and CBP/p300 was increased by Smad3 overexpression and was suppressed by si-Smad3. Our findings indicate that Smad3 has a stronger potential to stimulate the SOX9-dependent transcriptional activity by modulating the interaction between SOX9 and CBP/p300, rather than Smad2. This study suggests that the Smad3 pathway presents a key role for the SOX9-dependent transcriptional activation in primary chondrogenesis.

The transforming growth factor β (TGF-β) superfamily is a multifunctional growth factor for many cellular processes such as differentiation, proliferation, and apoptosis (1–3). In cellular differentiation, osteogenesis and chondrogenesis are modulated by bone morphogenetic proteins, which are members of the TGF-β superfamily (4–6). Bone morphogenetic proteins stimulate osteoblastic differentiation through the enhancement of runt-related gene 2 (Runx2)-based transcriptional activity. Bone morphogenetic protein-regulated Smads (Smad1/5/8) have been reported to progress osteogenesis by associating with Runx2, which is a key transcription factor in bone formation (7). However, the effects of TGF-β during chondrogenesis are still unclear. The differentiation of chondrocytes is regulated by the confictive effects of TGF-β. TGF-β promotes the differentiation of embryonic chick limb cartilage (8). Primary chondrogenesis derived from mesenchymal stem cells (MSCs) needs TGF-β signals (9). Conversely, TGF-β inhibits chondrocyte maturation (10, 11). These reports have prompted us to investigate an unknown regulatory mechanism of TGF-β in chondrogenesis. Several pathways after the activation of TGF-β receptor, such as Smad2/3 and mitogen-activated protein kinase (MAPK), have been identified as key signaling processes in response to TGF-β treatments (2, 12, 13). However, the mechanism of each TGF-β signaling process in primary chondrogenesis has not been fully clarified. We focus here on the function of TGF-β-regulated Smads (Smad2/3) during chondrogenesis derived from human MSCs.

Smad2 and Smad3 are phosphorylated by TGF-β type I receptor (TβR-I) and then form the heteromeric complexes by associating with Smad4 (14, 15). These Smad complexes move into the nucleus and regulate expression of their target genes through the direct binding to Smad-binding element on DNA sequence (16, 17). DNA-binding activity, which is lacking in Smad2, is dependent on the β hairpin in Mad homology 1 (MH1) domains of Smad3 and Smad4 (18). MH2 domain of Smad3 has been reported to associate with many transcription factors such as Runx2 and MyoD acting as a critical transcription factor for myogenesis (19, 20). In addition, MH2 domains of Smad2 and Smad3 interact with a co-activator termed cAMP-response element-binding protein binding protein (CBP) and its paralog p300, which has histone acetyltransferase activity (21, 22). However, the relationships between Smad2/3 and chondrogenesis-related molecules are still unclear. Therefore, we investigated the interactions between Smad2/3 and other molecules that induce chondrogenesis.

SOX9 (SRY-type high mobility group box 9), which encodes a high mobility group DNA-binding domain, has been identified as a master transcription factor in chondrocyte differentiation (23). The expression of α1 chain of type II collagen (Col2a1), the major component of cartilage extracellular matrix, closely parallels that of Sox9 (24). Col2a1 expression is regulated by Sox9 through the association with SOX9-binding site on the Col2a1.
enlargement region (25). However, the SOX9-dependent regulation during chondrogenesis seems to require additional cofactors (26). We described previously that CBP/p300 is an important co-activator for enhancing the transcriptional activity of SOX9 (27). In this study, we further analyzed the cross-talk among SOX9, CBP/p300, and Smad2/3 during chondrogenesis.

In the present study, we demonstrate that Smad3 stimulates primary chondrogenesis and modulates the association between Sox9 and p300. Moreover, the SOX9-dependent transcription is regulated by the transcriptional complex consisting of Smad3, SOX9, and CBP/p300.

**EXPERIMENTAL PROCEDURES**

**Cells and Cell Culture—**Human mesenchymal stem cells (MSCs) were obtained from Cambrex (Walkersville, MD) and maintained with mesenchymal stem cell growth media. According to the chondrogenesis protocol, 5 × 10⁵ cells were precipitated and cultured in TGF-β3 (BIO-SOURC, Camarillo, CA) for 1 week. MSCs between passages 3 and 5 were used. A human chondrosarcoma cell line (SW1353) was used as an immature chondrogenic cell line (27).

**RNA Isolation and Reverse Transcription-PCR—**Total cellular RNA was extracted using RNAeasy mini kits (Qiagen, Valencia, CA). RNA samples (1 μg) were reverse-transcribed to cDNA as described previously (28). The cDNAs were subjected to PCR amplification in the presence of the following specific primer sets: 5’-TGG AGC AGC TCT GGA GAC-3’ and 5’-CCC TCT CGC TTC AGG TCA-3’ for SOX9, 5’-ACG CCG TGT CCT CAC TGG TC-3’ and 5’-TGG GTT TGC AAC GGA TGG T-3’ for COL2A1, 5’-GGG TCG TCC ATC TGT CCA TTC AC-3’ and 5’-TTA TGA CAT GCT TGA GCA AC-3’ for Smad2, and 5’-GGG TGC TCC ATC CGT CTT AC-3’ and 5’-CTA AGA CAC ACT GGA ACA GC-3’ for Smad3. PCR fragments were normalized with the levels of glyceraldehyde-3-phosphate dehydrogenase signals (5’-ATC AGC /H11032 TCG TCC ATC CTG CCT TTC AC-3’/H11032 and 5’-ATC AGC /H11032 TGG GCA AGC TCT -TGG GCA AGC TCT /H11032 GGA GAC-3’/H9252-CGG TCT CGC TTC AGG TCA-3’/H9252 for GAPDH).

**Subcellular Fractionation—**After 30-min treatments with TGF-β3 (5 ng/ml), samples (1 g) were reverse-transcribed to cDNA as described previously. The expression of SOX9 and COL2A1 mRNA were increased along with primary chondrogenesis. TGF-β treatment (10 ng/ml) and three-dimensional culture were necessary for the augmentation of SOX9 and COL2A1 expressions. The 30-min treatments with TGF-β (5 ng/ml) stimulated the nuclear translocation of endogenous Smad2/3 in MSCs after 12-h culture with serum-starved medium. Red spots stained with anti-Smad2/3 antibodies increased in the nucleus of TGF-β-treated cells. Bar, 20 μm. C, phosphorylated Smad2/3 were transported into the nuclei by TGF-β treatments (5 ng/ml, 30 min). Equal amounts of protein from the whole cell, cytoplasmic, or nuclear fractions of monolayer-cultured MSCs were blotted. TGF-β induced the phosphorylation of Smad2/3 without changing the total amounts of endogenous Smad2/3. Left panel, blotted with anti-Smad2/3 antibody; right panel, anti-Phospho-Smad2/3 antibody.

**Luciferase Reporter Assay**—For immunoprecipitation, cells were transfected with immunoprecipitation buffer 0 (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Nonidet P-40, 1 mM EDTA, 2.5 mM EGTA, 10% glycerol, protease inhibitors) after indicated treatments. Cell extracts were then sonicated and centrifuged. Ten percent volume of supernatant was loaded as an input fraction. Remaining supernatants were incubated with protein G beads (Sigma) containing 1 μl of anti-FLAG M2 affinity gel (Sigma) or HA affinity matrix (Roche Applied Science) according to the manufacturer’s protocol. Cells were cultured for 48 h before immunoprecipitation assays. Indicated plasmids (500 ng) were used for transfection analyses.

**Histology and Immunohistochemistry—**Attached MSCs on CultureSlide (BD Biosciences) were fixed with cold acetone for 10 min at −20 °C. Pellet-cultured cells, maintained for 1 week, were fixed with 4% paraformaldehyde-buffered solution and embedded in paraffin. The following procedures for safranin O staining were performed as described previously (28). Immunohistochemical analyses using anti-Smad2/3 antibodies (Upstate) were performed according to the manufacturer’s instructions. Signals were visualized using Vectastain ABC-AP (Vector). Anti-Sox9 (27) and Vector red alkaline phosphatase substrate kit, and then the sections were stained with Vector hematoxylin QS (Vector, Burlingame, CA) for counterstaining. Rabbit IgG (Sigma) was used as a control.
Smad3 activates Sox9-dependent transcription with p300

RESULTS

TGF-β induces chondrogenesis and the nuclear translocation of Smad2/3 in human MSCs—TGF-β plays a key role to induce chondrogenic differentiation in pellet-cultured cells derived from human MSCs (9). In this three-dimension culture, TGF-β treatments stimulated the expression of α1 chain of type II collagen gene (COL2A1), which is the main chondrogenic differentiation marker. The expression level of the master transcription factor for chondrogenesis, SOX9, was also increased by TGF-β in pellet-cultured cells (Fig. 1A). However, COL2A1 and SOX9 expressions were not increased in monolayer-cultured cells (Fig. 1A). The expression of Smad2 and Smad3, which are the main transducers of TGF-β signal, showed no significant differences along with chondrogenesis (data not shown). These results indicated that both TGF-β signal and the three-dimension culture were necessary for the early chondrogenic differentiation. To assess TGF-β effects on the initiation of chondrogenesis via Smad pathway, we analyzed the localization of Smad2 and Smad3 in MSCs. TGF-β treatments stimulated the nuclear translocation of endogenous Smad2/3 even in MSCs (Fig. 1B). In addition, we detected phosphorylated Smad2/3 in the nuclear fraction after TGF-β stimulation (Fig.
These data prompted us to investigate the functions of Smad2/3 during chondrogenesis.

**Smad3 Accelerates the Early Chondrogenic Differentiation—**

Here, we focused on Smad signaling pathway activated by TGF-β/H9252 during chondrogenesis. To examine the effects of Smad2/3, we infected these Smads into pellet-cultured MSCs using adenovirus overexpression systems. Smad3 strongly stimulated the syntheses of proteoglycans in pellet-cultured cells (Fig. 2A, safranin O). Smad3 also increased the expression of COL2A1 mRNA (Fig. 2B). However, the SOX9 expression did not change in Smad3-overexpressed cells (Fig. 2B). Smad2-overexpressed cells showed no significant differences in both safranin O staining and COL2A1 expression (Fig. 2A, A and B). Simultaneously, we analyzed the knock-down models of Smad2/3 using their siRNAs. si-Smad3 inhibited MSC-derived chondrogenesis in safranin O staining (Fig. 2C) and reduced the expression of COL2A1 in reverse transcription-PCR analyses (Fig. 2E). However, si-Smad2 did not show remarkable inhibitory effects in our chondrogenic induction models. These observations suggest that Smad3 might have a stronger potential to induce the early chondrogenesis as the TGF-β signal transducer, rather than Smad2.

**Fig. 3.** Smad3 stimulates the SOX9-dependent transcriptional activity by associating with SOX9 in a TGF-β-dependent manner. A, 30-min treatments with TGF-β (5 ng/ml) enhanced the associations between endogenous Smad2/3 and SOX9 in immature chondrogenic SW1353 cells. Co-immunoprecipitation and Western blotting were performed using anti-Smad2/3 and -SOX9 antibody, respectively. The 10% volume of each sample was loaded as a control (Input). Serum starvation was performed for 12 h before TGF-β treatment. B, transfected FLAG-Smad3/2/3 associated with endogenous SOX9 in a TGF-β-dependent manner. Co-immunoprecipitated SOX9 with anti-FLAG M2 affinity gels were similar amounts between Smad2- and Smad3-transfected SW1353 cells. C, a schematic characterization of pKN185 Luc, 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). The black box denotes the SOX9-binding site on the enhancer region of Col2a1 intron 1. D, FLAG-Smad3 increased a relative luciferase activity to a level as high as 2.2-fold over the control (pDEF) in pKN185 systems. The additional transfection of constitutively active form of TβR-I(TD) induced an ~50% increase of the activity in Smad3-transfected SW1353 cells. FLAG-Smad7, which inactivates Smad2/3, decreased the Smad2/3-induced luciferase activity to the basal levels. Note that Smad3 and TβR-I(TD) synergistically activated the native Col2a1 reporter-dependent transcription. E, the activity of Gal4-TK was enhanced by the addition of Gal4-Sox9 corresponding to 182–507 amino acids (Gal4-Sox9 182–507) up to 7.4-fold levels of the Gal4 control. Smad3 increased the Gal4-based luciferase activity up to 20-fold higher level in the presence of Gal4-Sox9. TβR-I(TD) also induced 46% increase of the activity of Smad3-transfected cells in the presence of Gal4-Sox9. Note that the increase of luciferase activity by TβR-I(TD) was greater in Smad3-transfected cells than in Smad2-transfected cells. Relative luciferase activities were calculated using the activity of Gal4-TK as a control (100%). A schematic illustration of each reporter assay system is placed on the top of each figure (D and E). Statistical significances (p < 0.05) were observed among open bars in pKN185 or Gal4-Sox9 systems with the Mann-Whitney U test. Error bars, S.D.
Smad3 Up-regulates the Col2a1 Transcription and the Sox9-dependent Transcriptional Activity—We hypothesized that Smad3 might progress chondrogenesis through modulating the function of SOX9, which is the key transcription factor for COL2A1 expression. To investigate Smad functions in an early stage of chondrogenesis, we used SW1353 cells as immature chondrogenic cells. Interestingly, SOX9 was co-immunoprecipitated with Smad2/3 in a TGF-β-dependent manner (Fig. 3A). In addition, the associations between transfected Smad2/3 and endogenous SOX9 were increased by TGF-β treatments (Fig. 3B). To assess the effects of Smad2/3 on the SOX9-dependent transcriptional activity, we performed luciferase assays using pKN185, which includes a native Col2a1 promoter and enhancer, and Gal4-Sox9 systems. Transfected Smad3 increased the luciferase activity based on endogenous SOX9 in pKN185-transfected SW1353 cells (Fig. 3D). We also observed the transcriptional up-regulation of Gal4 reporter gene based on transfected Gal4-Sox9 by the co-transfection with Smad3 (Fig. 3E). These effects of Smad3 were higher than that of Smad2. In addition, TβR-I(TD) mainly enhanced the levels of reporter activities in Smad3-transfected cells (Fig. 3D and E). We also performed the co-transfection of Smad7, which is the main inhibitor of Smad2/3 phosphorylation and their nuclear localization (35, 36). Smad7 inhibited the positive effects of Smad2/3 and TβR-I(TD), especially in pKN185 systems (Fig. 3D). These results demonstrate that the nuclear-translocated Smad3 might have an important role to stimulate the SOX9-regulated COL2A1 expression.

Smad2/3 Associate with SOX9 on the COL2A1 Enhancer—To confirm the binding region of SOX9 against Smad2/3, we performed co-transfection analyses using SW1353 cells. HA-Smad2/3 similarly combined with the full-length of FLAG-Sox9 (Fig. 4, 1–507), and Smad2/3 (left panel). However, Gal4-Sox9 Δ328–423, which lacks the Smad2/3-binding domain, showed no significant differences in the presence of TβR-I(TD) and Smad2/3 (right panel). Relative luciferase activities were calculated using the activity of Gal4-TK as a control (100%). Statistical significances (p < 0.05) were observed among bars in Gal4-Sox9 1–507 systems with the Mann-Whitney test. Error bars, S.D. D, chromatin immunoprecipitation analyses using adenovirus overexpression systems and anti-FLAG M2 affinity gels in SW1353 cells. The PCR fragments, including the SOX9-binding site on the enhancer region of COL2A1 gene (located at +2405 through +2411, -CATTCAT-) were detected in both Smad2- and Smad3-infected cells (Enhancer). The other fragment, which contains a putative SRY-binding sequence with mouse IgG. The scheme shows the part of COL2A1 gene structure and the fragments amplified with the indicated primers (arrows). Numbers correspond to the transcription start site of human COL2A1 gene (National Center for Biotechnology Information, AC004801).

**FIG. 4.** Smad2/3 interact with the specific domain of Sox9 on the COL2A1 enhancer. A, a structural schema of rat Sox9. Bars under the schema represent each fragment of Sox9 and correspond to the next numbers of amino acids. The region, which might have a binding ability against Smad2/3, is represented as a bar on the schema. The P/Q and P/Q/S boxes denote the domain abundantly containing each amino acid, respectively. B, HA-Smad2/3 similarly combined with the full-length of FLAG-Sox9 (FLAG-Sox9 1–507). The deletion mutant of FLAG-Sox9 corresponding to 1–423 amino acids (DM1–423) and DM182–507 showed strong affinities against both HA-Smad2/3. Few affinities against HA-Smad2/3 were observed in SW1353 cells transfected with DM1–327 or Δ328–423, which lacks the region corresponding to 328–423 amino acids. Molecular weight markers are shown at the left of each panel. Upper and lower panels demonstrate the 10% input and immunoprecipitated FLAG-Sox9, respectively. C, to assess the putative Smad2/3-binding domain corresponding to 328–423 amino acids in Sox9, we performed luciferase assays using Gal4-Sox9 systems in SW1353 cells. The activity of Gal4-Sox9 1–507 was enhanced by the addition of TβR-I(TD) and/or Smad2/3 (left panel). However, Gal4-Sox9 Δ328–423, which lacks the putative Smad2/3-binding domain, showed no significant differences in the presence of TβR-I(TD) and Smad2/3 (right panel). Relative luciferase activities were calculated using the activity of Gal4-TK as a control (100%). Statistical significances (p < 0.05) were observed among bars in Gal4-Sox9 1–507 systems with the Mann-Whitney U test. Error bars, S.D. D, chromatin immunoprecipitation analyses using adenovirus overexpression systems and anti-FLAG M2 affinity gels in SW1353 cells. The PCR fragments, including the SOX9-binding site on the enhancer region of COL2A1 gene (located at +2405 through +2411, -CATTCAT-) were detected in both Smad2- and Smad3-infected cells (Enhancer). The other fragment, which contains a putative SRY-binding sequence on the enhancer region of COL2A1 gene (located at +2405 through +2411, -CATTCAT-) was not observed in both groups except inputs (Promoter). No fragments were detected in any control immunoprecipitated with mouse IgG. The scheme shows the part of COL2A1 gene structure and the fragments amplified with the indicated primers (arrows). Numbers correspond to the transcription start site of human COL2A1 gene (National Center for Biotechnology Information, AC004801).
These findings suggest that the domain corresponding to 328–423 amino acids of Sox9 is necessary for the associations with Smad2/3 and that Smad2/3 modulate the \( \text{COL2A1} \) expression by forming the SOX9-Smad2/3 complex on the \( \text{COL2A1} \) enhancer region.

**Fig. 5. Smad3 activates the Sox9-dependent transcription by modulating the association between Sox9 and p300.**

A, schematic diagrams of Smad2 and Smad3. Bars under the schema represent each fragment and correspond to the next numbers of amino acids. The region binding to Sox9 and CBP/p300 demonstrates as a bar on the schema. B, immunoprecipitation analyses using FLAG-Sox9 1–507 and HA-Smad2/3 in SW1353 cells. The full-length of Sox9 was co-immunoprecipitated with HA-Smad2/3 and -MH2 domains. No bands were detected each MH1-domain-transfected cells. C, transfected FLAG-Smad2/3 and -MH2 fragments associated with HA-p300 through their MH2 domains. MH1 domains of each Smad did not show significant affinities against p300. Note that the MH2 domain of Smad3 showed strong affinities against both Sox9 and p300. The association between Smad3 and p300 was slightly stronger than the Smad2-p300 interaction. D, the additional transfection of Smad2/3 slightly increased the association between Sox9 and p300 (left panel). Co-transfection of si-Smad3 (200 nM) or si-Smad2/3 (100 nM each) decreased the Sox9-p300 interaction. Note that the inhibitory effect of si-Smad2 (200 nM) against the Sox9-p300 binding was weaker than that of si-Smad3 (right panel). siRNAs were incubated with SW1353 cells for 12 h before the transfections of Sox9 and p300. E, we performed luciferase assays using pKN185 and Gal4-Sox9 systems in SW1353 cells. The SOX9-related co-activator, CBP/p300, stimulated the luciferase activities, especially in the presence of T\( \beta \)R-I(TD) and Smad3 (left panel). Similar results were observed in CBP-or p300-transfected cells, and in Gal4-Sox9 systems (right panel). F, si-Smad3 strongly suppressed the Gal4-Sox9 182–508 activity in the presence of T\( \beta \)R-I(TD) and CBP/p300. The additional inhibitory effect of si-Smad2 was not so remarkable. We obtained similar results using CBP instead of p300. Relative luciferase activities were calculated using the activity of Gal4-TK as a control (100%) in Gal4-Sox9 systems (E and F). Statistical significances (\( p < 0.05 \)) were observed between open and filled bars (E), and among bars (F), in each group using the Mann-Whitney U test. Error bars, S.D.

*Smad3 and CBP/p300 Synergistically Activate the Sox9-dependent Transcription by Associating with Sox9*—We described previously that CBP/p300 modulates the function of Sox9 as a co-activator (27). We further analyzed the relationships among Smad2/3, SOX9, and CBP/p300. Overexpressed Sox9 was co-
SOX9, a high mobility group domain-containing transcription factor, is known to play an essential role in establishing the pre-cartilaginous condensations and initiating chondroblast differentiation (37). Sox9 specifically binds to the Sox9-binding site within the intron 1 of Col2a1 gene to regulate Col2a1 transcription (34, 38). Mutations in Sox9 underlie the rare congenital dwarfism syndrome, campomelic dysplasia (39, 40). Expression of Sox9 nearly parallels Col2a1 expression during chondrogenesis (41), and an analysis of mouse chimaeras using Sox9(-/-) embryonic stem cells shows that Sox9(-/-) cells are excluded from cartilage tissues and unable to express chondrocyte-specific genes such as Col2a1 (42, 43). In the genital ridge, however, Col2a1 is not expressed despite abundant Sox9 expression (41). Overexpressed Sox9 in chondrocytes of mouse embryos produces a phenotype of dwarfism (44). We also observed that adenoviral-overexpressed Sox9 failed to induce a sufficient chondrogenesis using pellet-cultured MSCs (data not shown). These findings suggest the existence of additional regulatory mechanisms for Sox9 to promote Col2a1 gene expression in chondrogenic differentiation.

In addition to DNA-binding transcription factors such as SOX9, various co-activators are involved in transcriptional activation (45–47). The transcriptional co-activator CBP and its paralog p300 are recruited to promoter regions via direct interactions with various sequence-specific activators, including cAMP-response element-binding protein, activator protein 1, signal transducers and activators of transcription, and nuclear hormone receptors (48). Smad3, which recognizes the 4-bp Smad boxes (GTCT or AGAC), also associates with p300 in a TGF-β-dependent manner (49). These co-activators facilitate transcriptional activities by promoting interactions between sequence-specific activators and the RNA polymerase II transcriptional machinery (45–47).

In this regard, we have reported that CBP/p300 acts as the Sox9-related co-activator (27). However, the mechanisms whereby TGF-β-regulated Smad2/3 control primary chondrogenesis and the SOX9-dependent transcription activity on COL2A1 gene are still unclear. In this study, we demonstrated that Smad2/3 associate with SOX9 on the COL2A1 enhancer (Fig. 4D) and that Smad3 mainly stimulates primary chondrogenesis and COL2A1 expression (Figs. 2 and 3) by enhancing the interaction between Sox9 and p300 (Fig. 5, D and E). These findings suggest that CBP/p300 are recruited onto SOX9 by Smad3 in a TGF-β-dependent manner.

TGF-β stimuli on MSCs were converted into Smad2 and Smad3 signals, which are phosphorylated and translocated into nuclei (Fig. 1C). Here, we initially identified SOX9 as a nuclear target of Smad2/3 during chondrogenesis. In our experiments, both Smad2 and Smad3 equally associated with SOX9 in a TGF-β-dependent manner (Fig. 3B). However, Smad3 showed stronger effects on accelerating proteoglycan synthesis monitored by safranin O (Fig. 2A), COL2A1 expression (Fig. 2B), and the transcriptional activities of Col2a1-based reporter plasmid (Fig. 3D) than Smad2 did. Many reports have described the distinct abilities between Smad2 and Smad3 on nucleocytoplasm shuttling, DNA-binding, transcriptional regulation, and cellular differentiation (2, 3, 6, 14, 15). Although stable overexpression of Smad2 or Smad3 inhibits adipogenesis, the dominant negative form of Smad2 did not enhance adipogenesis (50). In transgenic Drosophila, using human Smad2/3 genes, Smad2 induces over-size wings while Smad3 causes cell death (51). The mechanism of the distinct effects of Smad2/3 is still unclear, but it may be explained by their differences of DNA-binding activity, co-operating factor, and/or proteolysis procedure. To explain different functions of Smad2/3 during chondrogenesis, we performed knock-down analyses using their siRNAs. si-Smad3 strongly inhibited the SOX9-dependent transcription (Fig. 2C, D and E), the SOX9-dependent transcriptional activity (Fig. 5F), and the Sox9-p300 interaction (Fig. 5D). However, si-Smad2 did not show remarkable inhibitory effects in our experimental models. These findings suggest that the distinct functions between Smad2 and Smad3 in chondrogenesis may depend on their abilities to stabilize the SOX9-CBP/p300 transcriptional complex. We propose the schematic model that SOX9-regulated chondrogenetic differentiation in Smad- and CBP/p300-dependent manners (Fig. 6). Several reports show that the multifunctional CBP/p300 co-activators stimulate the Smad-dependent transactivation by binding to MH2 domains of Smads and show stronger affinities against Smad3 rather than Smad2 (21, 22, 52–55). Further studies such as in vitro transcription and histone acetyltransferase assays using chromatin templates will be required to investigate the individual functions of SOX9-associated molecules during chondrogenesis.

Animal models for a loss of Smad function have revealed the role of specific Smads in physiological systems (56). Smad2 homozygous mutants exhibit early embryonic lethality due to
abnormalities in anterior-posterior axis formation (57). Mice trans-heterozygous for both Smad2 and Nodal mutations display craniofacial abnormalities (58). Smad3 null mice die within 10 months and show skeletal defects, including osteoarthritis (59). Haploinsufficiency of Smad3 causes an embryonic lethality due to endodermal defects and exhibits craniofacial defects (60). In this study, we analyzed the physiological functions of Smad2/3 using their siRNAs in the early stage of MSC-derived chondrogenesis. Gene silencing of Smad3 decreased proteoglycan synthesis and COL2A1 expression. In addition, double knock-down against Smad2/3 strongly inhibited primary chondrogenic differentiation (Fig. 2, C and E). These findings suggest that Smad2/3 cooperatively act as one of the important TGF-β signaling pathways to develop and/or maintain the phenotype of chondrocyte. In addition to the Smad2/3 pathways, TGF-β activates MAPK pathway during chondrogenesis (61). MAPK pathway stimulates the expression of Col2a1 (62, 63) and Sox9 (64) during chondrogenesis. The transcriptional cross-talk between Smad2 and MAPK pathways has an important role for the expression of aggrecan gene, the major proteoglycan in cartilage, in ATDC5 cells (65). These reports suggest that MAPK pathway would also be involved in chondrogenesis via Sox9 complex modification. It would be interesting to examine whether MAPK may act as a differentiation factor by modifying the formation of SOX9-related transcriptional complex and/or by promoting the expression of the other co-activators.

In conclusion, the present study demonstrates that Smad3 stimulates primary chondrogenesis through forming the transcriptional complex with Sox9 and CBP/p300. Our findings suggest that the functional modulation of Sox9 is a critical step for the initiation of chondrogenesis.

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