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Review

Histone Acetylation Influences the Activity of Sox9-related Transcriptional Complex

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Chondrocyte differentiation is the fundamental process in skeletal development. From the mesenchymal condensation of chondroprogenitors to the hypertrophic maturation of chondrocytes, chondrogenesis is sequentially regulated by cross-talk among transcription factors, growth factors, and chromatin structure. The master transcription factor Sry-type HMG box (Sox) 9 has an essential role in the expression of chondrogenic genes through the association with Sox9-binding sites on its target genes. Several transcription factors and coactivators, such as Scleraxis/E47 and p300, cooperatively modulate the Sox9-dependent transcription by interacting with Sox9. The Sox9-related transcriptional apparatus activates its target gene expression through p300-mediated histone acetylation on chromatin. The transforming growth factor (TGF)-β superfamily also plays a key role in chondrocyte differentiation. The TGF-β-regulated Smad3/4 complex activates Sox9-dependent transcription on chromatin by associating with Sox9 itself, and by recruiting p300 onto Sox9. These findings suggest that the epigenetic status including histone modification and chromatin structure, directly influences Sox9-regulated chondrocyte differentiation. In this article, we review the regulators of Sox9 expression itself, modulators of posttranslational Sox9 function, and Sox9-associating factors in the Sox9-dependent epigenetic regulation during chondrogenesis.

Key words: Sox9, TGF-β-Smad3, coactivator p300, scleraxis/E47, chondrogenesis

Mesenchymal differentiation regulates the development of musculoskeletal systems such as endochondral ossification and synovial joint formation. Chondrogenesis originating from the condensation of pluripotent mesenchymal stem cells (MSCs) has an essential role in skeletal development and articular cartilage formation. The steps of sequential differentiation and maturation from chondroprogenitors to hypertrophic chondrocytes are regulated by transcription factors and growth factors such as the Sry-type high-mobility group box (Sox) genes, the basic helix-loop-helix (bHLH) transcription factor Scleraxis (Scx), the runt-related Runx genes, and the transforming growth factor (TGF)-β superfamily [1–4]. The Sox E protein Sox9, which encodes a high-mobility group (HMG) DNA-binding domain, has been identified as the master transcription factor in chondrogenesis [5, 6]. Sox9 regulates the expression of its target genes through association with the Sox9-

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binding 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*, *cartilage oligomeric matrix protein (COMP)*, and *Cd-rap* [7–13]. Heterozygous mutations in the *SOX9* gene cause the congenital dwarfism syndrome, campomelic dysplasia [14]. Mouse embryonic stem cells derived from *Sox9* (-/-) chimeras are unable to express the *Col2a1* gene [15]. In the genital ridge, however, the Sox9-regulated gene *Col2a1* is not expressed despite abundant *Sox9* expression [16]. In addition, *Sox9* overexpression in chondrocytes produces a phenotype of dwarfism [17]. These reports suggest that additional mechanisms cooperatively regulate Sox9-dependent transcription during chondrogenesis, and that chondrocyte differentiation is not controlled by Sox9 alone.

The posttranslational modification of Sox9 also affects the Sox9-dependent transcription in chondrogenesis [18, 19]. Phosphorylation, sumoylation, and ubiquitination of the Sox9 protein influence the functional activity of Sox9. Protein kinase A-induced phosphorylation of Sox9 enhances Sox9-dependent transcription by increasing the DNA-binding affinity of Sox9 [20]. On the other hand, Sox9 activity is suppressed by PIAS1-mediated sumoylation of Sox9 [21]. The ubiquitin-proteasome pathway also inhibits Sox9 transcriptional activity by inducing the degradation of Sox9 [22]. These findings suggest that the expression and stability of Sox9 are regulated by many factors at each developmental stage during chondrogenesis. The group D Sox5 and Sox6, which possess a leucine zipper and a coiled-coil domain, cooperate with Sox9 to activate the expression of *Col2a1* and *aggrecan* genes [23]. Whereas *Sox5* and *Sox6* single-null mice are born with mild skeletal abnormalities, *Sox5/6* double mutants die with severe, generalized chondrodysplasia [24]. The transactivation of *Sox5/6* is necessary for the sequential chondrocyte differentiation in Sox9-expressing chondroprogenitors [25]. In the absence of Sox5/6, sclerotome MSCs are prevented from differentiating into chondrocytes, and switch their fate to *Scx*-expressing tendon/ligament progenitors [25]. However, the Sox5/6-dependent modulation of Sox9 function is not fully understood. The association between Sox9 and Sox5/6 has not been detected either. The gene transactivation by Sox5/6 and Sox9 is much stronger than the activation by Sox9 alone, while Sox5 and Sox6 do not influence the activity of gene expression in the absence of Sox9. The MYST family coactivator Tip60, which mainly acetylates H4, increases Sox9/Sox5-dependent *Col2a1* transcription by associating with Sox9 on chromatin [26]. Sox5/6 may stabilize Sox9 on its binding site through the bending of DNA and thereby stimulate Sox9-regulated gene expression [18, 19, 27].

The transforming growth factor (TGF)-β superfamily, including 2 major families, TGF-β and bone morphogenetic protein (BMP), comprises multifunctional growth factors for many cellular processes such as proliferation, differentiation, and apoptosis [28]. In chondrocyte differentiation, TGF-β stimulation is necessary for MSC-derived primary chondrogenesis [29]. On the other hand, chondrocyte maturation in the hypertrophic stage is inhibited by TGF-β [30]. Several pathways following the activation of TGF-β receptors such as Smad2, Smad3, and mitogen-activated protein kinase (MAPK) have been identified as key intracellular signals in response to TGF-β treatments [28, 31]. We have previously demonstrated that TGF-β-regulated Smad3 induces primary chondrogenesis through the association with Sox9 [32]. Smad3 also associates with other transcription factors, such as the osteogenic inducer Runx2, the myogenic factor MyoD, and the coactivator p300 [33–35]. In addition to the TGF-β-regulated Smad2/3 pathways, the MAPK pathway stimulates the expression of *Sox9* and *Col2a1* during chondrogenesis [36–38]. The Smad and MAPK pathways cooperatively regulate the expression of the Sox9-regulated *aggrecan* gene [39]. From these findings, chondrogenesis is considerably regulated by the transcriptional cross-talk between transcription factors and growth factor signals. However, the epigenetic cross-talk between Sox9 and TGF-β signaling has not been elucidated in the transactivation of chondrogenic genes on chromatin.

Epigenetics is defined as gene-regulating activity that does not involve changes in the underlying DNA information. Epigenetic regulation such as DNA methylation, histone modification, and chromatin remodeling has been highlighted. The fundamental unit of eukaryotic chromatin, the nucleosome, consists of 146 bp of genomic DNA wrapped around a histone octamer (2 sets each of H2A, H2B, H3, and H4 core histones) [40]. Posttranslational histone modification

including acetylation, methylation, phosphorylation, ubiquitination, and ADP-ribosylation determines the stability and/or instability of the chromatin structure [41, 42]. In condensed chromatin (heterochromatin), the expression of the transcription factor-regulated gene is inactivated. On the other hand, transcription factors and coactivators activate their target gene expressions in relaxed chromatin (euchromatin). The degree of chromatin folding directly influences the activity of DNA in transcription, replication, and recombination [43]. In this review, we focus on Sox9-dependent transcription and epigenetic regulation during chondrogenic differentiation.

The TGF-β Superfamily Regulates the Gene Expression of *Sox9* and *Scx* in Chondrogenesis

The expression of *Sox9* itself is modulated by transcription-related factors, growth factors, and cytokines [18, 19]. Several molecules have been reported to regulate the promoter activity of *Sox9*. Sonic Hedgehog, the key inductive signal in the patterning of the anterior-posterior limb axis, increases *Sox9* promoter activity [44]. Sp1 and CREB transcription factors enhance *Sox9* expression by associating with the Sp-1 and CRE sites in the Sox9 proximal promoter on chromatin, respectively [45]. In addition, hypoxia-inducible factor 1α is necessary for mesenchymal chondrogenesis by the direct induction of *Sox9* transcription [46]. *Sox9* expression also depends on complicated regulatory mechanisms in response to growth factors, cytokines, and organic compounds. Fibroblast growth factor (FGF) 1, FGF2, and insulin-like growth factor 1 up-regulate the expression of *Sox9* [36, 47]. Histone deacetylase (HDAC) inhibitors, including trichostatin A (TSA) and FK228, have the synergistic potential to induce *Sox9* expression via enhanced recruitment of nuclear factor Y (NF-Y) to the proximal promoter of *Sox9* [48]. On the other hand, *Sox9* expression is inhibited by inflammatory cytokines such as interleukin (IL)-1β and tumor necrosis factor α in chondrocytes [45, 49]. IL-1β treatment down-regulates *Sox9* transactivation by a reduction of Sp1 binding to the *Sox9* promoter [45].

Recent studies have revealed that the TGF-β superfamily has 2 contrary effects in chondrocyte differentiation. TGF-β treatments up-regulate *Sox9* expression in the developing limb mesenchyme, but the simultaneous induction of transcriptional repressor TGF-interacting factor 1 (Tgif1) inhibits this role [50]. BMP-2, an osteochondrogenic factor, stimulates *Sox9* expression by increasing the association between the NF-Y-p300 complex and the *Sox9* promoter. BMP-2 also induces histone hyperacetylation at the *Sox9* gene on chromatin [51]. On the other hand, the BMP-2 inhibitor Noggin represses *Sox9* expression in limb bud chondrogenic precursors while inducing the ligament/tendon-specific transcription factor *Scx* [52]. In micromass cultures of undifferentiated mesodermal cells, the TGF-β/Smad signal is a direct inducer of *Sox9* and *Scx*, but transcriptional repressors of the TGF-β signal such as Tgif1 and SnoN modulate the expression of *Sox9* and *Scx* [50]. In our previous studies, TGF-β3 and BMP-2 cooperatively regulated the expression of *Sox9* and *Scx* along with chondrogenesis [53]. The expression volume of *Sox9* and *Scx* also influenced *Col2a1* expression and the progress of chondrogenesis [4, 53]. Recent studies and our findings suggest that *Sox9* induction is positively regulated by TGF-β and BMP-2 in the early step of chondrocyte differentiation. On the other hand, *Sox9* expression is concurrently inhibited by TGF-β-induced negative signals. The TGF-β superfamily might modulate the expression balance between *Sox9* and *Scx*, partly by recruiting TGF-β-induced repressors of *Sox9* expression, according to each differentiation stage of chondrogenesis.

TGF-β-regulated Smad3, p300, and Scx/E47 Cooperatively Stimulate Sox9-dependent Transcription by a Direct Association with Sox9

The multifunctional coactivator p300 has an important role in gene expression and cellular differentiation. p300 acts as a protein scaffold and a bridging factor for the assembly of the transcriptional apparatus; in addition, the histone acetyltransferase (HAT) activity of p300 has the potential to facilitate transcriptional activity by modulating the chromatin structure [54]. In chondrogenesis, p300 stimulates transcription factor-mediated chromatin disruption. The CH3 domain of coactivator p300 directly associates with the C-terminal PQ-rich transactivation domain of Sox9, and activates Sox9-dependent transcription in chondrogenesis [55]. We have demonstrated that Sox9-dependent transactivation is induced

by p300-mediated histone acetylation of chromatin [56]. HAT analyses revealed that the histone acetylation of a chromatinized DNA template, which included multiple Sox9-binding sequences, was activated under the presence of Sox9 and p300 [56]. *In vitro* transcription and S1 nuclease assays also showed that Sox9-dependent transcription on assembled chromatin was up-regulated by a Sox9-p300 transcriptional complex [56]. These findings suggest that the Sox9-related coactivator p300 is necessary in the epigenetic initiation of chondrogenesis.

In our previous studies, TGF-β-regulated Smad3, but not Smad2, promoted MSC-derived primary chondrogenesis through the activation of the Sox9 function via p300 recruitment [32]. The MH2 domain of Smad3 interacted with the PQ-rich domain of Sox9 and the C-terminal transactivation domain of p300. Smad3 also stabilized the association between Sox9 and p300 by forming a transcriptional apparatus with Sox9 and p300 [32]. The transactivation of Sox9-regulated reporter genes was synergistically increased in the presence of Sox9, TGF-β, Smad3, and p300, but was suppressed by Smad3 si-RNA and Smad7, the main inhibitor of Smad2/3 phosphorylation [32, 57]. In addition, the bHLH transcription factor Scx and its partner E47 cooperatively stimulated Sox9-dependent transcription through the formation of a transcriptional complex with Sox9 and p300 [4]. The Scx/E47 heterodimer also associated with the conserved E-box sequence (CAGGTG) in the *Col2a1* promoter on chromatin [4]. These results indicate that the Sox9-associating coregulators such as TGF-β-activated Smad3, p300, and Scx/E47 have an essential role in Sox9-dependent chondrogenesis.

On the other hand, Sox9-induced Sox5/6 inhibited the expression of *Scx* in the middle stage of chondrogenesis [25]. In valvulogenesis, Sox9 and Scx were shown to be involved in heart valve compartmentalization [52]. BMP-2-induced Sox9 regulates the development of valve leaflets [52]. Although both *Sox9* and *Scx* are expressed in heart valve precursors, Scx regulates the differentiation of valve supporting structures [52]. These findings suggest that the cross-talk between Sox9 and Scx might be influenced by different developmental stages in chondrogenesis and valvulogenesis. Scx and E47 modulated the primary chondrogenic status by associating with the Sox9-related transcriptional apparatus, and by binding to the conserved E-box on the *Col2a1* promoter [4] (Fig. 1). After the induction of Sox5/6 (at the proliferation stage in chondrocyte differentiation), Scx might shift its transcriptional ability from a chondrogenic factor to a tendon/ligament differentiation factor.

Other Sox9-associating molecules have been investigated. Sox9 interacts with the Med12/Trap230 subunit of the mediator complex to stimulate RNA polymerase II-dependent transcription in chondrocytes [58]. Med12/Trap230 acts as an essential bridging factor between Sox9 and the RNA polymerase II transcriptional machinery. Peroxisome proliferator-activated receptor γ coactivator 1α (Pgc1α), which is involved in gluconeogenesis, stimulates Sox9-dependent transcription including *Col2a1* and *COMP* expression via direct association with Sox9 [59]. Sox9 and the homeobox transcription factor Barx2 cooperatively bind to adjacent sites in the *Col2a1* enhancer, and regulate chondrogenesis during limb development [60]. These reports suggest that the balance of Sox9-related factors, involved in the transcriptional complex formation and its DNA-binding activity, has an important role in Sox9-dependent transcription during chondrocyte differentiation (Fig. 1) [61].

Sox9, Smad3, and p300 Cooperatively Activate the Gene Expression Derived from Chromatin during Chondrogenesis

Epigenetics is an essential mechanism to control gene expression and fundamental cellular processes such as proliferation and differentiation [62–64]. Recent studies have revealed that multifunctional HAT complexes modulate the condensed chromatin structure by inducing histone acetylation [43, 65]. The acetylation of lysine residues in histone tails neutralizes their positive charge, thereby relaxing the chromatin structure. HAT complexes increase the accessibility of transcription factors to their target genes, and have a key role in epigenetic regulation [66, 67]. We previously reported that p300 potentiates Sox9-dependent transcription on a chromatinized DNA template and is associated with histone acetylation [56]. In addition, histone hyperacetylation using the HDAC inhibitor TSA enhanced Sox9-regulated cartilage matrix gene expressions (*COL2A1* and *aggrecan*) in human chondrocytes [56]. We have further ana-

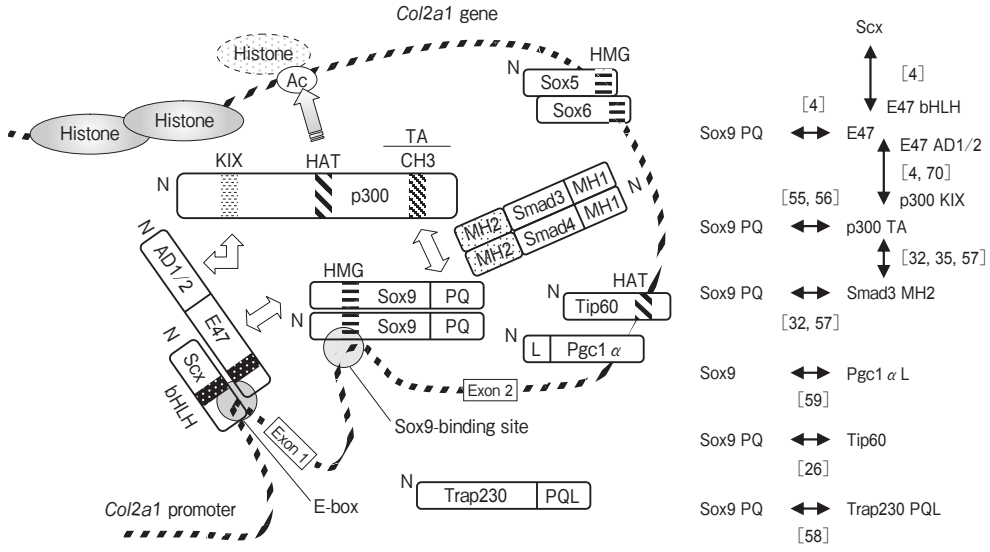

Fig. 1 Schematic illustration of Sox9-dependent transactivation on chromatin. The Sox9 homodimer recognizes the Sox9-binding site, which is conserved in the enhancer region of *Col2a1* intron 1. At this stage, however, *Col2a1* expression is not fully activated on the chromatin structure. The HAT activity of coactivator p300 has an essential role in inducing histone acetylation (Ac) and chromatin unfolding. The TGF-β-regulated Smad3/4 complex enhances Sox9-dependent transcription on chromatin through association with Sox9 and p300. The Scx/E47 heterodimer stimulates *Col2a1* expression by associating with the Sox9-related transcriptional apparatus, and by binding to the E-box on the *Col2a1* promoter. Other Sox9-related factors also play a key role in Sox9-dependent transactivation. Arrows denote the interaction between the indicated molecules (or domains). References are shown as numbers. TA, transactivation domain.

lyzed the cross-talk between Sox9-dependent transcription and TGF-β-regulated Smad3 in epigenetic regulation using an *in vitro* chromatin assembly model [57, 68]. The TGF-β-activated Smad3/4 complex directly associates with purified Sox9 and p300. *In vitro* transcription and S1 nuclease analyses revealed that Smad3/4, Sox9, and p300 cooperatively activated Sox9-dependent transcription on a chromatinized DNA template [57]. Our results suggest that the TGF-β signal Smad3 plays a key role in epigenetic regulation of chondrogenesis via its association with the Sox9/p300 transcriptional apparatus, and indicate that TGF-β treatment may be necessary for the transactivation of Sox9-regulated genes from the region of the inactivated (condensed) chromatin structure. From these studies, we consider that Sox9 may activate the transcription of its target genes in a multistep fashion, first inducing coactivator-dependent histone acetylation around Sox9-binding sites, then relaxing the chromatin structure and recruiting the Sox9-interacting activators and transcription apparatus for specific gene expression during chondrogenesis (Fig. 1) [69].

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