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REVIEW ARTICLE

Control of chondrogenesis by the transcription factor Sox9

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Abstract Cell-fate determination of pluripotent cells, cell proliferation, differentiation, and maturation, as well as the maintenance of stem cells, are essential cellular events during organogenesis. Previous reports show that some distinct cell-specific transcription factors are the master genes that control cell lineage commitment and the subsequent cell proliferation and differentiation. Some of these transcription factors generate hierarchical regulation of expression and act in concert to fulfill their roles. This review discusses the molecular properties and mechanisms of Sry-related high-mobility-group box (Sox) transcription factor, Sox9, in chondrogenesis.

Keywords Sox9 · Chondrogenesis

Introduction

Sox transcription factors were originally identified in Sry, the male sex-determination transcription factor, a gene localized on the Y chromosome. They contain the high-mobility-group box (HMG) domain that contributes to DNA binding in the minor groove, bending DNA, interaction with other transcription factors, and nuclear import or export. Twenty Sry-related high-mobility-group box (Sox) genes have been identified in the human and mouse, and these are divided into eight subgroups [1]. Along with Sox8 and Sox10, Sox9 belongs to group E, whose members have a well-conserved HMG domain and a transactivation domain. Sox5, Sox6, and Sox13 belong to group D, whose members have an HMG domain but no transactivation domain, suggesting that these Sox proteins act as architectural organizers.

Recent genetic approaches for mouse reveal that Sox proteins play pivotal roles in organogenesis in several organs. For example, Sox9 is required for testogenesis, chondrogenesis, terminal differentiation of oligodendrocytes, and endocardial cushion formation in cardiogenesis [2–5]. Sox5 and Sox6 are involved in proper chondrocyte differentiation and in the repression of specification and terminal differentiation of oligodendrocytes [6, 7].

Gene targeting of Sox9 in chondrogenesis

Sox9 was identified in 1994 as a causative gene of campomelic dysplasia [8, 9], a rare skeletal dysplasia associated with XY sex reversal [10]. Campomelic dysplasia represents an autosomal dominant condition caused by haploinsufficiency of the gene. Heterozygous mutation in and around Sox9 causes the distinct clinical features in patients including disproportionately short stature, bowing of the limbs, low ears, a depressed nasal bridge, talipes equinovarus, long philtrum, and micrognathis. Radiological findings show bowing of the long bones, hypoplasia of the scapula, narrow iliac wings, and a small thorax with slender ribs which suggest an important role of Sox9 in developing bone and cartilage.

During mouse embryogenesis, Sox9 is expressed in all chondroprogenitors and chondrocytes except hypertrophic chondrocytes, as well as in the male gonad, otic vesicle, heart, kidney, pancreas, intestine, and neural crest (Fig. 1). The expression of Sox9 overlaps largely with that of Col2a1, a major cartilage matrix protein, suggesting that

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Fig. 1 Sox9 expression during mouse embryogenesis

Sox9 controls Col2a1 expression [11, 12]. In vitro and in vivo analyses showed chondrocyte-specific regulatory sequences in the first intron of the Col2a1 gene, which includes four imperfect Sox-binding sites [13]. In chondrocytes, Sox9 binds as a homodimer to a pair of the consensus sequences of Col2a1, Col9a1, Col27a1, or Matrilin-1, and this binding is mediated by a dimerization domain located closer to the N-terminus than the HMG domain [14–18]. The mutants in this domain bind as a monomer, which reduces their binding ability and causes campomelic dysplasia.

Chondrogenesis is a multi-step cellular event. First, undifferentiated mesenchymal cells are committed to a chondrogenic cell lineage, aggregate with each other, and differentiate into chondrocytes. This is followed by unidirectional proliferation, production of matrix proteins, maturation, hypertrophic conversion, and finally replacement by bone. The chondrocytes form growth plates and contribute to the longitudinal skeletal growth. Bi et al. [19] created a null allele of Sox9 using a mouse genetics approach. Heterozygous Sox9 mutants exhibited the same skeletal abnormalities as campomelic dysplasia. Further close analysis revealed small and delayed chondrogenic mesenchymal condensation and enlargement of the hypertrophic zone in association with premature mineralization. These data provide strong evidence that an adequate level of Sox9 is required for both mesenchymal condensation and physiological inhibition of hypertrophic conversion of proliferating chondrocytes. Heterozygous Sox9 mutants die a couple of hours after birth because of respiratory distress, and homozygous mutants cannot be generated by the conventional mating scheme of heterozygous mutants with each other. To overcome this problem, mouse chimeras were generated using Sox9 homozygous mutant embryonic stem cells in which the LacZ gene was knocked in at both Sox9 loci [20]. Interestingly, Sox9-null cells are excluded from aggregated wild-type cells in mesenchymal condensation. In addition, Sox9-null cells do not express chondrogenic marker genes, such as Col2a1, Col9a2, Col11a2, and aggrecan. This chimera analysis strongly suggested that Sox9 is required

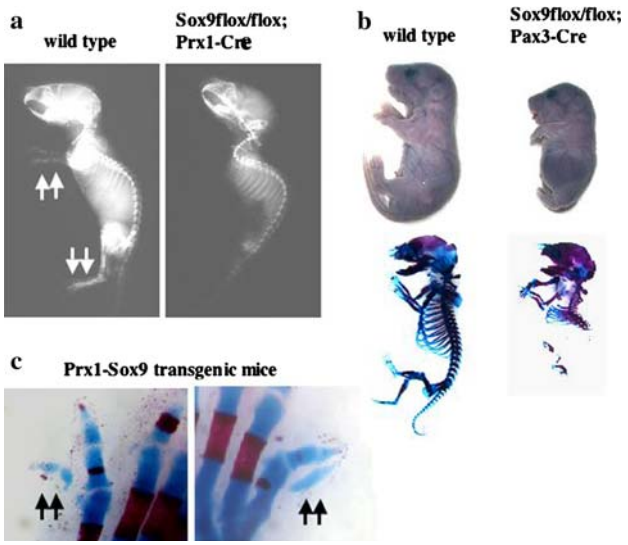

Fig. 2 **a** X-ray shows no bones in fore- and hindlimbs of Sox9flox/flox; Prx1-Cre mice. The *arrows* indicate fore- and hindlimbs of wild-type littermates. **b** Sox9flox/flox; Pax3-Cre mice lack some bones and cartilage derived from trunk neural crest. **c** Prx1-Sox9 transgenic mice exhibit ectopic cartilage formation (*arrows*)

for the commitment of a chondrogenic cell lineage and the formation of mesenchymal condensation. Conditional targeting of Sox9 genes using the Cre recombinase-loxP recombination system was used to analyze further the requirement for Sox9 in mesenchymal condensation (Fig. 2a, b). When Sox9 was inactivated before mesenchymal condensation using the Prx1-Cre transgene, mesenchymal condensation was totally absent and chondrogenic marker genes were not expressed [2]. In addition, the expression of Runx2, an osteogenic marker gene, was missing, suggesting that Sox9 is needed for the commitment of osteochondroprogenitors. Inactivation of Sox9 at and after mesenchymal condensation using the Col2a1-Cre transgene arrests differentiation of condensed mesenchymal cells at condensation and impairs the proper proliferation and maturation of differentiated chondrocytes [2]. These phenotypes of conditional mutants result mainly from the abolishment of Sox5 and Sox6 expression as the downstream targets of Sox9.

Loss-of-function analysis with Sox9-null alleles and the conditional allele demonstrate the essential roles of Sox9 in chondrogenesis. However, it is unknown how Sox9 controls chondrogenesis. The mutants in which Sox9 cDNA was knocked in to the Col2a1 locus and which overexpressed Sox9 in chondrocytes displayed chondrodysplasia through inhibition of chondrocyte proliferation [21]. In these mutants, overexpression of Sox9 downregulated cyclin D1 expression, which is mediated by the direct interaction between Sox9 and β-catenin at the protein level, suggesting crosstalk between the Sox9 and Wnt canonical pathway. The

conditional inactivation and overexpression of Sox9 in chondrocytes in mice cause similar skeletal phenotypes involving conditional stabilization and conditional inactivation of β-catenin in chondrocytes, respectively.

Sox9 as the master regulator of chondrogenesis

During cell lineage determination and subsequent cell differentiation in embryogenesis, a set of transcription factors act as the master regulators: the MyoD family in myogenesis [22, 23] and Runx2-Osterix in osteogenesis [24–26]. Both loss-of-function and gain-of-function analyses using mutant mice demonstrate that Sox9 is required for the commitment of osteochondroprogenitors, chondrogenic mesenchymal condensation and proper chondrocyte proliferation, differentiation, maturation and hypertrophic conversion, suggesting that Sox9-Sox5, Sox6 form the master regulatory axis of chondrogenesis. Overexpression of Sox9, Sox5, and Sox6 in cultured cells [27] and ectopic expression of Sox9 in mice induce the expression of type II collagen [28, 29]. Moreover, ectopic expression of Sox9 with retrovirus in chick limb bud induces alcian blue-stainable cartilage [30]. Transgenic mice in which Sox9 was ectopically expressed in limb bud mesenchyme using the Prx1 promoter exhibited ectopic cartilage formation in association with the induction of ectopic Sox5 and Sox6 expression without any patterning defects in limb bud development (Fig. 2c) [31]. These lines of evidence clearly indicate that Sox9-Sox5, Sox6 are the master regulators of chondrogenesis.

Regulation and modification of Sox9 expression and molecular functions

The mechanism underlying the regulation of Sox9 expression in chondrocytes is still unclear. Several studies have reported that fibroblast growth factor (FGF), insulin-like growth factor I (IGF-I), human cartilage glycoprotein 39, transient receptor potential vanilloid 4 (TRPV4), RAR agonists, and Src inhibitor increase Sox9 expression [32–37]. CCAAT-binding factor, Sp1, CREB, Sonic hedgehog, and hypoxia-inducible factor 1α directly transactivate the Sox9 proximal promoter [38–42]. Other transcription factors including Pax1, Pax9, Nkx3.1, Nkx3.2, and Barx2 control the level of Sox9 [43]. However, control of chondrocyte-specific Sox9 expression is more complicated. Mutation analysis of the Sox9 gene in campomelic dysplasia patients shows that some of the chromosome translocation breakpoints disperse over a large distance between 50 and 300 kb and beyond 900 kb from the transcription start site [44, 45]. In addition, promoter analysis using YAC transgenes suggests that chondrocyte-specific regulatory element is located between 75 and 350 kb proximal to the transcription start site [46].

The posttranslational modification of Sox9 protein affects the activity of Sox9. Sox9 is thought to inhibit hypertrophic conversion. This activity is regulated by phosphorylation of S64 and S211 of Sox9 by protein kinase A (PKA), a downstream intracellular signaling molecule of parathyroid hormone-related peptide (PTHrP)/PTHrP receptor [47, 48]. This phosphorylation increases the DNA-binding affinity and transactivation activity of Sox9. Translocation of Sox9 into the nucleus is controlled by Sox9-calmodulin interaction through the nuclear localization signal of the HMG domain within a consensus calmodulin-binding region [49]. Mutation in this region decreases the ability of Sox9 to activate transcription of cartilage genes and causes campomelic dysplasia. cGMP-dependent protein kinase type II reduces the activity of Sox9 by inhibiting the nuclear import of Sox9, a process that is mediated by phosphorylation of S181 [50]. Sox9 activity is also regulated by protein inhibitor of activated STAT1 (PIAS1)-mediated sumoylation [51–53]. PIAS1 enhances the sumoylation at K398 and represses Sox9 activity. The stability and degradation of Sox9 protein are determined by the ubiquitin-proteosome pathway [54].

Transcriptional mechanism of Sox9

L-Sox5, a large isoform of Sox5, and Sox6 have essential roles in chondrogenesis [6, 55, 56]. Both genes are coexpressed with Sox9 in chondrogenesis. Mutant mice with the conditional Sox9 alleles and the ectopic expression of Sox9 indicate that L-Sox5 and Sox6 are downstream targets of Sox9 [2, 31]. Sox5/Sox6 double-null mutant mice have normal mesenchymal condensation but no overt chondrocyte differentiation in association with severe reduction in the expression of Col2a1, Col9a2, Col11a2 [6]. Sox5 and Sox6 harbor two coiled-coil domains that mediate heterodimerization and homodimerization. In chondrocytes, Sox9 homodimer and Sox5/Sox6 hetero/homodimer may bind to the chondrocyte-specific enhancer in the first intron of Col2a1, which features four HMG domain-binding sites, and may activate Col2a1 gene in a chondrocyte-specific manner. The homeobox transcription factor Barx2 and c-Maf transcription factor also bind to the chondrocyte-specific enhancer of Col2a1 and cooperate with Sox9 to activate Col2a1 gene [57, 58].

DNA is wrapped around histone proteins to form the chromatin structure. The transcription of a gene into mRNA is dependent on activators that recruit the mediator coactivator complex, which bridges DNA-bound activators and the general transcriptional machinery, especially RNA

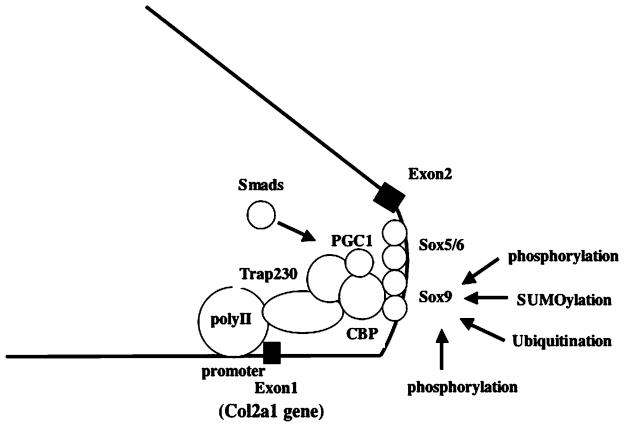

Fig. 3 Schema of the putative transcriptional mechanisms of Sox9 in chondrocytes

polymerase. Recent studies show that Sox9 uses a cAMP response element-binding protein-binding protein (CBP)/p300 to exert its effects, which is mediated by histone acetylation [59]. In chondrogenesis, Smad3, a signaling molecule of transforming growth factor-β,(TGF-β) stimulates the Sox9-dependent transcriptional activation by modulating the interaction between Sox9 and CBP/p300 [60]. In addition, peroxisome proliferation-activated receptor-gamma co-activator 1-α (PGC1-α) is a coactivator of Sox9 during chondrogenesis [30]. A yeast two-hybrid assay showed that Sox9 interacts with a component of the mediator complex, the thyroid hormone receptor-associated protein (Trap) 230/Med12 [61]. The Sox9a/Sox9b double mutant of zebrafish strongly resembles the zebrafish Trap230/Med12 ortholog mutant [62]. Thus, Sox9 is thought to be an activator that communicates with the general transcription machinery through the Trap complex (Fig. 3).

Possible clinical application of Sox9

Sox9 is indispensable for the commitment of the chondrogenic cell lineage and the expression of the chondrocyte-specific matrix proteins, including type II, IX, and XI collagen and aggrecan. Interestingly, Sox9 expression decreases in cartilage in individuals with osteoarthritis compared with age-matched controls [63]. The chondrocyte-specific matrix proteins are needed for maintenance of biomechanical properties of the articular cartilage. Static compressive force promotes type II collagen and aggrecan expression mediated by an increase in Sox9 expression [64]. These findings suggest that Sox9 has potential clinical value.

Several trials have studied treatment of hyaline articular cartilage damage. In one study, Sox9 cDNA was transfected into bone marrow-derived mesenchymal stem cells, and these cells were cultured in high-density micromass culture transplanted into athymic mice after being loaded into the diffusion chamber in vivo. In both the in vitro and the in vivo systems, these cells formed matrix-rich aggregates that stained with alcian blue and for type II collagen [65]. Other trials involve transduction with Sox9 in passaged osteoarthritic articular chondrocytes. In these cells, Sox9 stimulated type II collagen expression and glycosaminoglycan synthesis, and caused recovery of the features of chondrocyte phenotypes [66–68]. These lines of evidence support the concept of Sox9 gene therapy in the treatment of osteoarthritis.

Another trail focuses on the treatment of intervertebral degenerative disc diseases. Sox9 is expressed uniformly in newborn healthy disc cells, and Sox9 expression is lost during aging and disc degeneration, resulting in decreased expression of type II collagen [69]. Adenovirus vectors expressing Sox9 efficiently transduce degenerated human disc cells in vitro and rabbit intervertebral disc cells in vivo, and upregulate type II collagen production [70, 71]. Thus, a viral vector expressing Sox9 has potential in the treatment of human degenerative disc diseases.

No pharmacological approach to the regulation of Sox9 expression and function in chondrocytes has been reported. In breast cancer cells, retinoic acid receptor agonists stimulate Sox9 gene expression [36], and in embryonic gonads, prostaglandin D2 and its receptor, DP1, agonist stimulate Sox9 activity through induction of the nuclear import of Sox9 [72]. It is possible that pharmacological reagents will be developed to activate Sox9 in chondrocytes.

Rheumatoid arthritis and Sox9

Inflammatory cytokines, including IL-1, IL-6, and TNF-α, play critical roles in the pathology and destruction of articular cartilage in rheumatoid arthritis. The expression and roles of Sox9 in cartilage in individuals with rheumatoid arthritis remain unknown, but previous studies indicate that these inflammatory cytokines markedly decrease type II collagen expression and that this decreased expression correlates with the decrease in Sox9 expression [73, 74]. There is no clinical evidence that Sox9 mediates articular cartilage repair in rheumatoid arthritis. An in vitro study showed that FGF1, FGF2, and FGF9 increase the expression of Sox9 and antagonize inflammatory cytokine-mediated repression of Sox9 expression [75]. In addition, in vivo evidence shows anti-TNF-α antibody-induced repair of articular cartilage in polyarthritic transgenic mice [76]. Moreover, treating patients with rheumatoid arthritis with etanercept, anti-TNF-α antibody, results in remission

and repair of structural damage in rheumatoid arthritic joints [77]. These lines of evidence suggest that treatment with antibodies to inflammatory cytokines can repair the articular cartilage by increase Sox9 expression.

Conclusions

It is clear that Sox9 is required for the commitment of chondrogenic cell lineage, mesenchymal condensation, and, in cooperation with Sox5 and Sox6 downstream of Sox9, the subsequent chondrocyte proliferation, maturation, and matrix production. Future studies should help decipher the molecular targets, mechanisms, and interactions, and the transcriptional mechanisms of Sox9 in chondrogenesis. Clinical trials that target Sox9 are anticipated in the treatment of human joint diseases.

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