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Unraveling the transcriptional regulatory machinery in chondrogenesis

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Abstract

Since the discovery of *SOX9* mutations in the severe human skeletal malformation syndrome campomelic dysplasia in 1994, Sox9 was shown to be both required and sufficient for chondrocyte specification and differentiation. At the same time, its distant relatives Sox5 and Sox6 were shown to act in redundancy with each other to robustly enhance its functions. The Sox trio is currently best known for its ability to activate the genes for cartilage-specific extracellular matrix components. Sox9 and Sox5/6 homodimerize through domains adjacent to their Sry-related high-mobility-group DNA-binding domain to increase the efficiency of their cooperative binding to chondrocyte-specific enhancers. Sox9 possesses a potent transactivation domain and thereby recruits diverse transcriptional co-activators, histone-modifying enzymes, subunits of the mediator complex, and components of the general transcriptional machinery, such as CBP/p300, Med12, Med25, and Wwp2. This information helps us begin to unravel the mechanisms responsible for Sox9-mediated transcription. We review here the discovery of this master chondrogenic trio and its roles in chondrogenesis in vivo and at the molecular level, and we discuss how these pioneering studies open the way for many additional studies that are needed to further increase our understanding of the transcriptional regulatory machinery operating in chondrogenesis.

Keywords

Sox9; Sox5; Sox6; Transcription; Chondrogenesis

Introduction

Cartilage is a unique and essential tissue in vertebrates. More than 95% of bone tissue forms during embryonic and post-natal development through endochondral ossification, a process whereby bone is laid upon a cartilage template. In adulthood, cartilage remains mainly in the joints, where it allows articulation of bones and shock absorption. These properties are assumed by an abundant and highly hydrated extracellular matrix, composed of cartilage-specific collagens, proteoglycans, glycosaminoglycans, and glycoproteins. Abnormalities in this cartilage-specific extracellular matrix cause a variety of skeletal malformation

syndromes and adult-onset degenerative disorders, such as osteoarthritis. More complete understanding of these diseases requires clarification of the molecular mechanisms underlying chondrocyte differentiation and matrix production. In this review, we recall the history of seminal discoveries that have led in the past three decades to our current understanding of the transcriptional regulation of chondrogenesis.

From dawn to identification of chondrogenic master transcription factors

The search for chondrogenic master transcription factors began in the early 1980s based upon the following criteria: (1) the factors are expressed in all chondrocytes; (2) disruption of their genes causes severe chondrodysplasia; (3) the factors are required to activate cartilage-specific genes; and (4) their ectopic expression is sufficient to activate these genes. This search initially focused on transcriptional regulation of *Col2a1* because this gene encodes collagen type II that is an early, very abundant, and highly specific product of chondrocytes. The first study was reported by Yamada's group in 1987 [1]. This group demonstrated that an 800-base pair (bp) sequence located in the first intron of the rat *Col2a1* gene was sufficient to drive specific expression of the *Col2a1* promoter in chondrocytes. As a typical enhancer, this sequence was active in chondrocytes whether placed upstream or downstream of the promoter and whether inserted in forward or reverse orientation. Subsequently, in 1995, Yamada's group published in vitro data suggesting that *Col2a1* transcription possibly relied on the formation of a nuclear protein-mediated complex involving a *Col2a1*-binding zinc finger protein (CIIZFP) binding to the enhancer and Sp1 bound to a GC box in the promoter [2]. Within a year, they then delineated the minimal active region of the enhancer to a 100-bp sequence and showed that it contained several conserved motifs, including two AT-rich motifs, mediating the high-level, chondrocyte-specific activity of the enhancer [3]. Electrophoretic mobility shift assays (EMSA) revealed distinct chondrocyte-specific complexes forming with these elements. During the same period, de Crombrughe's group generated transgenic mouse lines, in which a mouse *Col2a1* sequence containing a 3 kb of the promoter, exon 1, and 3 kb of intron 1 was sufficient to drive high expression of the *Escherichia coli* β -galactosidase gene (*LacZ*) in chondrocytes in vivo and in primary culture [4]. The following year, the group reported on the identification of a 231-bp chondrocyte-specific enhancer in the first intron of *Col2a1* using a new highly stable and well-differentiated rat chondrosarcoma cell line [5]. Interestingly, this 231-bp enhancer overlapped with most, but not the entire sequence of the minimal 100-bp enhancer reported by Yamada's group.

At the same time, Scherer's group and Goodfellow's group demonstrated that the human skeletal dysmorphism syndrome, campomelic dysplasia, was caused by heterozygous mutations in and around the gene for the SRY-related high-mobility-group (HMG) box transcription factor 9 (*Sox9*) [6, 7]. This autosomal dominant condition is most lethal in the perinatal period due to respiratory distress. Its distinct clinical features include disproportionately short stature, bowing of the limbs, low-set ears, depressed nasal bridge, talipes equinovarus, long philtrum, and micrognathia. Radiological findings show bowing of the long bones, hypoplasia of the scapulae, narrow iliac wings, and a small thorax with slender ribs. In addition to skeletal defects, the disease is often accompanied with XY sex reversal and malformation of the heart and other internal organs. This finding jump-started research to identify the roles of *Sox9* in skeletogenesis and other developmental processes.

Sox9 was found to have a very specific expression pattern [8]. In the chondrocyte lineage, its expression starts at the mesenchymal osteochondroprogenitor stage. It remains high in all differentiated chondrocytes until the cells reach hypertrophy in the growth plate. Using EMSA in vitro and transgenic mice in vivo, Koopman's group and Cheah's group reported that *Sox9* bound to two sites in a 309-bp *Col2a1* intron 1 sequence and thereby likely

directed enhancer activity in chondrocytes [9–11]. Simultaneously, Lefebvre, one of the authors of this review, in de Crombrughe's group, delineated a 48-bp sequence in the mouse *Col2a1* intron 1 and demonstrated that Sox9 directly activated this minimal chondrocyte-specific enhancer [12]. This sequence was included in Yamada's 100-bp enhancer and overlapped with the 309-bp fragment reported by Cheah's group. Subsequently, de Crombrughe's group found that the 48-bp enhancer featured a total of four binding sites for HMG-domain proteins and that Sox9 specifically bound to two of them. Lefebvre and de Crombrughe showed that chondrocytes also expressed two other members of the Sox family, Sox5 and Sox6, which are closely related to each other, but are distant relatives of Sox9 [13]. The two proteins cooperatively bound to all four HMG-domain recognition sites and potentiated the ability of Sox9 to transactivate the enhancer. The Sox5 protein isoform expressed in chondrocytes was longer than the short Sox5 protein previously identified in testis. It was therefore called L-Sox5, but is currently most often referred to as Sox5. In 2003, Vilain and Harley's group, and Scherer and Wegner's group demonstrated that Sox9 homodimerized upon binding to DNA through a unique domain juxtaposed to its DNA-binding domain [14, 15]. Mutations that prevent homodimerization cause campomelic dysplasia, demonstrating the importance of homodimerization in Sox9 function in chondrogenesis. Homodimerization is also essential for Sox5 and Sox6 functions [13]. It is mediated by a coiled-coil domain unrelated to Sox9's homodimerization domain and occurs even in the absence of DNA binding. Subsequent studies by several groups clearly established that many genes for extracellular matrix components, including *Agc1*, *Col11a2*, *Col27a1*, *Cd-rap*, and *Matrilin-1*, owe their cartilage-specific expression to transactivation by the Sox trio upon binding to HMG-domain sites in their enhancers [16–22].

Sox9, Sox5, and Sox6 as chondrogenic master genes

Accumulating evidence that Sox9, Sox5, and Sox6 bind and cooperatively activate chondrocyte-specific enhancers in vitro raised the possibility that these proteins were chondrogenic master regulators, thus required for cartilage formation. *Sox9* heterozygous null mice almost perfectly reproduced the skeletal abnormalities of humans with campomelic dysplasia, and like many of these patients they died soon after birth [23]. Because they could not be used to generate *Sox9* homozygous null mutants and thereby study the functions of Sox9 in chondrogenesis, de Crombrughe's group collaborated with Behringer to create *Sox9*^{-/-} embryonic stem cells and used them to generate mouse embryo chimeras [24]. They replaced the Sox9 coding sequence by *LacZ* in these *Sox9*^{-/-} embryonic stem cells to be able to follow the fate of the mutant cells that expressed the *Sox9* null alleles (but not the Sox9 protein). They found such cells, intermingled with wild-type cells, in all skeletogenic mesenchyme in mid-gestation embryo chimeras, but failed to find any within precartilaginous condensations and cartilage primordia at later stages. A few clusters of cells were abutting cartilage primordia, but were unable to express chondrocyte markers, such as *Col2a1*, *Col11a2*, and *Agc1*.

In 2002, Akiyama [25], one of the authors in this review, in de Crombrughe's group, conditionally inactivated the *Sox9* gene at early stages of skeletogenesis using the Cre recombinase/*loxP* recombination system of bacteriophage P1 at early stages of skeletogenesis. Akiyama showed that inactivation of *Sox9* in limb bud mesenchyme before pre-cartilaginous condensation resulted in complete absence of mesenchymal condensation and of subsequent cartilage and bone formation. Inactivation of *Sox9* at and after pre-cartilaginous condensation blocked chondrocyte overt differentiation. In both types of mutants, inactivation of *Sox9* precluded expression of *Sox5* and *Sox6*, indicating that Sox9 is indispensable for chondrocyte differentiation at and prior to activation of these genes.

Lefebvre and de Crombrughe [26] showed that *Sox5*; *Sox6* double-null mice developed a severe, generalized chondrodysplasia characterized by the drastic underdevelopment of cartilage, whereas *Sox5*-null and *Sox6*-null mice were born with mildly impaired skeletons. In the double mutants, chondrocyte differentiation was virtually arrested at the stage of precartilaginous condensation, even though *Sox9* expression was normal, providing that *Sox5* and *Sox6* are essential to promote overt differentiation of chondrocytes together with or downstream of *Sox9*. Lefebvre's group went on to show that chondrocytes overtly differentiated in *Sox5/Sox6* partial mutants, but formed small cartilage growth plates [27]. They proliferated slowly in the columnar zone, underwent prehypertrophy prematurely and then skipped hypertrophy to directly proceed to terminal maturation. These data thus strongly suggested that *Sox5* and *Sox6* have important roles in chondrocytes beyond early differentiation. Soon afterwards, Akiyama reported that transgenic mice, in which *Sox9* was ectopically expressed in limb bud mesenchyme, formed ectopic cartilage in association with ectopic expression of *Sox5* and *Sox6* [28].

Together, loss-of-function and gain-of-function experiments in the mouse thus demonstrated that *Sox9* and *Sox5/6* are both needed and sufficient for chondrogenesis as master transcription factors.

Untying the knot of *Sox9*-dependent transcriptional mechanisms in chondrogenesis

Since *Sox9* was identified as a chondrogenic master gene, several studies have focused on uncovering the mode of action of *Sox9* in chondrogenesis. Post-translational modifications of the *Sox9* protein, including phosphorylation, acetylation, and SUMOylation, have been shown to affect *Sox9*-dependent transcription in chondrogenesis. The catalytic subunit of cyclic AMP-dependent protein kinase A (PKA) and the Rho-associated coiled coil-forming kinase (ROCK) interacted with *Sox9* and directly phosphorylate it at serine 181 [29, 30]. This modification caused nuclear accumulation of *Sox9*, increased the efficiency of *Sox9* binding to DNA, and/or increased transcriptional activity. It occurred principally in the prehypertrophic zone of the growth plate, which is the major site of expression of the parathyroid hormone-related peptide (PTHrP) receptor, suggesting that PTHrP may inhibit conversion of proliferating cells into prehypertrophic cells, at least in part, through activating PKA and thereby the activity of *Sox9* [31]. One report indicated that the histone acetyl transferase Tip60 increased *Sox9/Sox5*-dependent transcription in association with acetylation of *Sox9* at lysine 61, 253, and 398. Tip60, together with *Sox9* and *Sox5*, was also present in the chromatin of the *Col2a1* enhancer; however, it remains unknown whether this stimulatory effect of Tip60 on *Sox9* transcriptional activity depends on *Sox9* or histone acetylation [32]. The *Sox9* protein is also modified by protein inhibitor of activated STAT (PIAS)1-mediated SUMOylation, but it is unclear whether PIAS1 represses or stimulates *Sox9* activity [33, 34]. Additional in vitro and in vivo studies are thus needed to clarify the functional consequences of post-translational modifications of *Sox9*.

At the onset of endochondral ossification, chondrocytes derive from the same mesenchymal progenitors as osteoblasts [35]. These cells, called osteochondroprogenitors, are bipotent because they express *Sox9*, the Runt-related factor 2 (*Runx2*), and β -catenin. *Runx2* is a transcription factor required for osteoblast cell fate determination and early differentiation [36, 37]. β -catenin is a transcriptional transducer of the Wnt canonical pathway. Its protein level is upregulated in preosteoblasts and downregulated in prechondrocytes. Conditional inactivation of β -catenin in osteochondroprogenitors demonstrated that β -catenin promoted osteoblast differentiation and suppressed chondrocyte differentiation [38, 39]. *Sox9* binds to β -catenin and inhibits the transcriptional activity of β -catenin, in part through inducing β -catenin degradation [40]. *Sox9* also binds to the components of the β -catenin destruction

complex, glycogen synthase kinase 3 (GSK3) and β -transducin repeat-containing protein, to promote their nuclear localization and subsequent β -catenin phosphorylation [41]. Sox9 has been proposed to exert a dominant inhibitory function over Runx2 [42]. It may also promote Runx2 degradation through a proteasome-dependent pathway or through lysosomal degradation in a phosphorylation-dependent manner [43]. These mechanisms may greatly impact the determination of osteochondroprogenitors to the osteoblast and chondrocyte lineages.

While Sox5/Sox6 have been suggested to potentiate transactivation of chondrocyte-specific genes by Sox9 through cooperative binding to multiple recognition sites on enhancers [18], new searches have been started to identify additional factors that likely assemble with Sox9 into functional transcriptional complexes. It is believed that these factors bind to Sox9 directly or indirectly, are recruited with Sox9 on chondrocyte-specific genes, and cooperate with Sox9 to activate transcription. In addition, loss-of-function mutations of their genes should cause chondrodysplastic phenotypes. Several nuclear proteins have already been proposed to pair off with Sox9 in chondrocytes. They include c-Maf, the peroxisome proliferation-activated receptor-gamma coactivator 1alpha (Pgc-1 α), Znf219, and the AT-rich interactive domain 5A (Arid5a) [44–47]. Another protein is the P54^{nrb}, 54-kDa nuclear RNA-binding protein, which physically interacted with Sox9 to increase transactivation of *Col2a1* and promoted splicing of the *Col2a1* mRNA [48]. Transgenic mice expressing a P54^{nrb} protein lacking two RNA recognition motifs in chondrocytes exhibited dwarfism associated with impairment of chondrogenesis.

Transcription factors initiate their job by recruiting factors that induce histone modifications and subsequent chromatin remodeling. They also recruit a mediator complex that bridges them with the general transcriptional machinery around the RNA polymerase II at transcriptional start sites. Asahara's group reported that histone deacetylase inhibition increased Sox9-dependent gene expression in human chondrocytes, suggesting that Sox9 activates the transcription of its target genes by inducing coactivator-dependent histone hyperacetylation around the enhancers that it binds to [49]. This group showed direct association between the cAMP response element-binding protein (CREB)-binding protein (CBP)/p300 and Sox9, subsequent chromatin modification, and activation of *Col2a1* transcription [50]. In 2002, using a yeast two-hybrid system, Berta's group identified thyroid hormone receptor-associated protein (Trap)230 (Med12) as a Sox9-associated protein [51]. Trap230 (Med12) is expressed in chondrocytes and its interacting domain acts in a dominant-negative manner against the Sox9 transactivation domain, suggesting that Sox9 communicates with the general transcription machinery through the TRAP complex in chondrocytes. In 2006, Neumann's group [52] found that zebrafish mutants with disruption of the Trap230 (Med12) ortholog or inactivation of *Trap230* (*Med12*) by microinjection of its morpholino oligonucleotides exhibited a skeletal phenotype similar to that of *Sox9a*/*Sox9b* double mutants. Thus, these studies revealed a critical function of Trap230 (Med12) as a coactivator of Sox9. Akiyama's group recently reported that physical interaction between Sox9, Wwp2, and Med25, a component of the Mediator complex, underlies important functional interactions in chondrogenesis [53]. In zebrafish, morpholino-mediated knockdown of either *Wwp2* or *Med25* disrupted palatal chondrogenesis, as seen in *Sox9* mutants. Finally, recent studies have shown that Med25 is required for recruitment of CBP/p300 and RNA polymerase II to promoters [54–56]. These studies shed light on the transcriptional machinery of Sox9 in chondrogenesis.

Conclusion

Human and genetic approaches have revealed that Sox9 plays an essential role in determining chondrocyte fate and differentiation, and molecular approaches have started to

reveal that multiple factors participate in the formation of transcriptional complexes with Sox9. These factors provide the specificity that Sox9 needs to bind and transactivate the gene sets that constitute the chondrocyte genetic program. Additional work is needed to further uncover these mechanisms and thereby provide new insights into the principles of cartilage development and into much needed strategies for tissue regeneration in cartilage degenerative diseases.

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