The role of TGFβs and Sox9 during limb chondrogenesis

Yasuhiro Kawakami1, Joaquín Rodríguez-León2 and Juan Carlos Izpisúa Belmonte1,2

The majority of the skeletal elements, except the flat bones of the skull, are formed by endochondral ossification, in which cartilage is replaced by bone. The formation of cartilage is a multi-step process termed chondrogenesis, during which undifferentiated mesenchymal cells condense and undergo differentiation towards chondrocytes. Notwithstanding recent advances, our knowledge of the detailed mechanisms implicated in cartilage and bone formation is still scarce. Recent genetic, cellular and biochemical studies have highlighted the importance of TGFβ signaling and the activity of the transcription factor Sox9 during the early stages of vertebrate limb chondrogenesis.

Addresses
1 Gene Expression Laboratory, Salk Institute for Biological Studies, 10010 N. Torrey Pines Rd., La Jolla, CA 92037, USA
2 Center of Regenerative Medicine in Barcelona, Dr. Aiguader, 80, 08003 Barcelona, Spain

Corresponding author: Belmonte, Juan Carlos Izpisúa (belmonte@salk.edu)

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Introduction
The formation of the skeleton in vertebrates involves the differentiation of mesenchymal cells to cartilage. This process, called chondrogenesis, is a tightly regulated event involving multiple steps, including condensation of the precartilaginous mesenchyme, commitment to the chondrogenic lineage, and differentiation into chondroblasts and, eventually, into chondrocytes. All these events are regulated by the concerted action of extracellular and intracellular cues, including extracellular factors specific for cartilage differentiation (e.g. type IIa1 Collagen and Aggrecan), and more general pathways that, while not exclusively specific to this process, play an important role at various stages of chondrogenesis, for example the bone morphogenetic protein (BMP), Hedgehog, Wnt and fibroblast growth factor pathways. The intracellular events elicited by the activation of these and other pathways lead to the transcriptional regulation of chondrogenesis-specific genes (e.g. the gene for Sox9), enabling the embryonic differentiation of mesenchymal cells towards chondrocytes. Here, using the vertebrate developing limb as a paradigm, we will review recent studies on how members of the transforming growth factor β (TGFβ) superfamily and Sox9 regulate the early process of chondrogenesis.

Extracellular regulation of chondrogenesis by members of the TGFβ superfamily
TGFβ-related proteins form a large family of secreted molecules including, among others, TGFβs, activins and BMPs [1]. These molecules form either homodimers or heterodimers, and exert their activity through type I and type II serine/threonine kinase receptors. These receptors are grouped into a large family that includes BMP and activin receptors.

The fact that distinct ligands can bind and activate multiple receptors in a context-dependent manner [2], points towards the importance of TGFβ signaling and the complex way in which it directs and modulates different chondrogenic events.

Among a number of ligands, activins and TGFβs are expressed at different stages of limb development. These molecules can induce ectopic chondrogenesis, leading to complete extra-digit formation, when applied to the chick interdigital area (Figure 1), a region of the developing limb where cells normally undergo apoptosis [3,4]. Four BMP genes, namely BMP2, BMP4, BMP5 and BMP7, are expressed in the developing limb, and all of them show chondrogenic abilities [5]. Their activity is, however, context-dependent because BMPs can also induce apoptosis in mesenchymal cells [6,7]. Studies on their receptors have shown that BMPR1A and BMPR1B have distinct roles during chondrogenesis [8–11]. Moreover, mice lacking both BMPR1A and BMPR1B exhibit severe generalized chondrodysplasia. Double mutants do not express Sox9, a key regulator of chondrogenesis, or other molecules known to be important for chondrogenesis such as L-Sox5 and Sox6, and showed impaired cartilage matrix gene expression [12**]. Therefore, mesenchymal BMPR1A and BMPR1B are functionally redundant and BMP signaling is required for early chondrogenesis.

Intracellular regulation of chondrogenesis by Sox9
Among several intracellular factors, Sox9, a transcription factor, is a key molecule in early chondrogenesis. Sox9 belongs to the SRY (sex-determining region on the Y chromosome) family and contains the HMG
(high mobility group) box DNA binding domain. Sox9 is expressed in association with chondrogenic aggregates (Figure 1) [13,14] and a variety of mouse genetic studies, as well as human mutations, have shown that Sox9 is a master regulator of chondrogenesis. Sox9^{-/} mouse embryos die perinatally and exhibit severe hypoplasia of the cartilage that is associated with lower levels of cartilage matrix genes [15]. Similarly, SOX9 haploinsufficiency in humans causes campomelic dysplasia, characterized by severe skeletal dysmorphology [16]. Further analyses with conditional inactivation of Sox9 at varying times during mouse limb development have revealed that it is required for mesenchymal condensation and subsequent chondroblast differentiation [17,18]. Of particular interest is the observation that elimination of Sox9 before mesenchymal condensation leads to absence of cartilage and bones, suggesting that Sox9 is required for osteogenesis initiation. A genetic lineage tracing experiment using a Sox9^{-ires-Cre} knock-in mouse has demonstrated that Sox9-expressing cells give rise to both chondroblasts and osteoblasts, indicating that progenitors of these lineages (formally called osteochondroprogenitors) are derived
from Sox9-expressing cells [19**]. It has been suggested that the osteogenic lineage cells segregate from osteochondroprogenitors during mesenchymal condensations, which then leads to chondroblasts that express Sox9 and subsequently to osteoblasts that express Runx2, a gene that is required for osteogenesis of endochondral and intramembranous bones [20]. Further studies have revealed that Sox9-expressing cells are progenitors for a variety of cell types, including tendons and synovium in the limb bud as well as progenitors of the spinal cord, intestine, testis and pancreas.

Gain-of-function studies in chick embryos suggest that osteochondroprogenitor differentiation proceeds via a delicate balance in the levels of Sox9 and Runx2, where higher Sox9 levels lead to chondrogenesis and higher Runx2 levels lead to osteogenesis [21]. Recent mouse genetic analyses have demonstrated that Wnt/β-catenin signaling is involved in regulating this balance. Wnt signaling induces osteoblastic differentiation and suppresses chondrogenic differentiation [22–24]. The Wnt family comprises >20 genes with multiple roles during embryonic development and organogenesis [25]. Among the various intracellular Wnt signaling pathways, the best-characterized is the canonical pathway; in this pathway, binding of Wnt to cognate receptors induces stabilization and accumulation of cytosolic β-catenin, which is then transported into the nucleus where it mediates transcription of target genes [26]. Conditional inactivation of β-catenin results in osteochondroprogenitors differentiating into chondroblasts rather than osteoblasts, leading to an arrest of osteoblast differentiation. The inhibitory effect of Wnt/β-catenin signaling on chondrogenesis is, at least in part, explained by a mutual antagonism between β-catenin and Sox9 (described below). Since Runx2 directs osteochondroprogenitors into the osteoblastic lineage [20,22], it would be of particular interest to ascertain whether β-catenin activity may interact with Runx2 function during chondrogenesis.

**Regulation of chondrogenesis-specific expression of Sox9**

Given that Sox9-expressing cells mark osteochondroprogenitors as well as progenitors in a variety of tissues, it is of great interest to elucidate how Sox9 expression is regulated. Identification of chondroprogenitor-specific regulatory elements would shed light on how chondroprogenitors emerge from undifferentiated mesenchymal cells, and might eventually help to guide therapeutic approaches to artificially engineer chondrocyte differentiation.

Several lines of evidence indicate that BMP signaling regulates Sox9 expression. First, as discussed above, inactivation of both BMPRIA and BMPRIB in mice results in a lack of Sox9 expression [12**]. Second, in the developing chick limb bud interdigital region, TGFβ can induce chondrogenesis by regulating cell condensation after 12 h [27]. In this in vivo system, kinetic analysis of the expression of genes known to be involved in chondrogenesis (Sox genes, Col2a1, Aggrecan and genes for BMP signaling components) has demonstrated that Sox9 expression is induced as early as 30 min after TGFβ-bead implantation, the earliest induction among several markers known to be induced during chondrogenesis [27]. These observations suggest that Sox9 expression during chondrogenesis might be under direct control of TGFβ/BMP activity, and that factors downstream of TGFβ/BMP pathways, such as Smads and TAK1 (TGFβ-activated kinase), control chondrogenesis-specific enhancers of the Sox9 gene [28,29].

Notwithstanding the importance of these observations, analysis of the Sox9 locus reveals that Sox9 expression during chondrogenesis in the limb bud requires more complex regulation than was previously suspected. While in vitro studies demonstrate that the basal promoter activity for the Sox9 locus involves a CCAAT-binding factor acting on the Sox9 proximal promoter region [30], chondrogenesis-specific regulatory element(s) had not been precisely characterized. Comparison of the Sox9 locus in human and pufferfish (Fugu rubripes) as well as transgenic analyses in mice, using a 740 kb area of the human Sox9 locus linked to a LacZ reporter, have identified several tissue-specific enhancers, including enhancers specific to cranial neural crest cells, gut and the pancreas during embryogenesis, but have failed to identify a chondrogenesis-specific enhancer [31,32*]. From the analyses of campomelic dysplasia patients’ chromosomal rearrangements as well as YAC transgenesis, it is likely that chondrogenesis-specific enhancers lie ~350–250 kb upstream of the SOX9 gene [16]. Given that campomelic dysplasia patients show variations in their phenotypes, spatial and temporal regulatory elements are more likely to be scattered over a couple of hundred kb. Other genes essential for organogenesis during limb development, such as the Hoxd cluster and Gremlin, exhibit similar regulation, with a ‘global control region’ that could contain multiple elements for spatial and temporal expression, as well as scattered regulatory elements, which probably act in combination to regulate proper expression [33,34].

**Sox9 post-translational regulation**

Increasing evidence suggests that the levels of Sox9 protein are to be strictly controlled in order for normal chondrogenesis to occur. A Sox9 gain-of-function experiment in which the Sox9 transcript level was increased by ~20% caused dwarfism [18*], and conversely, 50% down-regulation in Sox9+/− mice and human campomelic dysplasia patients resulted in chondrodysplasia [15].

Sox9 protein levels are controlled via a self-regulated balance between degradation and synthesis of protein,
maintained by the K398 residue of the Sox9 protein (Figure 2). A yeast two-hybrid screen identified PIAS (protein inhibitor of activated STAT) family proteins as those interacting with Sox9 \[35**\]. PIAS acts as a sumo-ligase, mediating sumoylation of Sox9, which results in an increase in Sox9 stability \textit{in vitro}. It also affects subnuclear localization, leading to diffusion of Sox9 distribution in nuclei. Interestingly, K398 is also a target of ubiquitination, through which Sox9 is subjected to proteosome-mediated degradation \[36\]. There are two more possible sumoylation amino acid residues (K61 and K253) which may have similar mechanisms acting on them. Since Sox9 marks progenitors in a variety of tissues, further study \textit{in vivo} may help to elucidate the role of these residues and the degradation/stabilization of Sox9 during embryonic development.

Canonical Wnt signaling also serves to regulate Sox9 at the post-translational level through \(\beta\)-catenin by interacting with and antagonizing Sox9 \[18*\]. This is, at least in part, a mechanism by which Wnt/\(\beta\)-catenin signaling negatively regulates Sox9 function and chondrogenesis \[25,37\]. On the other hand, Sox9 inhibits Wnt/\(\beta\)-catenin signaling, and therefore inhibits expression of its targets, such as \textit{cyclinD1}, a cell cycle regulator, thereby negatively regulating cell proliferation at the hypertrophic cartilage.

Another post-translational modification known to regulate Sox9 activity is the phosphorylation of S64 and S211 by the PHTRP–PKA pathway \[38\], which enhances the DNA binding affinity of Sox9 as well as its transcriptional activity. Recent findings have demonstrated that cGMP-dependent protein kinase type II (cGKII) phosphorylation also attenuates Sox9 function in chondrocytes \[39\]. cGKII phosphorylates and inhibits nuclear entry of Sox9, thereby preventing its activity. Although S181 is the consensus amino acid phosphorylated by cGKII, S181 is dispensable for the cGKII attenuation of Sox9. Therefore, it is possible that cGKII may also phosphorylate other amino acid residue(s) to attenuate Sox9 function. Since phosphorylation of these serine residues is likely to affect the structural conformation of Sox9, and hence its interaction with other proteins, determining the conformational changes caused by phosphorylation of each amino acid residue may help to enhance our understanding of the molecular mechanisms regulating Sox9 function post-translationally.

**Transcriptional complex of Sox9 regulates chondrogenesis**

Given that Sox9 acts as a transcriptional activator, not only its modification but also its molecular interactions may help to regulate its transcriptional activity \[40\]. Several
cofactors have been shown to interact with Sox9 through its transactivation domain. Among them, p300/CBP, a general coactivator, has been reported to interact with and enhance the transactivation activity of Sox9 in vitro [41]. These proteins are shown to act synergistically on artificially chromatinized DNA and elicit high levels of transcriptional activity on the Col2α1 reporter when mixed with nuclear extracts [42*]. Modification of the chromatin structure by p300/CBP harboring histone acetyltransferase (HAT) activity could be an important mechanism to regulate gene expression during chondrogenesis. Further analysis in vivo would enhance our understanding of the importance of chromatin modification and gene regulation.

TRAP230, which is ubiquitously expressed, encodes a component of the thyroid hormone receptor-associated protein complex that acts as a coactivator for Sox9. Both biochemical and in vivo analyses indicate that TRAP230 also acts as coactivator during chondrogenesis [43,44,45*]. tpd mutant zebrafish embryos that harbor mutations in the trap230 gene exhibit reduced levels of col2α1 expression. Furthermore, injection of Sox9 mRNA into tpd mutant embryos is not able to upregulate expression of Sox9-target genes, upregulation that can be accomplished by injection into wildtype embryos. TRAP230 is shown to have Sox9-independent activities and thus could regulate multiple transcription factor activities.

PGC-1α, initially identified as a coactivator for nuclear receptors such as PPARγ and RXR, associates with Sox9 and enhances its transcriptional activity [46**]. Interestingly, Sox9 expression is detected in undifferentiated human mesenchymal stem cells and PGC-1α expression is induced upon induction of chondrogenesis by TGFβ. Furthermore, overexpression of both Sox9 and PGC-1α induced ectopic chondrogenesis both in human mesenchymal stem cells and the developing chick limb bud. These observations highlight how the spatiotemporal regulation of cofactors during embryogenesis may have a crucial role in the transcriptional activation of Sox9 and possibly other cartilage-specific transcriptional regulators. The identification of other Sox9 cofactors and elucidation of their precise molecular mechanisms of action both in vitro and in vivo will be needed to further enhance our understanding of how chondrogenesis during limb development is elicited.

Conclusions

Differentiation of limb mesenchymal cells into cartilage is a well-studied model system of vertebrate embryogenesis, in which condensation, lineage determination towards chondroblasts and subsequent differentiation into chondrocytes are regulated in time and space to allow definitive bone formation. We have reviewed our current knowledge on the role of several extracellular members of the TGFβ family of secreted factors, together with the intracellular activities modulated by Sox9 during the early stages of limb chondrogenesis. A variety of in vitro and in vivo studies have started to uncover a complex network of molecular interactions that regulates the spatial-temporal expression, protein stability and post-translational modifications of Sox9. Further in vivo studies as well as the development of in vitro model systems will not only lead us to a better understanding of the cellular and molecular basis of cartilage formation during normal development and physiology, but may also point us towards therapeutic approaches for cartilage and bone repair.

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References and recommended reading

Papers of particular interest, published within the annual period of review, have been highlighted as:

- of special interest
- of outstanding interest

mesenchyme regulates distal outgrowth and patterning. 

**Cell differentiation**

Eames BF, Sharpe PT, Helms JA: Regulation of skeletal development by the Runx genes and BMP signaling. 


It is demonstrated that two type I BMP receptors, Bmpr1a and Bmpr1b, are expressed in cartilage condensation. Eliminating both receptors in mice results in severe chondro dysplasia with loss of Sox9, L-Sox5 and Sox6 expression. Increased apoptosis and reduced cell proliferation causes reduced condensation size. The authors concludes that BMP signaling through BMPR1A and BMPR1B is required for chondrocyte proliferation, survival and differentiation.


This study demonstrates that a slight increase (~20%) in Sox9 protein level results in dwarfism. It also illustrates the antagonism between Sox9 and β-catenin.


The authors generate a Sox9-ires- Cre knock-in into the Sox9 locus, and cross it with the ROSA26 reporter line to determine the cell fate of Sox9-expressing cells. This analysis reveals that Sox9-expressing mesenchymal cells in the limb bud give rise to both chondrocytes and osteoblasts.


24. Day TF, Guo X, Garrett-Beal L, Yang Y: Wnt/β-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. Dev Cell 2005, 8:739-750.


On artificially chromatinized DNA, p300 harboring HAT activity potentiated Sox9-dependent transcription, an effect associated with hyper-acetylated histones. This study demonstrates how the interaction between Sox9 and p300 regulates transcription through chromatin modification.


The authors identify a zebrafish mutant, tpd, which exhibits similar defects to Sox9 zebrafish mutants. The tpd mutation is identified in the Trap230 gene, a component of the mediator complex that acts as a coactivator.


A transcriptional coactivator, PGC-1α, is shown to associate with Sox9 and enhance its transcription. Expression of PGC-1α correlates with chondrogenesis in the developing limb and in human mesenchymal stem cells. Overexpression of both Sox9 and PGC-1α causes chondrogenesis in the developing limb and differentiating human mesenchymal stem cell. These data demonstrate that the specific interaction between Sox9 and PGC-1α regulates chondrogenesis.