Original Article

SOXC transcription factors induce cartilage growth plate formation in mouse embryos by promoting noncanonical WNT signaling†

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ABSTRACT

Growth plates are specialized cartilage structures that ensure the elongation of most skeletal primordia during vertebrate development. They are made by chondrocytes that proliferate in longitudinal columns and then progress in a staggered manner towards prehypertrophic, hypertrophic and terminal maturation. Complex molecular networks control the formation and activity of growth plates, but remain incompletely understood. We investigated here the importance of the *SoxC* genes, which encode the SOX4, SOX11 and SOX12 transcription factors, in growth plates. We show that the three genes are expressed robustly in perichondrocytes and weakly in growth plate chondrocytes. $SoxC^{Prx1Cre}$ mice, which deleted *SoxC* genes in limb bud skeletogenic mesenchyme, were born with tiny appendicular cartilage primordia because of failure to form growth plates. In contrast, $SoxC^{Col2Cre}$ and $SoxC^{ATC}$ mice, which deleted *SoxC* genes primarily in chondrocytes, were born with mild dwarfism and fair growth plates. Chondrocytes in the latter mutants matured normally, but formed irregular columns, proliferated slowly and died ectopically. Asymmetric distribution of VANGL2 was defective in both $SoxC^{Prx1Cre}$ and $SoxC^{ATC}$ chondrocytes, indicating impairment of planar cell polarity, a noncanonical WNT signaling pathway that controls growth plate chondrocyte alignment, proliferation and survival. Accordingly, *SoxC* genes were necessary in perichondrocytes for expression of *Wnt5a*, which encodes a noncanonical WNT ligand required for growth plate formation, and in chondrocytes and perichondrocytes for expression of *Fzd3* and *Csnk1e*, which encode a WNT receptor and casein kinase-1 subunit mediating planar cell polarity, respectively. Reflecting the differential strengths of the SOXC protein transactivation domains, SOX11 was more powerful than SOX4, and SOX12 interfered with the activity of SOX4 and SOX11. Altogether, these findings provide novel insights into the molecular regulation of skeletal growth by proposing that SOXC proteins act cell- and non-cell-autonomously in perichondrocytes and chondrocytes to establish noncanonical WNT signaling crosstalk essential for growth plate induction and control.

Keywords
Cartilage growth plate
Mouse genetics models
Noncanonical WNT signaling
Perichondrium
SOXC transcription factors

INTRODUCTION

Most of the bones that compose the vertebrate skeleton develop upon cartilage templates.[1] These templates arise when mesenchymal progenitor cells coalesce at various skeletogenic sites in the embryo and commit to the chondrocyte lineage. Nascent chondrocytes surround themselves with an abundant, cartilage-specific extracellular matrix and actively proliferate. They then organize growth plates, specialized structures responsible for the rapid and considerable elongation of skeletal elements during fetal and postnatal development.[2,3] In order to do so, chondrocytes undergo a series of terminal differentiation steps in a staggered manner. Chondrocytes located in the center of cartilage primordia initiate this progression and are followed first by proximal and then by distal neighbors. The result is a highly organized structure featuring at any time point layers of chondrocytes at successive stages of differentiation. Immature chondrocytes form tightly packed longitudinal columns, within which they progressively slow down proliferation. Prehypertrophic chondrocytes growth-arrest and drastically alter their differentiation program, and hypertrophic cells enlarge massively. Terminal chondrocytes undergo apoptosis or convert to the osteoblast lineage and join efforts with perichondrium-derived osteoblasts, osteoclasts, blood vessels, and marrow precursors to achieve endochondral ossification, i.e., replace cartilage by bone tissue and marrow.[4] Many types of regulatory factors have been identified that control the spatial and temporal progression of growth plate chondrocytes. They include systemic and local hormones and growth factors, extracellular matrix components, extracellular and intracellular enzymes, and epigenetic and transcription factors. These factors act either cell-autonomously, i.e., they are produced and act within chondrocytes, or non-cell-autonomously, e.g., they are produced by perichondrocytes and signal to chondrocytes to alter cell differentiation, morphology, proliferation or survival.[3,5,6]

Genetic and non-genetic disturbances in the expression and activity of many of these factors have been shown to underlie various types of growth plate dysfunctions in humans. Severe forms can lead to neonatal death or incapacitating dwarfism, and mild forms to osteoarthritis and other skeleton degenerative diseases.[7,8] Today, gaps remain in our knowledge of the factors that control growth plate formation and activity. Filling these gaps is crucial to uncover the causes of still unexplained skeletal dysplasias and to design efficient treatments for all types of cartilage diseases.

The family of SOX transcription factors has key roles in determining cell fate and differentiation in virtually every lineage.[9] All SOX proteins feature a DNA-binding domain closely related to that of SRY, the family founder encoded by the Sex-determining Region on the Y chromosome. SOX proteins are distributed into eight groups (A to H) according to sequence identity. A SOX trio composed of SOX9 (SOXE protein), SOX5 and SOX6 (SOXD proteins) is well known for its pivotal roles in chondrocyte specification and differentiation.[10,11] SOX9 directly transactivates cartilage-specific genes, and SOX5 and SOX6 boost SOX9's ability by securing its DNA binding. We recently unearthed that another SOX trio has key roles in skeletogenesis. This trio is made of the three SOXC group members: SOX4, SOX11 and SOX12. These proteins are virtually identical in the SOX domain, but only share partial identity in a group-specific transactivation domain.[12,13] This domain is most potent in SOX11 and least potent in SOX12. The *SoxC* genes overlap in expression in the precursor cells of many organs, such as the heart, nervous system, kidney and pancreas.[12-18] They are essential in the development of these organs, primarily by ensuring cell survival, but were also described to participate in differentiation of neuronal and B cell precursors.[19,20] With respect to skeletogenesis, the *SoxC* genes are largely co-expressed in the mesenchymal progenitors of cartilage and bone primordia.[17] They are

dispensable for cell specification and proliferation, but instrumental for cell survival. They act at least in part by transactivating *Tead2*, which encodes a transcription factor involved in Hippo signaling mediation. They remain strongly expressed in perichondrium and presumptive joint cells when cartilage primordia overtly develop.[21] Co-inactivation of the three genes in limb bud mesenchyme of mouse embryos using the *Prx1Cre* transgene (*Sox4*$^{fl/fl}$*11*$^{fl/fl}$*12*$^{-/-}$*Prx1Cre*, referred to as *SoxC*^{Prx1Cre}) results in fusion of appendicular cartilage primordia because perichondrium and joint cells can neither decline chondrogenesis nor overtly differentiate. Molecular dissection of the underlying mechanism revealed that SOXC proteins stabilize β-catenin and thereby boost canonical WNT signaling and contribute to repress *Sox9*.

The appendicular cartilage primordia of *SoxC*^{Prx1Cre} embryos remain tiny throughout fetal life, and histological analysis has suggested that they were unable to develop growth plates.[21] We investigated this defect and its underlying mechanism in the present study. We show that SOXC proteins act at the top of a molecular cascade led by noncanonical WNT signaling to induce and ensure the proper organization and activity of growth plates.

MATERIALS AND METHODS

Mice

Mice were used according to federal guidelines and as approved by the Cleveland Clinic Institutional Animal Care and Use Committee. They harbored *SoxC* wild-type, conditional or null alleles[17,22] and a *Prx1Cre*[23], *ATC*[24] or *Col2Cre*[25] transgene. Unless otherwise indicated, females carrying $SoxC^{ATC}$ fetuses received doxycycline (Sigma) at 2 mg/ml in drinking water also supplemented with 5% sucrose from day 12.5 of pregnancy. All mouse lines were inbred for multiple generations on a mixed 129xB6 genetic background.

Skeletal preparations and in situ assays on embryo sections

Whole-mount skeletons were stained with alcian blue (cartilage) and alizarin red (mineralized tissue), as described.[21] Seven-µm-thick paraffin sections of embryo legs were generated following fixation of tissues in 4% paraformaldehyde (Electron Microscopy Sciences). Staining with alcian blue and nuclear fast red, TUNEL assay, and RNA in situ hybridization were performed as described.[21] The *Tcf7l2* (*Tcf4*) probe corresponded to nucleotides 869 to 1579 in NM_001142924.1. The *Fzd3* probe corresponded to nucleotides 3766 to 4510 in NM_021458.2. The *Csnk1e* probe corresponded to nucleotides 1219 to 2005 in NM_013767.6. The cDNA for these probes were amplified by PCR, cloned in pCR4-TOPO (Invitrogen), and sequence-verified. Other probes were as described.[12,24,26] Immunostaining was performed on 10-µm-thick frozen sections or 7-µm-thick paraffin sections of embryo legs fixed in 4% paraformaldehyde. Proteins were revealed using primary antibodies against cleaved caspase 3 (1/100, Cell Signaling 9661S), β-catenin (1/100, Cell Signaling 8480) or acetyl-alpha-tubulin (1/500, Cell Signaling 5335), and Alexa Fluor 594-conjugated goat anti-rabbit IgG as secondary antibody (1/200, Invitrogen

A11037). They were also revealed using anti-VANGL2 primary antibody (1/50, Santa Cruz Technologies sc-46561) in combination with Alexa Fluor 488-conjugated donkey anti-goat IgG (1/100, Invitrogen A11055) for frozen sections and with streptavidin Alexa Fluor 594 (1/1000, Invitrogen S32356) with aid of TSA Plus Biotin kit (PerkinElmer NEL749A001KT) for paraffin sections. Cell nuclei were stained with Hoechst 33258 (2 µg/ml, Invitrogen). Cell proliferation was assayed using Click-iT®EdU kit (Life Technologies) as described.[27] Pregnant females were injected intraperitoneally with EdU solution 2 h before embryo harvesting. Data were visualized with Leica CTR5000 microscope, captured with LEICA DFC310 FX digital color camera, and processed with Adobe Photoshop CS4 software. Confocal images were visualized with Leica TCS SP2 confocal microscopy system. Z-stack images were acquired at 0.5 µm intervals for 5 µm and then projected for analysis.

Quantitative RT-PCR

Growth plates were isolated from fetal mice by manually dissecting out cartilage epiphyses, bone, ligaments and muscle tissue, but preserving perichondrium. When compensatory regulation was analysed, growth plates were collected along with cartilage epiphyses and perichondrium. Tissue samples were collected in RNAlater (Life Technologies) and homogenized in TRIzol (Life Technologies). Cultured cell samples were directly collected in TRIzol. Total RNA extracted using TRIzol was further purified using RNeasy Mini Kit (Qiagen). cDNA was synthesized using Superscript III First-Strand Synthesis System (Life Technologies) and amplified with specific primers (Table S1) using SYBR Green PCR Master Mix (Applied Biosystems). PCR conditions were 1 cycle at 95°C for 10 min, and 40 cycles of 95°C for 15 sec and 60°C for 60 sec.

Relative mRNA levels were normalized to *Gapdh* RNA level and calculated using the 2^-ΔΔCt method.

Wnt5a analysis and reporter construction

We used the genome browser of the University of California at Santa Cruz (http://genome.ucsc.edu/) to identify sequences within and around *Wnt5a* that are highly conserved in vertebrate genomes and that present marks of promoter and enhancer activity. These marks included epigenetic signatures in E14.5 mouse embryo limbs, as detected in chromatin immunoprecipitation followed by high-throughput sequencing (ChIP-seq) by the ENCODE/LICR project. They also included DNase I hypersensitivity in E11.5 mouse limb buds, as detected by the ENCODE project at the University of Washington. *Wnt5a* enhancer (>mm9_dna range: chr14:29,314,218-29,316,377) and promoter region (>mm9_dna range= chr14:29,317,415-29,319,242) were amplified by PCR from mouse genomic DNA and cloned upstream of the *lacZ* gene in the pWHERE reporter plasmid (InvivoGen). PCR products were sequence-verified upon cloning.

Cell cultures, transient transfection and adenovirus infection

COS-7 cells and MC3T3-E1 cells were cultured in 10% FCS-supplemented DMEM and αMEM, respectively. Transient transfection with the 6FXO-p89Luc reporter was performed by exposing 3 x 10^5 cells plated in 10-cm^2 dishes to mixtures containing 1 µg plasmid and 3 µl FuGENE6 (Roche). Plasmid mixtures included the reporter (150 ng), a pSV2βGal plasmid control for transfection efficiency normalization (50 ng), SOXC expression plasmids (200 or 400 ng) and an empty expression plasmid (600 or 400 ng)[12]. Transfections with *Wnt5a* reporters were

performed using 3 x 10^4 cells plated in 2-cm^2 dishes and exposing the cells to mixtures containing 0.5 µg plasmid and 1.5 µl FuGENE6. Plasmid mixtures included a reporter (300 ng), pGL-3Luc plasmid (100 ng), and SOXC expression plasmids or an empty expression plasmid (100 ng). Cell extracts were made 24h or 48 h later in Tropix buffer (Applied Biosystems). Reporter activities were assayed using Dual-Light system (Life Technologies). SDS-PAGE and semi-dry western blotting on PVDF membranes (Bio-Rad) were performed under standard conditions. Membranes were incubated with anti-FLAG-HRP antibody (1/1000, Sigma A8592) in blocking solution containing 5% non-fat dry milk. Signals were detected using ECL Prime Western Blotting Reagent (Life Technologies). Calvarium osteoblasts and rib chondrocytes were prepared from newborn mice, and SOX11, CRE, and *lacZ* adenovirus preparations were obtained and used at 100 plaque-forming units/cell as described.[21]

Electrophoretic mobility shift assays

Electrophoretic mobility shift assays (EMSA) were performed essentially as previously described[12] Briefly, EMSA probes (Table S2) were end-labelled with [α-^{32}P]-dCTP. They were then incubated in DNA-binding buffer (25 mM HEPES, pH 7.9, 40 mM KCl, 1 mM EDTA, 1 mM MgCl2, 0.5 mM DTT, 0.1% IGEPAL, 5% glycerol) along with 100 ng of recombinant SOX4 protein (OriGene TP309139). After a 30-min incubation at 30°C, protein-DNA mixtures were resolved by electrophoresis in 4% polyacrylamide native gels. Dried gels were exposed to X-ray films for 1 h to visualize signals.

Quantitative data presentation and statistical analysis

In vivo data were analyzed using control and mutant littermates. At least three measurements per animal and three pairs of animals were made to show reproducibility of phenotypes. In vitro data were analyzed using technical triplicates per condition, and experiments were repeated at least twice to demonstrate reproducibility of biological effects. Unless otherwise indicated, data are presented as average of experimental values with standard deviation. The two-tailed Student's t-test was used for statistical analysis. Differences were considered significant when the p value was less than 0.05.

RESULTS

SoxC genes are necessary for growth plate chondrocyte differentiation

We started this study by asking whether the severe brachysyndactyly of $SoxC^{Prx1Cre}$ fetuses was due to growth plate chondrocyte differentiation defects (Fig. 1A).[21] RNA in situ hybridization of tibia sections from E18.5 fetuses showed that *Sox9*, *Col2a1* and *Acan* expression was restricted to epiphyseal, columnar and prehypertrophic chondrocytes in control fetus tibias, as expected, but was encompassing all chondrocytes, perichondrium and joint cells in mutant tibias (Fig. 1B). This expression pattern of *Sox9* at E18.5 was as previously described for E14.5 $SoxC^{Prx1Cre}$ embryos.[21] Occasionally, mutant fetuses featured an ectopic pocket of chondrocytes bulging from the perichondrium towards the central region of the tibia and always located on the external side. This cell pocket was not expressing *Sox9*, *Col2a1* (encoding collagen type II) and *Acan* (aggrecan core protein). *Pth1r* (receptor for parathyroid hormone and parathyroid hormone-related peptide) and *Ihh* (Indian hedgehog), which mark prehypertrophic chondrocytes, and *Col10a1* (collagen type X), which marks pre- and hypertrophic chondrocytes, were not expressed in mutant tibias, except for weak expression of *Pth1r* and *Ihh* in the ectopic bulge. *Pthlh* (parathyroid hormone-related peptide) was properly expressed in mutant tibias in sub-articular cartilage regions at both E14.5 and E18.5 (Fig. 1B and S1). *Runx2* and *Sp7* (also known as *Osx*), which are weak markers of growth plate chondrocytes and robust markers of developing osteoblasts, were not or weakly expressed in mutant tibias. We thus concluded that *SoxC* genes are necessary to allow chondrocytes in cartilage primordia to promptly mature, form growth plates and induce endochondral ossification.

SoxC genes are expressed in growth plate chondrocytes and perichondrocytes

Prx1Cre is active in the entire limb bud mesenchyme that gives rise to chondrocytes, perichondrocytes and joint cells. Since these cell types crosstalk in order to coordinate all aspects of skeleton patterning, growth and maturation, the question arose as whether $SoxC^{Prx1Cre}$ fetuses were lacking growth plates as a consequence of *SoxC* absence in mesenchymal cells or in any of their derivatives. We previously showed that *SoxC* genes are actively expressed in skeletogenic mesenchymal cells, including perichondrium and presumptive joint cells, and more weakly expressed in E14.5 cartilage primordia.[12,21] RNA in situ hybridization of E18.5 fetus sections revealed here that growth plate chondrocytes were readily expressing *Sox11* at the epiphyseal stage and *Sox4* at the terminal stage, but were weakly expressing the three *SoxC* genes in other chondrocytes compared to articular joint, perichondrium and bone cells (Fig. 2). Thus, *SoxC* genes could direct growth plate formation through roles in precursor cells, chondrocytes, or adjacent cells.

SoxC expression in growth plate chondrocytes promotes skeletal growth

We tested whether *SoxC* genes have direct roles in growth plate chondrocytes by conditionally inactivating them using *ATC*. This bi-transgene expresses CRE recombinase in fully differentiated chondrocytes under the control of an *Acan* enhancer, but only in response to doxycycline, a member of the tetracycline antibiotics family.[24] Since *SoxC* genes have redundant functions in early skeletogenesis and many other processes, we generated triple mutants, i.e., $Sox4^{fl/fl}11^{fl/fl}12^{-/-}ATC$ mice, hereafter referred to as $SoxC^{ATC}$ mice. When pregnant females were treated with doxycycline from E12.5, a stage at which most cartilage primordia overtly develop, *SoxC* inactivation could be detected in growth plate chondrocytes from E14.5

(Fig. S2). *SoxC*^{ATC} pups looked grossly normal at birth but most died within a few hours (data not shown). Whole-mount preparations showed that all skeletal elements were overall well formed, except that the thoracic cage was narrow (possibly causing lethal respiratory distress) and that long bones were on average 10% shorter than normal (Fig. 3A). Histology analysis of tibias at E14.5 and E15.5 did not reveal remarkable abnormalities (Fig. 3B). The total length of these elements was normal and so were the epiphyseal, columnar, and hypertrophic chondrocyte zones and the endochondral bone region. By E16.5, the cartilage columnar zone and endochondral bone region showed a trend towards shortening. At E18.5, all zones of the tibias were shorter, except epiphyses. Similarly, E18.5 *SoxC*^{Col2Cre} fetuses, in which *Col2Cre* allowed inactivation of *Sox4* and *Sox11* in chondrocytes earlier than *ATC* and in few joint and perichondrium cells,[25] showed similar defects (Fig. 3C). Thus, *SoxC* expression in chondrocytes is not required to form growth plates, but contributes to promoting skeletal elongation.

SOXC proteins are dispensable in chondrocytes for growth plate differentiation

RNA in situ hybridization and qRT-PCR assays revealed that the distribution pattern and level of *Sox9* RNA were normal in *SoxC*^{ATC} growth plates (Fig. 4A, B). Likewise, prehypertrophic (*Pth1r, Ihh, Runx2),* hypertrophic (*Bmp6, Col10a1, Vegfa, Tcf7l2*), and terminal (*Mmp13*) chondrocyte markers were expressed with normal patterns and at normal levels in mutant growth plates. These data thus demonstrated that SOXC proteins do not control the growth plate chondrocyte differentiation program, and together with data obtained in *SoxC*^{Prx1Cre} fetuses, argued that SOXC proteins likely control this program through non-cell-autonomous actions.

SOXC proteins promote growth plate chondrocyte survival, proliferation, morphology and columnar organization

Having demonstrated that the shortness of *SoxC*^{ATC} growth plates was not due to chondrocyte differentiation defects, we looked for other deficiencies in these mutant structures. Using TUNEL assay in proximal tibia growth plates, we observed as expected that control fetus chondrocytes were dying almost exclusively in the terminal zone (Fig. 5A). In contrast, *SoxC*^{ATC} chondrocytes were dying in a significantly higher proportion in the columnar (4.9% versus 0.1%), hypertrophic (9.5% versus 0.6%), and terminal zones (17.2% versus 11.4%). This untimely death of columnar and hypertrophic mutant chondrocytes was confirmed by cleaved caspase-3 immunostaining (Fig. 5B). Other tibia and femur growth plates gave similar results (Fig. S3A and B). We then measured cell proliferation through DNA incorporation of the thymidine analogue EdU in cells at the S phase of mitosis. *SoxC*^{ATC} chondrocytes were proliferating at a normal rate in epiphyses, but instead of plateauing in the proximal half of the columnar zone, they immediately started to reduce their proliferation rate and they then growth-arrested slightly earlier than control cells in the prehypertrophic zone (Fig. 5C). Last but not least, high-magnification pictures uncovered chondrocyte morphological and organizational defects in *SoxC*^{ATC} growth plates (Fig. 5D). Whereas control chondrocytes were flattened and forming straight, compact columns, often comprised of more than 6 cells, mutant chondrocytes were irregular in shape, often started prematurely to round up and enlarge, and were forming 3.5 times fewer columns of more than 6 well-aligned cells. Thus, *SoxC* expression in growth plate chondrocytes guarantees cell survival, optimal proliferation, fit shape and columnar organization.

SoxC genes are necessary for noncanonical WNT signaling in the growth plate

We then sought to decipher the mechanism(s) whereby SOXC proteins induce the formation and subsequently control the activity and organization of growth plates. We previously showed that SOXC proteins synergize with canonical WNT signaling to stabilize β-catenin in perichondrium and joint surrounding nascent cartilage primordia.[21] However, immunostaining revealed here that the level of β-catenin was normal or slightly increased in $SoxC^{ATC}$ chondrocytes (Fig. S4). Therefore, SOXC proteins must promote growth plate organization and activity through a mechanism that is independent of, and possibly even represses, canonical WNT signaling in chondrocytes. Since noncanonical WNT signaling antagonizes canonical WNT signaling[28,29] and since the skeletal phenotype of $SoxC^{Prx1Cre}$ fetuses strikingly resembled that of $Wnt5a^{-/-}$ fetuses, which lack the noncanonical WNT5A ligand,[30,31] we explored the possibility that WNT5A signaling was impaired in *SoxC* mutants. RNA in situ hybridization indicated that E14.5 $SoxC^{Prx1Cre}$ fetuses were expressing *Wnt5a* at a greatly reduced level in perichondrocytes and chondrocytes (Fig. 6A). *Fzd3* and *Csnk1e* expression was strongly reduced too, whereas *Ror2* expression was unchanged. *Fzd3* and *Ror2* encode WNT receptors that contribute to noncanonical WNT signaling.[32,33] *Csnk1e* encodes the casein kinase 1ε subunit, which may mediate canonical WNT/β-catenin and noncanonical WNT Fzd/planar cell polarity signaling, while the related casein kinase 1δ subunit may phosphorylate VANGL2 downstream of noncanonical WNT signaling.[33,34] We strengthened these findings by performing additional experiments. We used primary cells from newborn mouse calvarium and cultured them under conditions that keep the cells at an osteoblastic precursor stage. We chose these cells because they resemble, but are more abundant and easier to isolate than perichondrocytes. Cells infected with a SOX11-expressing adenovirus exhibited 4.2 fold more *Wnt5a* RNA than cells infected

with a *lacZ*-encoding control adenovirus (Fig. 6B). Moreover, they also contained 16.7 fold more *Fzd3* RNA, 2.3 fold more *Csnk1d* RNA and 7.0 fold more *Csnk1e* RNA. Again, the level of *Ror2* RNA was unchanged. On the other hand, RNA in situ hybridization indicated that $SoxC^{ATC}$ mutants were expressing *Wnt5a* at a normal level in perichondrium and cartilage (Fig. 6C). A small decrease in mRNA level was seen for *Fzd3* and *Csnk1e* in mutant growth plates, but not for *Ror2*. RT-PCR assays in primary chondrocytes from $SoxC^{fl/fl}$ mice confirmed that *SoxC* inactivation in these cells did not significantly change the level of *Wnt5a* expression, but resulted in a mild decrease in *Fzd3*, *Csnk1d* and *Csnk1e* expression (Fig. 6D). Consistent with these results, forced expression of SOX11 in primary chondrocytes increased the RNA levels of *Wnt5a*, *Fzd3*, and *Csnk1e*, but with lower amplitude than in primary osteoblasts. Taken together, these findings support the conclusion that growth plate agenesis in $SoxC^{Prx1Cre}$ embryos likely results from failure to express *Wnt5a* and noncanonical WNT signaling mediators and that growth plate disturbances in $SoxC^{ATC}$ fetuses likely result from reduction in noncanonical WNT signaling. To further strengthen this conclusion and because irregular stacking of columnar chondrocytes is suggestive of a defect in planar cell polarity, a major event downstream of noncanonical WNT signaling, we analyzed the asymmetric localization of VANGL2, an important contributor to planar cell polarity in early-stage chondrocytes downstream of WNT5A signaling.[32,33] Immunostaining data revealed that significantly fewer chondrocytes were exhibiting asymmetrically localized VANGL2 in $SoxC^{Prx1Cre}$ and $SoxC^{ATC}$ tibias than in matched controls (Fig. 6E and F). Furthermore, primary cilia, which are also involved in planar cell polarity, were preferentially localized in the middle of columnar chondrocytes in control growth plates, as expected,[35] but on the lateral sides of the cells in $SoxC^{ATC}$ growth plates (Fig. 6G).

We then asked whether SOXC proteins might directly activate the *Wnt5a* gene. Since the regulatory elements involved in the activation of the gene are unknown, we analyzed the entire gene and surrounding domains in order to identify putative enhancers. The gene promoter and transcribed region were carrying marks of activity in E14.5 mouse embryo limbs, as revealed by H3K4me3 (promoter), H3K27ac (active enhancer) and H3K4me1 (active and poised enhancers) histone modifications. They were also carrying marks of activity in E11.5 limb bud and mesoderm, as detected by DNase I hypersensitivity (nucleosome-free chromatin) (Fig. 6H and S5A). Besides these regions, a 500-bp domain located 4 kb upstream of the gene stood out by a high degree of conservation and by H3K4me1, H3K27ac, and DNase I hypersensitivity marks. These features strongly suggested that this domain could be an important enhancer. To test this possibility and the ability of SOXC proteins to activate this enhancer, we cloned the *Wnt5a* putative enhancer and promoter regions upstream of *lacZ* in the pWHERE reporter (Fig. S5B). Transient transfection of MC3T3-E1 cells (undifferentiated osteoblasts) with *Wnt5a* Prom/pWHERE and Enh/Prom/pWHERE reporters along with SOXC expression plasmids showed that the enhancer had no activity without SOXC protein overexpression (Fig. 6I). Interestingly, SOXC proteins were not able to significantly modulate the activity of the promoter-only reporter, but were able to increase it by 2 to 5 fold when the enhancer was included in the reporter. As seen for other reporters,[12] SOX11 was the most potent of the three proteins. We then asked whether SOXC proteins mediate this effect by directly binding to the enhancer. Inspection of the most conserved region of the enhancer revealed multiple sequences closely resembling DNA motifs preferred by SOX proteins, including SOX4 and SOX11.[20,36,37] We selected three regions containing such sequences (Fig. S5C and Table S2) and used them as DNA probes in electrophoretic mobility shift assay (Fig. 6J) along with FXO+, a positive control

probe.[21] Purified SOX4 protein efficiently bound probes 1 and 3, which had the best fit SOX binding motifs, but did not bind probe 2, which only contained loose SOX motifs. Taking all data into account, we concluded that SOXC proteins induce growth plate formation and organization at least in part by eliciting noncanonical WNT signaling via direct activation of *Wnt5a* expression.

SOX12 tempers the beneficial action of SOX4 and SOX11 on growth plate chondrocytes

Since *SoxC* genes showed overlapping but not identical expression patterns in the growth plate, we asked whether they have redundant or distinct roles in this structure. Because $SoxC^{ATC}$ fetuses treated with doxycycline were often delivered prematurely, we generated and compared single, double, and triple mutants at E16.5. $Sox12^{-/-}$ fetuses were not significantly different from $Sox12^{+/+}$ littermates with regards to the length of tibias and the organization of growth plates (Fig. 7A). $Sox4^{ATC}$ fetuses had shorter tibias than control littermates, with shorter columnar zones and a trend towards a shorter endochondral bone region, but $Sox4/12^{ATC}$ fetuses were similar to $Sox12^{-/-}$ littermates. $Sox11^{ATC}$ fetuses were more affected than $Sox4^{ATC}$ fetuses, showing shorter epiphyseal, columnar and endochondral bone zones, and an enlarged hypertrophic zone. The latter defect was transient, due to a slight delay in initiating endochondral ossification (data not shown). In contrast, $Sox11/12^{ATC}$ fetuses were not markedly different from $Sox12^{-/-}$ littermates. $Sox4/11^{ATC}$ fetuses were as affected as $Sox11^{ATC}$ mutants, and more affected than $Sox4/11/12^{ATC}$ ($SoxC^{ATC}$) fetuses at both E16.5 and E18.5 (Fig. 7A and S6A). $Sox4^{ATC}$, $Sox11^{ATC}$, and $Sox4/11^{ATC}$ fetuses showed the same growth plate organizational defects and increase in cell death rate as $SoxC^{ATC}$ fetuses (Fig. S6B and C). As seen in triple SOXC mutants, no major change in *Wnt5a* and *Fzd3* expression could be detected in single and double mutants by RNA in

situ hybridization (data not shown). In contrast, these partial mutants exhibited impairment in asymetrically distributing VANGL2 in direct proportion to the severity of their growth plate phenotype (Fig. S7). The percentage of cells localizing VANGL2 asymetrically was indeed reduced two fold in *Sox4*^{ATC} and *Sox11*^{ATC} mutants and by three fold in *Sox4/11*^{ATC} mutants, and was only slightly decreased in *Sox11/12*^{ATC} mutants. This result strengthened the notion that SOXC proteins have key roles in controlling planar cell polarity in the growth plate.

To test whether inactivation of one or two SOXC genes affected the expression of the remaining wild-type SOXC genes through a positive or negative feedback loop, we measured mRNA levels in growth plates by qRT-PCR (Fig. S8). Each group of mutant samples showed significant reduction in expression of the SOXC genes deleted in growth plates by ATC but, while expression of the remaining SOXC alleles tended to decrease, there was no statistically significant difference.

Taken together, these data suggest a model whereby SOX12 tempers positive actions of SOX4 and SOX11 in growth plate chondrocytes; the combined levels of SOX4 and SOX11 proteins are amply sufficient to promote growth plate activity and neutralize the negative effect of SOX12; hence, SOX12 deletion in an otherwise wild-type background does not affect growth plates, but when either SOX4 or SOX11 is deleted, the level of the remaining protein is insufficient to neutralize SOX12 and exert maximum effect on growth plates; it is sufficient to maximally promote growth plate activity only when SOX12 is also deleted. To add weight to this model, we tested the ability of SOX12 to interfere with the activity of SOX4 and SOX11 in vitro. Since the differential importance of the three proteins in vivo matched their relative transactivation strengths, we used a transactivation assay. We transfected COS-7 cells with SOXC expression plasmids and 6FXO-p89Luc, a reporter containing six tandem copies of a SOXC-binding site.[12]

We used amounts of expression plasmids leading to saturating levels of reporter activation in order to establish competition conditions for the SOXC proteins. As expected,[12,13] SOX4 and SOX11 were more potent transactivators than SOX12 (Fig. 7B). When SOX12 was co-expressed with SOX4 or SOX11, it prevented its relatives from exerting maximum activity. We obtained similar results using the *Wnt5a* Enh/Prom/pWHERE reporter (Fig. S9). These results thus support the conclusion that SOX12 likely acts in vivo as a rheostat to temper transactivation of target genes in cells expressing high levels of SOX4 and/or SOX11.

DISCUSSION

This study sheds new lights on molecular networks that govern fetal induction and activity of growth plates. It consolidates the notion that crosstalk between perichondrocytes and chondrocytes pivotally control skeletal growth. Specifically, it newly proposes that SOXC proteins have decisive roles upstream of noncanonical WNT signaling in chondrocytes and perichondrocytes and adds support to the view that this molecular path is chiefly involved in crosstalk between these cells (Fig. 8). Key points in the model that we propose are that SOXC proteins are robustly expressed in perichondrocytes, in which they activate *Wnt5a*, and are weakly expressed in chondrocytes but influential enough to allow WNT5A signaling to spark growth plate chondrocyte differentiation and, through planar cell polarity, to keep chondrocytes alive, proliferating, and aligning into straight, packed columns. Of the three proteins, SOX4 and SOX11 are the most potent and their actions are fine-tuned by SOX12.

Together, the phenotypes of $SoxC^{Prx1Cre}$, $SoxC^{Col2Cre}$ and $SoxC^{ATC}$ fetuses convincingly demonstrated that SOXC proteins are required for growth plate development, but that their main action does not take place in chondrocytes, but most likely in perichondrocytes. Although conditional strategies to specifically delete genes in perichondrocytes remain unavailable to definitively confirm this point, several pieces of data strongly support it. They include marked expression of the *SoxC* genes in perichondrocytes and an abnormal phenotype of these cells in $SoxC^{Prx1Cre}$ fetuses when chondrocytes commit to growth plate differentiation. This abnormal phenotype was shown in our previous study to comprise lingering expression of *Sox9* and deficient canonical WNT signaling.[21] We showed here that it also includes scarce expression of *Wnt5a* and reduced expression of noncanonical WNT signaling mediators. Supporting the

argument that SOXC proteins elicit production of a signal traveling from perichondrocytes to chondrocytes is the development of a small pocket of growth plate chondrocytes on the external side of the tibia in *SoxC*^{Prx1Cre} fetuses. This phenomenon suggests that the skin (in which *SoxC* genes are not inactivated) might substitute for the perichondrium in providing an inducing signal. It also nurtures the concept that the requirement of SOXC proteins for growth plate formation is not a secondary consequence of actions in cartilage primordia development, but truly involves live actions in perichondrocytes to induce growth plates.

Our study points to activation of noncanonical WNT signaling driven by WNT5A as the main non-cell-autonomous action of SOXC proteins on growth plates. This proposition is made at a time when the principle that perichondrocytes and chondrocytes entertain active crosstalk remains incompletely deciphered. This crosstalk has been convincingly demonstrated to include Indian Hedgehog and TGFβ, BMP, FGF, and canonical WNT family members.[1,3,38] However, none of these factors has been shown to be necessary and sufficient to initiate growth plate formation. Participation of noncanonical WNT signaling in this crosstalk has been proposed in several studies, but not definitively proven. *Wnt5a*$^{-/-}$ embryos were shown to fail to form growth plates, but whether this phenotype is a consequence of early cartilage primordia patterning and growth defects or a consequence of WNT5A absence at the time of growth plate formation remains unknown.[39] Forced expression of noncanonical WNT pathway mediators was shown to facilitate the columnar organization of growth plate chondrocytes in the chick and in cultured cells.[40,41] Thus, although the direct importance of endogenous noncanonical WNT signaling in the growth plate remains untested, all data concur to propose that WNT5A could be at the top of the cascade leading to cartilage growth plate induction. This proposition is further endorsed by the observation that *SoxC*^{Prx1Cre} embryos were unable to express *Wnt5a*, were impaired in their

ability to express mediators of the pathway in perichondrocytes and chondrocytes, and strongly resembled *Wnt5a*$^{-/-}$ embryos in their inability to form growth plates. Concomitant expressions of *Wnt5a* and *SoxC* genes in perichondrocytes suggest the possibility that SOXC proteins directly transactivate *Wnt5a*. Validating this hypothesis, we identified here an enhancer located 4 kb upstream of *Wnt5a* and demonstrated that SOXC proteins are able to bind this enhancer to highly conserved sequences and to activate it. These data, along with recently put forward evidence that *Csnk1e* is a direct target of SOX4 in early B cells,[20] establish a direct link between SOXC proteins and noncanonical WNT signaling and suggest that this link could be pivotal in multiple processes besides skeletogenesis. This conclusion is not depreciated by the fact that the overall phenotype of *SoxC*^{Prx1Cre} embryos was not as dramatic as that of *Ror2*$^{-/-}$ and *Wnt5a*$^{-/-}$ mice since SOXC genes were only deleted in cells expressing *Prx1Cre* and daughter cells.

All cell type-specific transcription factors required for growth plate chondrocyte differentiation that have been identified as of today act in a cell-autonomous manner. They include SOX5/6/9 in early, columnar and hypertrophic chondrocytes[24,26] and RUNX2/3 and MEF2C from the prehypertrophic stage.[42-44] To our knowledge, SOXC proteins are the first cell type-specific transcription factors identified that control growth plate formation in a non-cell-autonomous manner. In addition to actions in perichondrocytes, we have also shown that SOXC proteins have significant roles in chondrocytes, but we ruled out major roles in the cell differentiation program.

We presented evidence that the contribution of SOXC proteins in chondrocytes in fetuses results in a 10-15% accrual in length of major bones. This gain corresponds to the difference between the 5th and 95th percentiles in human newborn length (45.5 and 54 cm) and is therefore not negligible. Considering that numerous genes contribute to skeletal growth, this finding fits with the notion that SOXC proteins intervene in pivotal regulatory pathways. SOXC proteins were

found to facilitate growth plate chondrocyte columnar organization, proliferation and survival, as were WNT5A and VANGL2.[32,33] Thus, the defects of *SoxC* mutant growth plates could be explained by reduced PCP signaling downstream of WNT5A. SOXC proteins were shown in many processes to ensure cell survival and several mechanisms have been proposed, such as facilitation of Hippo signaling in mesenchymal and neural cells and PI3K signaling in leukemia cells.[17; 45-49] SOXC proteins may thus promote growth plate chondrocyte survival through promoting noncanonical WNT signaling and other pathways.

We previously showed that SOXC proteins amplify canonical WNT/β-catenin signaling in joint and perichondrium cells,[21] whereas we showed here that they boost noncanonical WNT signaling, but do not affect or destabilize β-catenin in growth plate chondrocytes. SOXC proteins can thus act differently or with different consequences in distinct cell types. A likely explanation for the different action of SOXC proteins on canonical WNT signaling in perichondrium/joint cells and chondrocytes is that the relative levels of SOX9 and SOXC proteins vary considerably between these cells. SOX9 was shown to repress β-catenin signaling in early-stage and growth plate chondrocytes and was proposed to achieve this action by stimulating β-catenin destruction in the APC/Axin complex.[24,50,51] We recently showed that SOXC proteins compete with SOX9 to enter the β-catenin destruction complex and in contrast to SOX9 prevent β-catenin degradation.[21] While SOXC proteins likely outnumber SOX9 in perichondrium and joint cells, resulting in β-catenin stabilization, the inverse situation likely occurs in chondrocytes, resulting in β-catenin degradation. As canonical and noncanonical WNT signaling are antagonistic, low canonical WNT signaling in chondrocytes allows SOXC proteins to fully reveal their actions on noncanonical WNT signaling, leading to a further drop in β-catenin level.

We previously showed that *Sox12* inactivation (*Sox12*$^{-/-}$) worsened the phenotype of *Sox4*$^{-/-}$*11*$^{-/-}$ embryos and *Sox4*$^{fl/fl}$*11*$^{fl/fl}$*Prx1Cre* embryos,[17,21] whereas we showed here that it rescued the growth plate phenotype of *SOX4/11*^{ATC} embryos. In vitro experiments provided a molecular explanation to this unexpected finding by showing that under saturating conditions, SOX12 reduces activation of a SOXC-dependent reporter by SOX4/11. The underlying cause is that the three proteins have virtually identical DNA-binding domains, but SOX12 has a significantly weaker transactivation domain than SOX4/11. By competing with SOX4/11 for binding to cis-acting elements, SOX12 thus prevents its relatives from acting at full power. Since the *SoxC* genes are expressed robustly in perichondrocytes, but at low levels in chondrocytes, *Sox12* inactivation could have resulted in an indirect rather than direct rescue of the *SOX4/11*^{ATC} growth plate phenotype, i.e., it could have empowered perichondrocytes to signal more efficiently to chondrocytes and thereby to compensate for the diminished ability of mutant chondrocytes to respond to the perichondrium-derived signal. Thus, SOX12 likely acts as a "SOXC rheostat": it may join forces with SOX4 and SOX11 in order to achieve a maximum output in cells expressing low level of the proteins, but it may prevent SOX4 and SOX11 from activating genes too strongly in cells expressing high levels of the proteins.

Humans with *SOX4* or *SOX12* mutations have not been identified yet, but heterozygous mutations in *SOX11* were recently found to cause Coffin-Siris syndrome.[52] This condition has many features, including growth deficiency. Although the latter could have multiple causes, our findings suggest a primary defect in skeletal growth. They also suggest that other conditions comprising growth defects may exist in humans due to mutations in any of the *SOXC* genes.

In conclusion, SOXC proteins are critically involved in skeletal growth. They act in perichondrocytes and growth plate chondrocytes to promote noncanonical WNT signaling,

including planar cell polarity. This results in growth plate initiation and establishment of well-organized columns of actively proliferating chondrocytes. Given the wide expression of SOXC proteins and implication of noncanonical WNT signaling in many processes, this novel SOXC/noncanonical WNT molecular axis may be decisive not only in skeletal growth, but also in many other developmental, physiological and pathological conditions.

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FIGURE LEGENDS

Fig. 1. Chondrocytes do not undergo growth plate differentiation in *SoxC*^{Prx1Cre} fetuses. (*A*) Longitudinal sections of tibias from E18.5 *Sox4*$^{fl/fl}$*Sox11*$^{fl/fl}$*Sox12*$^{-/-}$ (control) and *Sox4*$^{fl/fl}$*Sox11*$^{fl/fl}$*Sox12*$^{-/-}$*Prx1Cre* (*SoxC*^{Prx1Cre}) littermates were stained with alcian blue (cartilage-specific dye) and nuclear fast red. Epi, epiphysis; Col and Hyp, columnar and hypertrophic zones of the tibia proximal growth plate, respectively; Endo Bone, endochondral bone in the primary ossification center. (*B*) Adjacent sections from the proximal tibia growth plate of E18.5 control fetuses and the entire tibia of *SoxC*^{Prx1Cre} littermates were hybridized with RNA probes, as indicated (red signals), and counterstained with the Hoechst 33258 DNA blue dye. *, Eccentric pocket of chondrocytes undergoing growth plate differentiation. White arrows, perichondrium.

Fig. 2. *SoxC* genes are expressed in growth plate, perichondrium, and bone tissue. Adjacent sections from an E18.5 mouse proximal tibia were stained with alcian blue and nuclear fast red (AB/NFR) or hybridized with SOXC RNA probes. AC, articular; EC, epiphyseal; PC, proliferating; pHC, prehypertrophic; HC, hypertrophic and TC, terminal chondrocytes; Pc, perichondrium; OF, ossification front cells.

Fig. 3. *SoxC*^{ATC} and *SoxC*^{Col2Cre} mice exhibit mild dwarfism. (*A*) Skeletal preparations of newborn (P0) control and *SoxC*^{ATC} pups and relative lengths of long bones in mutants expressed as percentage of those of littermates. **, $p < 0.01$ in the Student's t-test (n = 3). (*B*) Histology analysis and measurements of tibias in control and *SoxC*^{ATC} littermates collected at the indicated

gestation stages. Sections were stained with AB/NFR. *, $p < 0.05$; **, $p < 0.01$ (n = 3 or 4). (*C*) Histology analysis and measurements of tibias in E18.5 control and *SoxC*^{Col2Cre} littermates.

Fig. 4. Chondrocyte differentiation is not affected in *SoxC*^{ATC} growth plates. (*A*) Adjacent sections of proximal tibia growth plates from E18.5 control and *SoxC*^{ATC} littermates were stained with AB/NFR and hybridized with RNA probes as indicated. (*B*) qRT-PCR in proximal tibia growth plates from E18.5 control and *SoxC*^{ATC} littermates. Data are presented as averages with standard deviation for three fetuses per genotype. *, $p < 0.05$ in the Student's t-test. Note that *Sox4* and *Sox11* expression is only partially reduced in mutants, likely because the perichondrium was included in the samples.

Fig. 5. *SoxC* expression in chondrocytes helps develop functional growth plates. (*A*) TUNEL assay in proximal tibia growth plates of E16.5 control and *SoxC*^{ATC} littermates. Left, representative images of data. Dying cells are seen as green dots; cell nuclei are seen as blue dots. Boxed regions were used for quantification. Right, quantification of the percentage of TUNEL-positive cells in the columnar (Col), hypertrophic (Hyp) and terminal (Ter) zones. Data are presented as the average with standard deviation of values obtained using three non-adjacent sections per fetus. *, $p < 0.05$; **, $p < 0.01$ in the Student's t-test (n = 3). (*B*) Immunostaining of cleaved caspase 3 in proximal tibia growth plates from E16.5 control and *SoxC*^{ATC} littermates. Cleaved caspase 3 signal is seen in red; cell nuclei are seen in blue. Arrows point to dying cells. (*C*) EdU incorporation in DNA-synthesizing cells in proximal tibia growth plates from E18.5 control and *SoxC*^{ATC} littermates. Left, representative images of EdU-stained sections. Proliferating cells are seen as green dots; cell nuclei are seen in blue. Cells were counted in 12 adjacent regions. Regions 1 to 5 correspond to the epiphysis and regions 6 to 12 to the columnar

zone. Right, graph showing the percentage of EdU-positive cells in each area. *, $p < 0.05$ in the Student's t-test (n = 3). (*D*) Cell morphology in proximal tibia growth plates from E18.5 control and $SoxC^{ATC}$ fetuses. Entire growth plates are shown on the left. Columnar zone regions indicated with boxes are magnified in the middle pictures. The graph shows the numbers of such columns per surface area five times bigger than shown by the yellow box. Data are presented as average with standard deviation of values obtained for three non-adjacent sections per fetus. *, $p < 0.05$ in the Student's T-test (n = 3).

Fig. 6. SOXC proteins promote noncanonical WNT signaling in the growth plate. (*A*) RNA in situ hybridization of zeugopods from control and $SoxC^{Prx1Cre}$ embryos at E14.5. (*B*) qRT-PCR in primary osteoblasts infected with *lacZ* or SOX11 adenovirus for 24h. Data are shown for a representative experiment as averages of triplicates with standard deviation following normalization. *, $p<0.05$; **, $p<0.01$ in the Student's T-test. (*C*) RNA in situ hybridization in proximal tibia growth plates from E18.5 control and $SoxC^{ATC}$ fetuses. (*D*) qRT-PCR in $SoxC^{fl/fl}$ primary chondrocytes treated with *lacZ* or CRE adenovirus *(left)* or *lacZ* and SOX11 adenovirus (right). Data were obtained and are presented as in panel *B*. (*E-F*) VANGL2 immunostaining in frozen sections (green signal) of tibias from E14.5 control and $SoxC^{Prx1Cre}$ littermates (*E*) and from E16.5 control and $SoxC^{ATC}$ littermates (*F*). Cell nuclei were stained with Hoechst 33258 (blue). Left pictures show the proximal tibia growth plate. Right pictures are confocal microscopic images of the boxed area. White arrows point to asymmetrically localized VANGL2. Graphs show the percentage of cells with asymmetrically localized VANGL2. Cells were counted in three non-adjacent sections per embryo. *, $p<0.05$; **, $p<0.01$ in the Student's T-test (n = 3). (*G*) Detection of cilia by acetyl-alpha-tubulin immunostaining (red signal) of growth

plate chondrocytes from E16.5 control and *SoxC*^{ATC} littermates. Confocal microscopy pictures were taken in the middle of the proliferating zone. Arrows and arrowheads point to cilia present in the middle and lateral side of cells, respectively. The graph shows the percentage of cells exhibiting a cilium. Data were obtained and are presented as described in panels E and F. *(H)* UCSC genome browser analysis of the mouse *Wnt5a* proximal and promoter regions (chr14:29314215-29319243). The top schematic represents the first exon of *Wnt5a*. The next rows show peaks for the histone modification signature of promoters (H3K4me3), for DNase I hypersensitivity in E11.5 limb buds, and for sequence conservation in mammalian genomes. Double arrows at the bottom of the panel represent the enhancer and promoter segments used to make reporter plasmids and EMSA probes. *(I)* Assay of *Wnt5a* reporters transfected in MC3T3-E1 cells along with expression plasmids encoding no protein (-) or a SOXC protein, as indicated. Normalized reporter activities are shown for a representative experiment as averages of triplicates with standard deviation. Statistical significance of differences obtained for the reporter activities upon expression of each SOX protein was calculated using the Student's t-test (*, $p<0.05$; **, $p<0.01$). *(J)* EMSA of recombinant SOX4 incubated with *Wnt5a* probes 1 to 3 and FXO+. An arrow indicates the level of the wells. The migration levels of the free probes and probes bound by SOX4 are shown.

Fig. 7. Comparison of the activities of SOXC proteins in growth plates and on reporter genes. (*A*) *Top*, longitudinal sections of tibias from E16.5 control and *SoxC*^{ATC} mutants were stained with AB/NFR. # indicates that controls were null for *Sox12*. *Bottom*, the lengths of mutant tibia zones are presented relative to controls as the average with standard deviation of values obtained using three non-adjacent sections per fetus. *, $p<0.05$; **, $p<0.01$ in the Student's t-test (n = 3).

(*B*) Competition between SOXC proteins in activating a reporter. The 6FXO-p89Luc reporter was transfected in COS-7 cells along with two doses of 200 ng of expression plasmids encoding no protein (-) or a FLAG-SOXC protein, as indicated. Normalized reporter activities are shown for a representative experiment as averages of triplicates with standard deviation. Statistical significance of differences was calculated using the Student's t-test. A western blot shows the levels of SOXC proteins and a non-specific protein recognized by the FLAG antibody. A non-specific band is also seen at the level of SOX12. This blot shows that differences in reporter activities obtained for SOX4 and SOX11 could be due differences in relative amounts of proteins made in cells, whereas the low transactivation activity of SOX12 and inhibitory effect of SOX12 on SOX4 and SOX11 activities are not due to differences in protein amounts.

Fig. 8. Model for the roles of SOXC proteins in cartilage growth plates. See the discussion for details. Arrows of variable thickness schematize the relative transactivation force of the SOXC proteins.

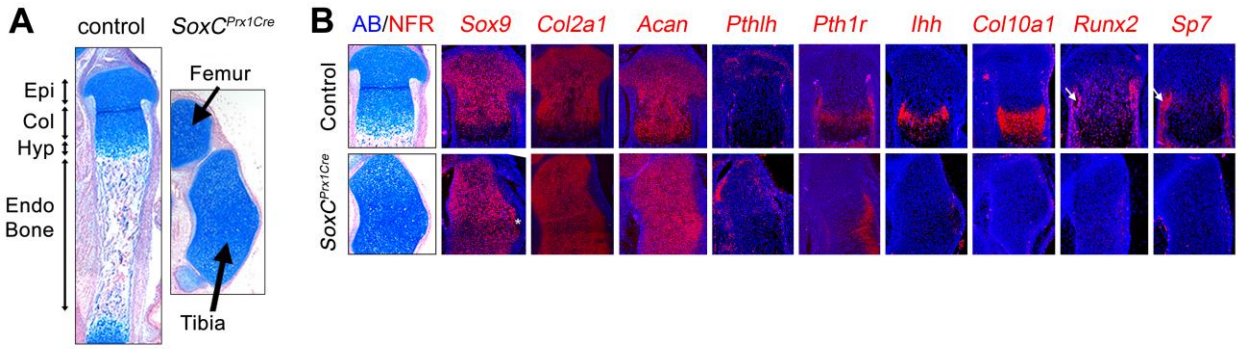


Figure 1

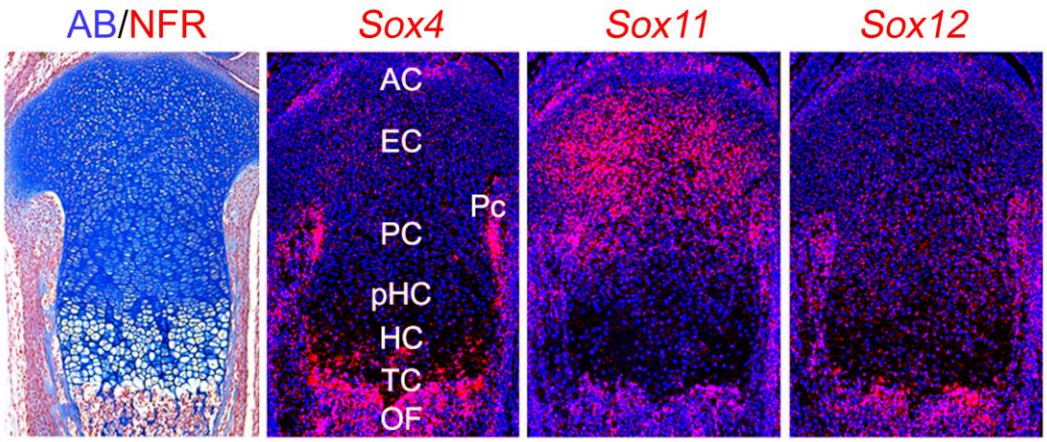
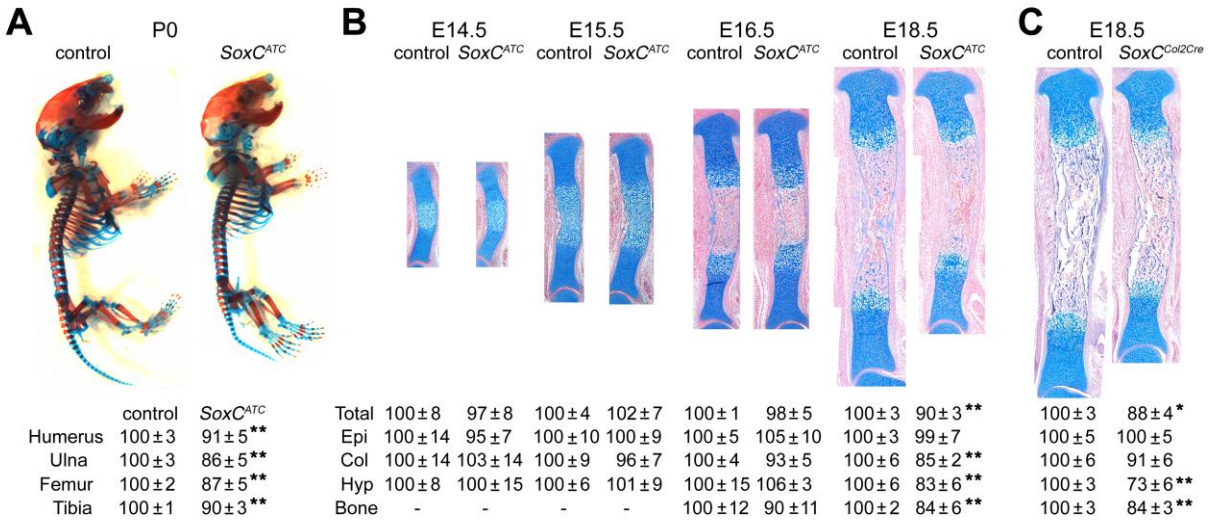


Figure 2



	control	$SoxC^{ATC}$
Humerus	100 ± 3	91 ± 5**
Ulna	100 ± 3	86 ± 5**
Femur	100 ± 2	87 ± 5**
Tibia	100 ± 1	90 ± 3**

	E14.5 control	E14.5 $SoxC^{ATC}$	E15.5 control	E15.5 $SoxC^{ATC}$	E16.5 control	E16.5 $SoxC^{ATC}$	E18.5 control	E18.5 $SoxC^{ATC}$
Total	100 ± 8	97 ± 8	100 ± 4	102 ± 7	100 ± 1	98 ± 5	100 ± 3	90 ± 3**
Epi	100 ± 14	95 ± 7	100 ± 10	100 ± 9	100 ± 5	105 ± 10	100 ± 3	99 ± 7
Col	100 ± 14	103 ± 14	100 ± 9	96 ± 7	100 ± 4	93 ± 5	100 ± 6	85 ± 2**
Hyp	100 ± 8	100 ± 15	100 ± 6	101 ± 9	100 ± 15	106 ± 3	100 ± 6	83 ± 6**
Bone	-	-	-	-	100 ± 12	90 ± 11	100 ± 2	84 ± 6**

E18.5 control	E18.5 $SoxC^{Col2Cre}$
100 ± 3	88 ± 4*
100 ± 5	100 ± 5
100 ± 6	91 ± 6
100 ± 3	73 ± 6**
100 ± 3	84 ± 3**

Figure 3

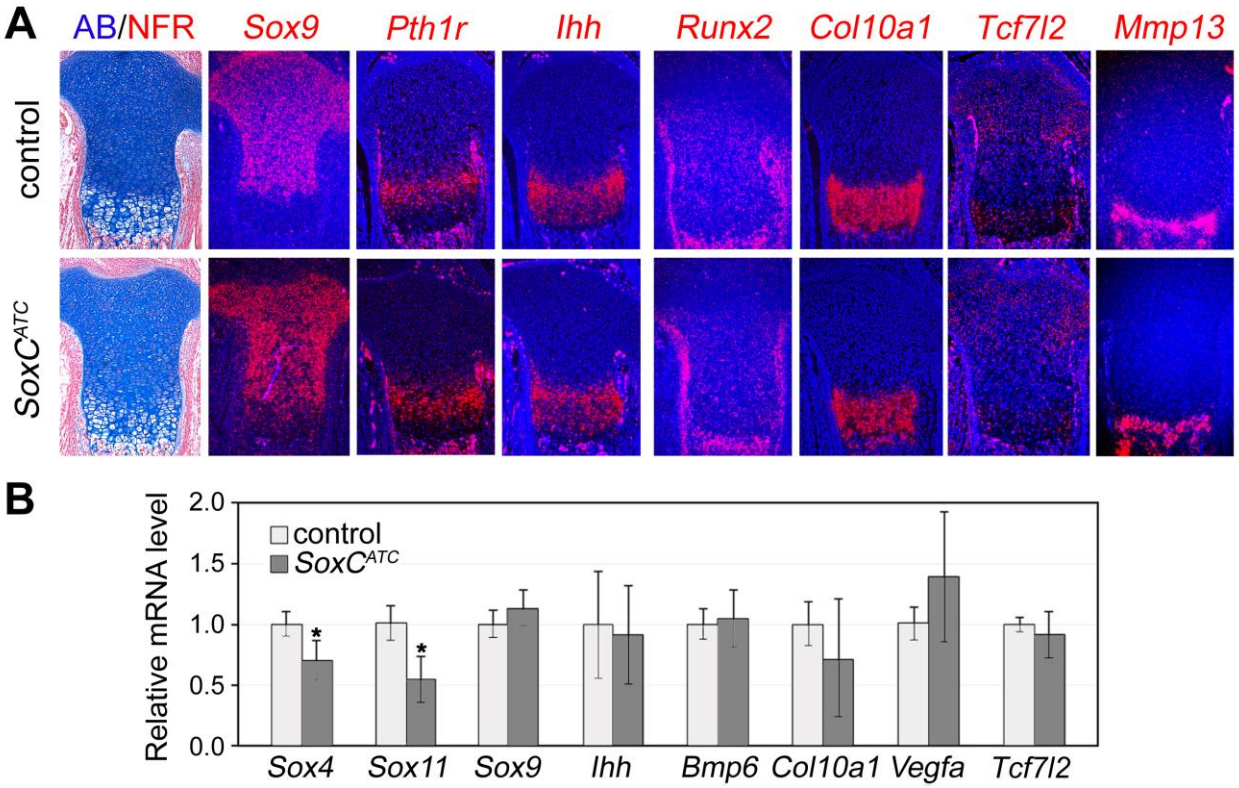


Figure 4

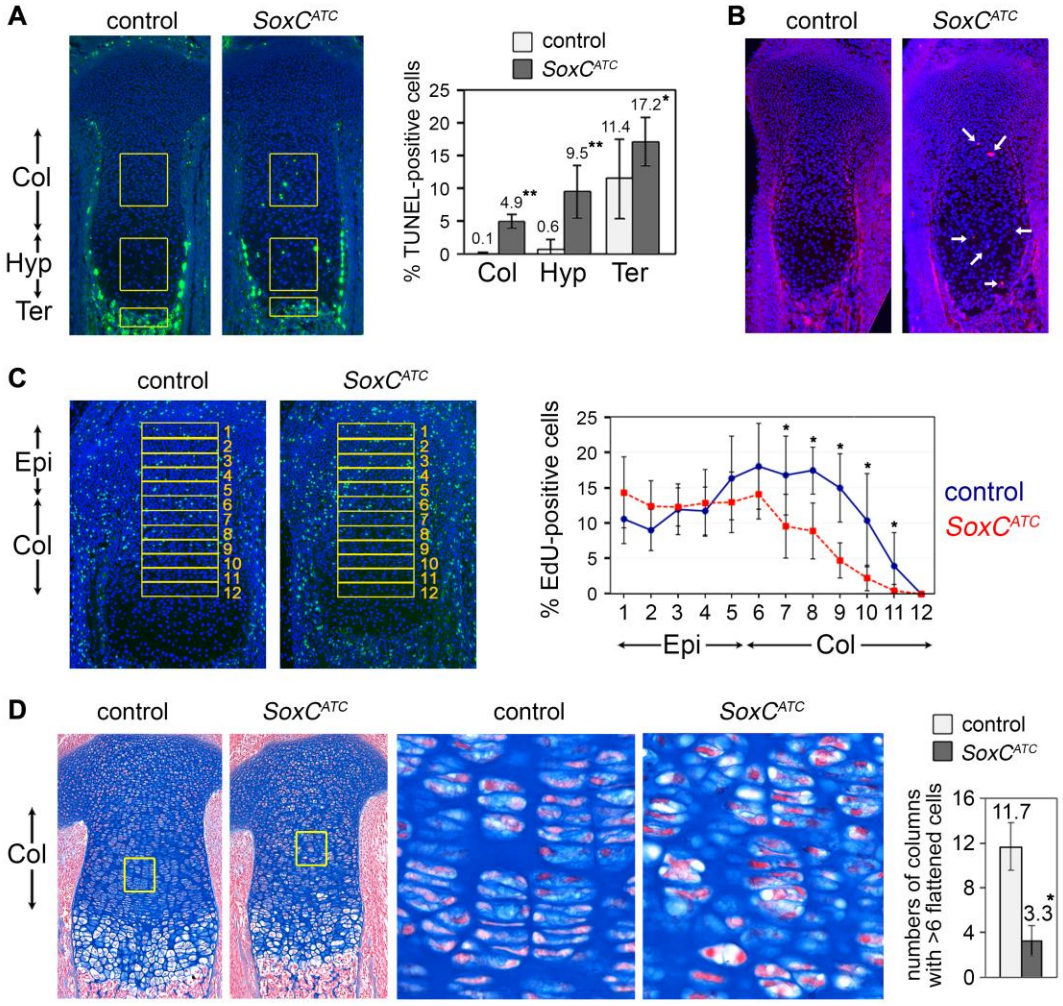


Figure 5

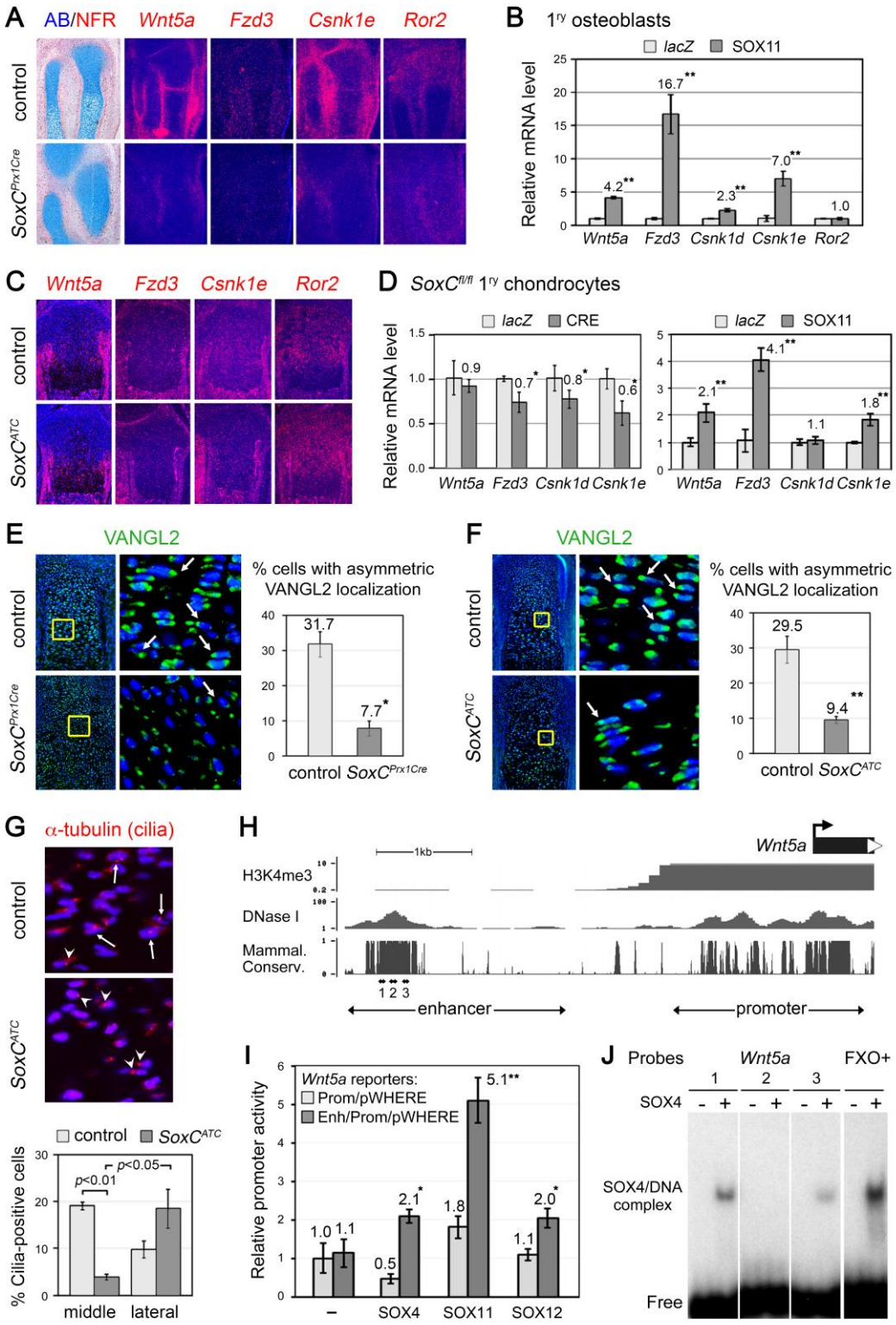


Figure 6

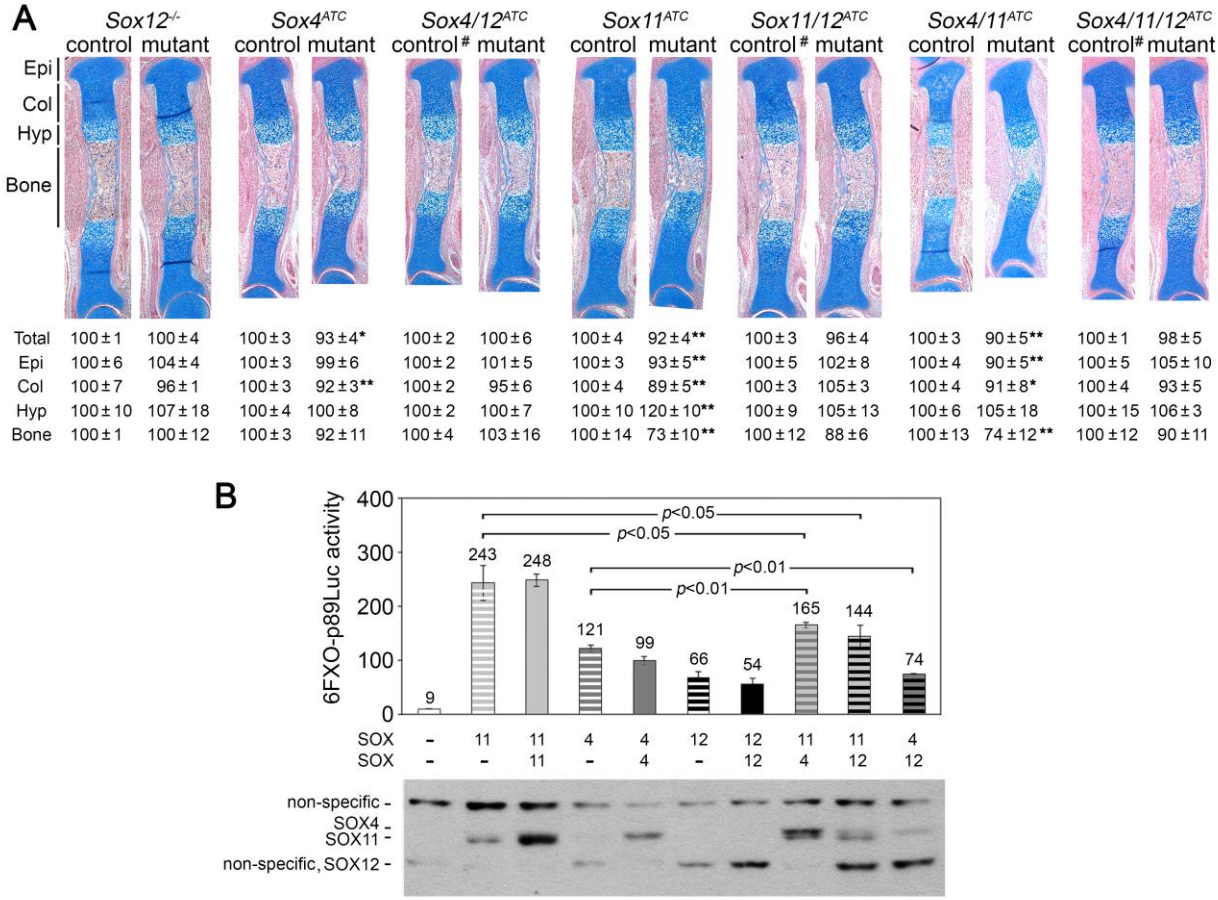


Figure 7

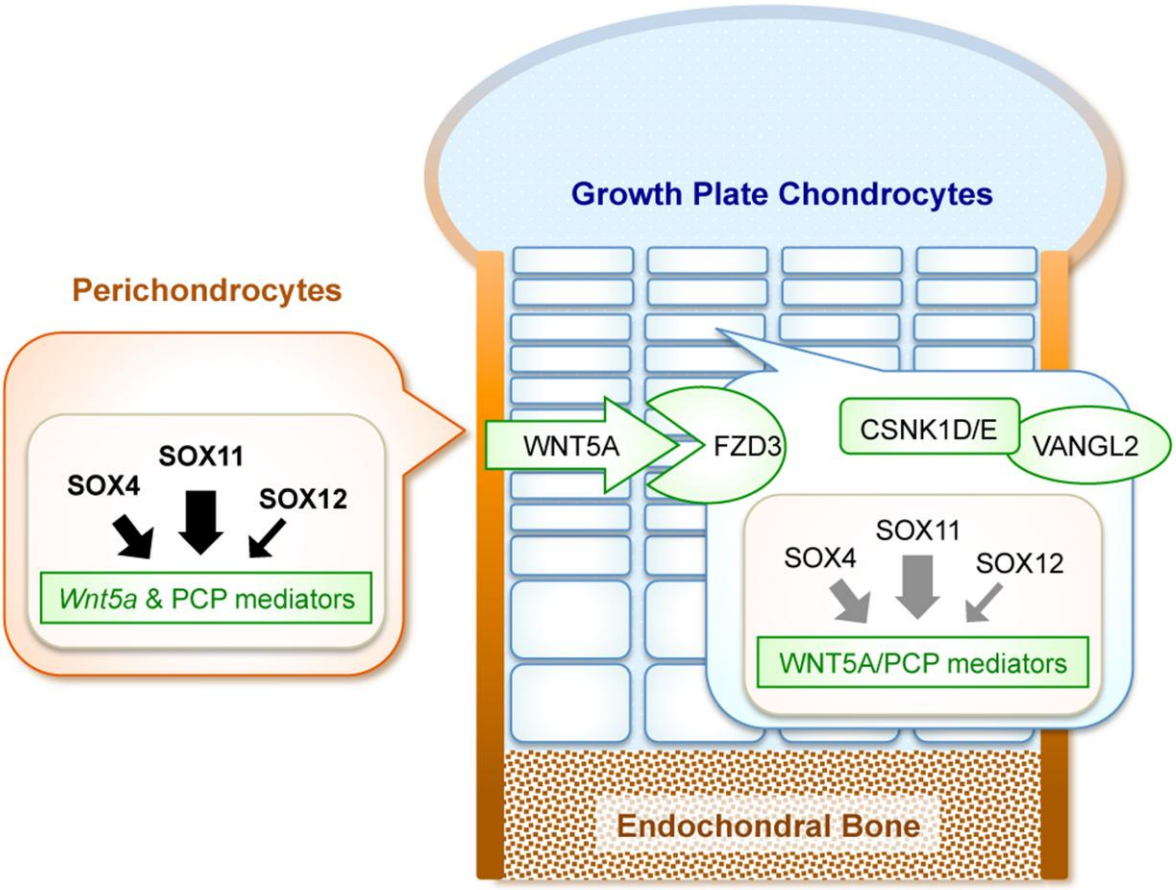


Figure 8