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SOX9 Governs Differentiation Stage-Specific Gene Expression in Growth Plate Chondrocytes via Direct Concomitant Transactivation and Repression

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Abstract

Cartilage and endochondral bone development require SOX9 activity to regulate chondrogenesis, chondrocyte proliferation, and transition to a non-mitotic hypertrophic state. The restricted and reciprocal expression of the collagen X gene, Col10a1, in hypertrophic chondrocytes and Sox9 in immature chondrocytes epitomise the precise spatiotemporal control of gene expression as chondrocytes progress through phases of differentiation, but how this is achieved is not clear. Here, we have identified a regulatory element upstream of Col10a1 that enhances its expression in hypertrophic chondrocytes in vivo. In immature chondrocytes, where Col10a1 is not expressed, SOX9 interacts with a conserved sequence within this element that is analogous to that within the intronic enhancer of the collagen II gene Col2a1, the known transactivation target of SOX9. By analysing a series of Col10a1 reporter genes in transgenic mice, we show that the SOX9 binding consensus in this element is required to repress expression of the transgene in non-hypertrophic chondrocytes. Forced ectopic Sox9 expression in hypertrophic chondrocytes in vitro and in mice resulted in down-regulation of Col10a1. Mutation of a binding consensus motif for GLI transcription factors, which are the effectors of Indian hedgehog signaling, close to the SOX9 site in the Col10a1 regulatory element, also derepressed transgene expression in non-hypertrophic chondrocytes. GLI2 and GLI3 bound to the Col10a1 regulatory element but not to the enhancer of Col2a1. In addition to Col10a1, paired SOX9–GLI binding motifs are present in the conserved non-coding regions of several genes that are preferentially expressed in hypertrophic chondrocytes and the occurrence of pairing is unlikely to be by chance. We propose a regulatory paradigm whereby direct concomitant positive and negative transcriptional control by SOX9 ensures differentiation phase-specific gene expression in chondrocytes. Discrimination between these opposing modes of transcriptional control by SOX9 may be mediated by cooperation with different partners such as GLI factors.

Introduction

Chondrogenesis and the formation of bone by endochondral ossification depend on progressive steps of cell differentiation. Mesenchymal cells condense and differentiate into chondrocytes in a pattern that will define the eventual shape of the different skeletal elements. These chondrocytes proliferate, mature, exit the cell cycle and become prehypertrophic. The differentiation program culminates in the terminal differentiation and apoptosis of post-mitotic hypertrophic chondrocytes [1]. This differentiation program is controlled by members of the SOX and RUNX families of transcription factors and the integration of multiple signaling pathways mediated by Indian hedgehog (Ihh), parathyroid hormone-related protein (PTHrP), Wnts, BMPs, and Notch (reviewed in [2]). PTHrP and Ihh are two important players which interact to form a feedback loop that controls the pace of the differentiation program [3].

Sox9 is essential for chondrogenesis and chondrocyte differentiation [4–6]. It is essential for mesenchymal condensation prior to chondrogenesis, and in its absence chondrocyte differentiation fails. Inactivation of Sox9 in chondrocytes at different stages of differentiation suggests that its expression is essential for the survival of chondrocytes so that they can progress to hypertrophy [5–7]. Mutations in Sox9 are associated with the human skeletal malformation syndrome, campomelic dysplasia, in which skeletal abnormalities can be attributed to the disruption of the chondrogenic differentiation program due to failure to express Sox9 target genes. Upon hypertrophy, chondrocytes down-regulate Sox9 expression [8,9], which is believed to mark the end of Sox9 control in the growth plate.

Despite the wealth of information about spatial and temporal gene expression patterns in the developing growth plate, it is not clear how transcriptional controls achieve appropriate and specific
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Author Summary
Chondrogenic differentiation is a key process in the formation of endochondral bone. Despite the wealth of information about gene expression patterns and signaling pathways important for this process, it is not clear how differentiation state-specificity of transcription is controlled. The transcription factor SOX9 regulates chondrocyte differentiation, proliferation, and entry into hypertrophy and is highly expressed in immature/proliferating chondrocytes. It directly transactivates Col10a1, enhancing this gene’s expression in immature/proliferating chondrocytes. The Col10a1 gene is specifically expressed in hypertrophic chondrocytes in which Sox9 is downregulated. How is differentiation phase-specific transcription of genes controlled in chondrocytes, particularly during hypertrophy? We found that SOX9 directly represses Col10a1 expression in immature/proliferating chondrocytes of the growth plate, so that its expression is restricted to hypertrophic chondrocytes. Discrimination of this concomitant opposing transcriptional control may involve cooperation between SOX9 and different partners such as GLI factors (effectors of hedgehog signaling). SOX9 control of chondrocyte maturation therefore may be integrated with hedgehog signaling. Mutations in human SOX9 cause the skeletal malformation syndrome campomelic dysplasia, which is attributed to the disruption of the chondrogenic differentiation program because of failure to express SOX9 target genes. This interpretation should be revised to include inappropriate expression of genes normally repressed by SOX9.

gene expression during chondrocyte differentiation. SOX9 activates many genes expressed in proliferating chondrocytes, including the extracellular matrix (ECM) genes Col2a1, Col9a1, Col11a2, Acan (aggrecan) and Cd-rap/Mia1 [10–15]. For the Col2a1 gene, which is expressed most strongly in proliferating chondrocytes, SOX9 directly transactivates the gene in vivo via a conserved enhancer sequence within the first intron [10,11].

The collagen X gene, Col10a1, is a hypertrophic chondrocyte specific marker. The specificity and reciprocity of Sox9 and Col10a1 expression epitomise the strict control of temporal and differentiation phase-specific gene expression in the growth plate. Col10a1 is ideal for studying transcriptional regulation because as well as its highly specific expression pattern, over-expression or loss-of-function does not disrupt chondrocyte differentiation. These properties simplify interpretation of changes in gene expression resulting from perturbing transcriptional control [16–18]. Here, we examined the transcriptional controls that restrict Col10a1 expression to hypertrophic chondrocytes. We found that SOX9 coordinates gene expression during chondrocyte differentiation through both transcriptional activation and repression. Discrimination between these opposing actions is probably achieved by cooperation between SOX9 and different partners such as GLI factors.

Results
Proliferating and hypertrophic chondrocytes show overlapping and different protein binding domains in the Col10a1 enhancer
Previous cell transfection studies identified an enhancer element upstream of human COL10A1 [19]. This element is highly conserved in mammals and corresponds to a 640 bp region between −4.3 and −3.6 kb of the mouse Col10a1 gene (designated element A) (Figure 1A and 1B). We used DNase I footprinting assays to test the configurations in which the element A sequences could be directly bound by nuclear factors derived from chondrocytes at different differentiation states (Figure 2A). Extracts from hypertrophic chondrocytes MCTs, but not fibroblasts COS-1 or osteoblasts MC3T3-E1, protected six blocks of sequence (H1–H6). Noticeably, four different blocks (P1–P4) that partially overlap H1–H4 were protected from extracts from the proliferating chondrocyte/chondrosarcoma cell line CCL (Figure 1C and Figure 2A). Since proliferating chondrocyte/chondrosarcoma cells do not express Col10a1 (Figure S1A), these results suggest that in these cells, the proteins that bind to element A may contribute to the repression of Col10a1.

SOX9 binds to Col10a1 element A in proliferating chondrocytes
We and others previously showed that SOX9 regulates COL2A1/Col2a1 gene via a functional in vivo binding site in the intron 1 enhancer element [10,11]. We identified the same SOX9-binding sequence within the Col10a1 element A (Figure 1C and Figure 2B). This site, COL2A1, lies on a region in block P3 that is not protected in hypertrophic chondrocytes, and is adjacent to a stretch of thymidine/guanine-rich (TG-rich) sequence. Electromobility shift assays revealed that SOX9 bound to this SOX9/TG-rich motif with a similar affinity as to the COL2A1 enhancer element (Figure S1C, S1E, S1F) [10] and the interaction involved dimeric binding (Figure 2C, cf. lane 3–4). SOX9 also interacted with the TG-rich motif but with a lower affinity than with the consensus SOX9 site. Mutation of the TG-rich motif reduced the overall SOX9 binding to the P3 element (Figure 2C, cf. lanes 6–10). The TG-rich motif resembles a RUNX binding consensus sequence, but we found that RUNX2 did not interact with this motif effectively compared with its binding to the RUNX site in the Bglap (osteocalcin) enhancer (Figure 2D). Chromatin immunoprecipitation (ChIP) assays using extracts from E13.5 mouse limb, a stage at which the cartilage anlagen is largely composed of immature chondrocytes, confirmed specific SOX9 binding to the Col10a1 element A and the Col2a1 enhancer in vivo (Figure 2E).

SOX9/TG-rich motif is required for appropriate Col10a1 expression
The paired SOX9 binding sequences in element A are separated by 4 bp, a distance similar to that between the paired SOX-like consensus sequences in Col2a1, Col9a1, and Acan that mediate transactivation of expression [15]. We tested the in vivo role of element A and the effects of SOX9/TG-rich motif mutations on the expression of Col10a1 mini-genes (Figure 3A) in transgenic mice. We have previously shown that a Flag-tagged Col10a1 vector Col10a1Flag [formerly known as FColX] is expressed in P10 hypertrophic chondrocytes [18]. Here, we show that in E15.5 humeri, the Col10a1Flag transgene was expressed in islands in prehypertrophic and hypertrophic chondrocytes in the upper hypertrophic zone (Figure 3B, c). In two independent mouse lines, a transgene comprising element A fused to the Col10a1Flag (Col10a1Flag-E) was expressed in a similar pattern as Col10a1Flag but was significantly more strongly expressed than Col10a1Flag in all hypertrophic chondrocytes (Figure 3B, d), reflecting the enhancer activity of element A (see also Figure S2). However, mutation of the SOX9 site in element A (Col10a1Flag-EΔ1) resulted in marked expansion of the expression domain of the transgene, extending from the hypertrophic zone to the proliferating zone in the majority of transgenic fetuses (71.4%) (compare Figure 4, a with
Figure 1. Conserved non-coding regions in COL10A1. (A) Global alignment of COL10A1 loci was performed using mammalian genomes. The translated regions in exons 2 and 3 of COL10A1 are highly conserved (deep blue). Element A of mouse Col10a1, which corresponds to the human enhancer, is located in the nearest conserved upstream non-coding region (pink). The untranslated regions (light blue) are poorly conserved in opossum. The mouse genome serves as the base sequence for alignment. Op: opossum; Ca: canine; Bo: bovine; Hu: human. (B) The 640 bp element A is located between −4286 and −3646 bp of mouse Col10a1. (C) Conserved non-coding regions in mammalian COL10A1. Alignment showed that the functional SOX9 binding site (COL2C1) is conserved in the mammalian COL10A1 enhancers together with the adjacent TG-rich sequence. The
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positions of the chondrocyte-specific binding motifs H1–H4 and P1–P4 deduced from DNase I footprinting are indicated. The primer sequences used in the chromatin immunoprecipitation are indicated in blue boxes. Consensus GLI and TCF binding sites were identified near the SOX9/TG-rich motif. Sequences involved in mutagenesis are shaded in blue. Only the mismatched nucleotides are shown in the aligned sequence.
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Figure 3B, e and in almost all the Sox9-expressing chondrocytes in the rest (Figure 4, e). Expansion of transgene expression in proliferating chondrocytes was also noted but was less marked when the TG-rich motif in element A was mutated (Col10Flag-EA2) (compare Figure 4, m and i with a and e). Mutation of either SOX9 or the TG-rich motif did not abrogate transgene expression in hypertrophic chondrocytes. Together, these observations suggest that element A contains both positive and negative regulatory sequences, and that mutations in the SOX9/TG-rich motif in element A might disrupt SOX9-mediated repression in immature chondrocytes.

SOX9 is a negative regulator of Col10a1

To test whether SOX9 negatively regulates Col10a1, we established a cell line from hypertrophic chondrocytes MC3Ts which expressed the Col10Flag-E transgene at the non-permissive (growth-arrest) temperature (Figure 5A, findings for dedifferentiated chondrocytic cells MC615, over-mediated repression in immature chondrocytes. Negative regulatory sequences, and that mutations in the SOX9/TG-rich motif in element A might disrupt SOX9-mediated repression in immature chondrocytes.

GLI factors bind and regulate Col10a1 in proliferating chondrocytes

The specificity of SOX9 protein action is known to be achieved through interaction with cell-specific partners [23,24]. We questioned whether concomitant transactivation of Col2a1 and repression of Col10a1 by SOX9 in proliferating chondrocytes could be mediated by different combinations of cofactors. ChiP assays in E13.5 mouse limb chondrocytes or CCL cells revealed similar interactions of TRAP230/MED12, a mediator of SOX9 activity [25], and of TRPS1, a GLI3-interacting repressor [26,27], with both the Col10a1 element A and the Col2a1 enhancer (Figure 6A, upper panel). On the other hand, the transcriptional co-repressor, histone deactylase HDAC4 [28] immunoprecipitated neither element. GLI1, GLI2 and GLI3 are effectors of Ihh signaling which controls chondrocyte proliferation and maturation [29]. GLI1 is a transactivator expressed in proliferating chondrocytes and perichondrial tissue flanking the prehypertrophic and hypertrophic zones [30] whereas GLI2 and GLI3 can act as repressors and are predominantly expressed in non-hypertrophic chondrocytes and are down-regulated in hypertrophic chondrocytes [29,31]. Since there is a conserved GLI-binding site near the SOX9/TG-rich motif in the same footprint block P3 (Figure 1C), we examined whether GLI1, GLI2 and GLI3 can interact with the element A. Strikingly, while SOX9 bound to both the Col10a1 element A and Col2a1 enhancer, GLI2 and GLI3 associated with only Col10a1 element A (Figure 6A, lower panel). GLI3 interacted the most with element A, while GLI1 interaction was much less. Quantitative ChiP assays confirmed the preferential interaction (Figure 6B). From these results we hypothesized that GLI proteins may repress Col10a1 expression. To test this in vivo, we examined the impact on transgene expression of mutating the GLI-binding site in element A (Col10Flag-EA3). Consistent with our hypothesis, the majority of fetuses (7 out of 10) expressing Col10Flag-EA3 showed distinct islands of transgene misexpression in non-hypertrophic chondrocytes (Figure 6C, e, f). Mutating all three sites (GLI, SOX9, TG-rich) in the transgene (Col10Flag-EA4) did not restrict the expansion of the expression domain to proliferating chondrocytes in all the expressing transgenic fetuses obtained (Figure 6C, i, j). Indeed in the majority of these expressing fetuses (3 out of 5), transgene expression extended throughout the entire cartilage zones. Thus mutation of the GLI site alone had a similar derepressing effect as mutating the SOX9/TG-rich motif and mutating all the motifs did not restrict expression but resulted in more extensive mis-expression. This is consistent with a model whereby SOX9 and GLI act cooperatively to repress Col10a1 transcription.

To assess whether the cooperation of SOX9 and GLI2/3 is a potential common mechanism for restricted or preferential gene expression in hypertrophic chondrocytes, we searched in silico for this configuration of binding sites in genes, other than Col10a1, that have strong and specific up-regulation in hypertrophic chondrocytes (HC genes) in the growth plate. Six of 11 HC genes analyzed, namely Col10a1, Bmp2, Hidac4, Mef2c, Runx2, and Sox4, possessed the linked SOX9 and GLI sites (<100 nt spacing) in the inter- or intragenic conserved non-coding regions (Figure 7A and Figure S3). In contrast, these sites were absent from most of the genes tested (12 out of 14) that were expressed in proliferating but not (or down-regulated) in hypertrophic chondrocytes (PC genes). These include known SOX9 targets: Col2a1, Col9a1, Col11a2, Acan, and Mati (see Figure 7A legend for all negative genes). The exceptions were Sox3 and Sox6 (Figure 7A and Figure S3). To investigate whether the over-representation of linked SOX9/GLI sites in the HC genes but not the PC genes occurs by chance, we performed a hypergeometric test to calculate the probability of finding 6 or more SOX9/GLI site-containing genes out of 11 genes randomly sampled from the mouse genome. For the HC genes, the results showed that the occurrence of 6 or more genes with associated conserved SOX9/GLI sites is unlikely to occur by
chance ($p = 0.0000201$) (Figure 7B). For the PC genes, the $p$-value was 0.17, which is comparable to random occurrence. Furthermore the frequency of the presence of SOX9-GLI sites for HC genes (6/11) was significantly higher than that for PC genes (2/14) (Fisher’s test $p = 0.043$, one tailed). This suggests that the linked SOX9-GLI sites are preferentially associated with the HC genes.
Discussion

The positive and negative mechanisms mediating the stage-specific transcription of genes within the growth plate are not well defined, partly because of the difficulty in distinguishing direct effects on transcription from the consequences of abnormal differentiation. In this study we have exploited the specificity of Col10a1 expression in hypertrophic chondrocytes and the fact that manipulating its expression in vivo has no overt effect on differentiation, to dissect these transcriptional controls. We provide new insight into how differentiation stage-specific gene expression is achieved in the growth plate, presenting in vitro and in vivo evidence that SOX9, in addition to its known role as a transactivator of many genes preferentially expressed in non-hypertrophic chondrocytes, such as Col2a1, directly represses expression of Col10a1 at a stage prior to the onset of hypertrophy and subsequently in proliferating chondrocytes. This discovery extends our understanding of the mecha-
Figure 4. Mutation of SOX9 binding consensus results in derepression of Col10a1 transgene expression in vivo. The expression pattern of mutant Flag-tagged Col10a1 mini-genes, in which the SOX9 site (Col10Flag-EΔ1) or the TG-rich motif (Col10Flag-EΔ2) within the element A was mutated, was examined by in-situ hybridization of proximal humeri in E15.5 expressing fetuses. In a major (5/7) portion of Col10Flag-EΔ1 fetuses (a–d), the expression in the proliferating zone (*) was relatively higher than in the hypertrophic zone. In a minor (2/7) portion (e–h), the expression was predominant in nearly all immature chondrocytes (*). For Col10Flag-EΔ2, a minor (2/6) portion of fetuses (m–p) showed up-regulated expression in the non-hypertrophic zone (*), while a major (4/6) portion showed relatively slight mis-expression (i–l). The prehypertrophic and hypertrophic zones are encircled. PC, proliferating chondrocytes; PHC, prehypertrophic chondrocytes; HC, hypertrophic chondrocytes.

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Figure 5. SOX9 directly represses Col10a1 in hypertrophic chondrocytes. (A) Clonal cell line MCTS-F expressing the Col10a1Flag-E was established from MCTS. (a) Immunohistochemistry indicated up-regulated Flag-tagged collagen X in growth arrested MCTS-F cells. (b) Flag signal in the cytosolic vesicles. (B) MCTS-F cells transfected with SOX9 expression vector (pSG-Sox9), or empty vector (pSG5). Expression of Col10a1 and Col10a1-Flag (36 bp longer in amplicon) was significantly reduced upon exogenous Sox9 expression (*, p<0.02). No RNA (−) or RNA from E16.5 Col10a1Flag-E expressing fetus (+) was used as control. (C) Strategy for activating ectopic Sox9 expression in hypertrophic chondrocytes. The pZ/Sox9

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nisms by which SOX9 controls chondrocyte differentiation phase-specific gene expression.

We have identified a conserved regulatory sequence, element A, that acts as an enhancer of Col10a1 expression in both cultured cells and in vivo. This element contains a SOX9 binding sequence that, when bound by SOX9, represses Col10a1 expression in immature and proliferating chondrocytes. Since Sox9 is expressed in nonhypertrophic chondrocytes but not in hypertrophic chondrocytes, this repressive action of SOX9 restricts Col10a1 expression to hypertrophic chondrocytes.

SOX9 has been proposed to direct chondrogenic fate in osteochondroprogenitor cells in part by interacting with RUNX2 [32,33]. SOX9 may inhibit chondrocyte hypertrophy in part via activation of Bapts1 which represses Runx2 [34,35]. Previous in vitro and in vivo studies suggest that Col10a1 expression is regulated positively by Mef2c, Runx2/Obf1, and AP-1 members, which are expressed in hypertrophic chondrocytes [19,36–38]. Runx2 has been shown to directly regulate the expression of Col10a1 [37]. The element A that we identified contains no conserved consensus RUNX site. The RUNX2 site revealed by Zheng et al. [37] is located within a poorly conserved region outside the element. Our data showed that the ectopic expression of Col10a1 transgene in non-hypertrophic chondrocytes does not require co-expression of Runx2. In addition, RUNX2 is not expressed in the costal hypertrophic chondrocytes and cultured hypertrophic chondrocytes MCTs (which is derived from costal cartilage), where Col10a1 expression is strong. Although real-time PCR showed levels of Col10a1 was markedly reduced in P1 Runx2-null mice [37], hypertrophic chondrocytes with strong Col10a1 expression do develop in many cartilages in Runx2 null fetuses [39,40]. Collectively existing data suggest that RUNX2 together with other factors regulate Col10a1 in vivo via promoting chondrocyte hypertrophy or otherwise functions to initiate a cascade of regulatory pathways that sustain Col10a1 expression in hypertrophic chondrocytes.

Previous in vitro studies in chicken have suggested that a combined action of positive and negative DNA elements may contribute to the hypertrophic chondrocyte-specific expression of Col10a1 [41,42]; however, these chick Col10a1 elements are not conserved in mammals. The enhancer element we identified is highly conserved in mammals, but not in chicken, which agrees with previous data [43]. This suggests that in both mammals and chicken, Col10a1 transcription is restricted to hypertrophic chondrocytes by repression, though by different cis-acting elements. In the chicken, this repression may extend to non-hypertrophic cell types [41]. We found no evidence to support such a mechanism in the mouse since when we abolished the interaction of SOX9 with the repressive element, we observed no ectopic Col10a1 expression in non-chondrogenic cells.

Consistent with a role for SOX9 in repressing Col10a1 in vivo, we have shown in Col10a1-Cre;Sox9 compound mutants at E17.5. Ectopic Sox9 expression (i), encoded down-regulated Col10a1 (k, encircled) and Cre (g) in hypertrophic chondrocytes, especially in the extended hypertrophic zone. The reduction of Col10a1 is not associated with change in Runx2 expression (l) in hypertrophic chondrocytes. PC, proliferating chondrocytes; PHC, prehypertrophic chondrocytes; HC, hypertrophic chondrocytes.

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SOX9 cannot regulate chondrocyte differentiation appropriately without sonic hedgehog (Shh), which mediates the generation of chondrogenic precursor cells [46], and Indian hedgehog (Ihh), which regulates their proliferation and maturation [47]. GLI proteins are the effectors of Hh signaling. Double knockout mutants indicate that GLI2 has overlapping functions with GLI1 and GLI3 in skeletal and CNS development [48,49]. Binding of Ihh to its receptor, Patched, blocks the proteolytic processing of the GLI transcription factors from active (GLI1<sup>A</sup>) to repressive (GLI1<sup>R</sup>) forms, and the balance between these forms modulates hedgehog target gene expression [50]. In the growth plate, GLI2<sup>A</sup> can positively regulate chondrocyte hypertrophy and control vascularization of the hypertrophic cartilage in endochondral ossification [29,51]. GLI3, which acts mainly as a repressor, has been suggested to inhibit chondrocyte hypertrophy [29,52] and it is interesting that the highest interaction of element A was with GLI3. Mau et al. reported that GlI2/3 null mutations altered the expression domain of collagen X, but it was not possible to distinguish whether this was due to a direct effect on Col10a1 transcription or more general perturbation of hypertrophy [29].

How the GLI factors interact with other regulatory factors or genes in the chondrocyte differentiation program is not clear. Our results are consistent with cooperation between SOX9 and the Hh signaling pathway and suggest that SOX9 acts in synergy with GLI2 and GLI3, probably their repressive forms GLI1<sup>R</sup>, to repress transcription in chondrocytes. Thus, reduced synergy between GLI1<sup>R</sup> and SOX9 may explain the accelerated chondrocyte hypertrophy seen when Ptk2 is inactivated [53]. Over-representation of the SOX9-GLI paired consensus in a number of genes that are preferentially expressed in hypertrophic chondrocytes and
Figure 6. GLI as SOX9 partner in Col10a1 repression. (A) ChIP assays with CCL cells (upper panel) and E13.5 mouse limbs (lower panel) using various antibodies. The products were amplified with primers for element A of Col10a1 or enhancer element of Col2a1 in intron 1. Extract was immunoprecipitated with rabbit IgG or without antibody (−) as negative reference. Extract was directly used in PCR as positive control (input). (B) The
not in proliferating chondrocytes, suggests that SOX9 may use this partnership to repress transcription of several genes in other chondrocyte types. However this partnership may not be the exclusive mechanism by which SOX9 acts to repress expression in chondrocytes.

Hattori et al. have recently shown that SOX9 directly represses Vegfa in cultured primary chondrocytes [44] by interacting with the 5′ untranslated region of the gene. This agrees with our findings of a repressive role of SOX9, however, we found no linked SOX9-GLI binding sites near the Vegfa gene and a recent SOX9 ChIP-on-chip study reported no in vivo interaction in Vegfa exon 1 [54]. A different mode by which SOX9 may repress gene expression in chondrocytes has been proposed by Huang et al. [55]. In their model, SOX9 negatively regulates Ccn2 expression in non-hypertrophic chondrocytes via binding to overlapping binding sites for SOX and TCF/LEF, thereby interfering with binding of a TCF/LEF/β-catenin transcription complex. Reduction of SOX9 upon hypertrophy allows this TCF/LEF/β-catenin complex to activate Ccn2 expression [55]. However, Ccn2 is also expressed in resting zone chondrocytes in the epiphyses of the growth plate and it is not clear why SOX9 does not repress the gene in these cells. We identified a conserved TCF consensus site in Col10a1 element A, but this is unlikely to interfere with SOX9 binding since it is located 59 bp downstream of the functional SOX9-GLI motif, unlike in Ccn2 where the SOX and TCF/LEF sites overlap (Figure 1C). This suggests that the model proposed by Huang et al. does not apply to Col10a1 element A-mediated repression of transcription.

It is also possible that SOX9 and GLI cooperate to activate or repress transcription depending on context. While our data implicate a cooperation of SOX9 with GLI factors in transcriptional repression, this association between SOX9 and GLI may not be restricted to negative regulation. Amano et al. have recently reported that GLI2 cooperates with SOX9 to transactivate the Pthlh gene [56]. However, the expression patterns of Sox9 and Pthlh are mutually exclusive in the developing growth plate, Pthlh being expressed mainly in the perichondrium and only at extremely low levels in proliferating chondrocytes [57,58]. This contradiction may reflect differences between in vitro assays and regulation in vivo, and it is also unclear whether the expressed GLI2 was processed to a repressor form or not in these cells. The observed stimulation of Pthlh promoter activity in the cultured chondrocytes could therefore be attributable to over-expression of GLI2 which persisted largely as the activated form GLI2A. However this report does raise the possibility of a context dependent SOX9-GLI partnership that mediates either transactivation or repression.

Sox9 has been suggested to act upstream of Sox5 and Sox6 in chondrogenesis [6,46]. The presence of conserved SOX9-GLI sites in the Sox3 and Sox6 genes suggests their expression in proliferating chondrocytes may be positively controlled via cooperation of SOX9 with GLI3 or GLI1, an activator that reinforces GLI3 function. Hence, the roles played by SOX9 in transcriptional regulation may be determined by context—partnering with GLI3/GLI1 favours transactivation, with GLI8 favours repression. Alternatively, as discussed above, the mode of regulation might depend on whether intermediate factors are present to interfere with the SOX9-GLI interaction. Interestingly, while there is a linked SOX9-GLI motif in Sox6, a conserved TCF site occurs between the SOX9 and GLI sites (Figure S3). Cooperation between SOX9 and TCF/LEF/β-catenin might therefore abrogate cooperative repression by SOX9 and GLI and transactivate Sox6 in proliferating chondrocytes.

Validation of these different modes of cooperative regulation by SOX9 and GLI factors in vivo would require the generation and analyses of compound null or conditional knockout mutants; however, the consequent dysregulation of chondrogenesis and impact on cell survival would make it impossible to distinguish changes in transcriptional control from effects on differentiation. For example, Sox9 is essential for chondrogenesis and Sox9 conditional null chondrocytes undergo apoptosis and as a consequence, hypertrophy with the characteristic activation of Col10a1 expression, fails to occur [4,7,59]. Because inactivation of Col10a1 does not disrupt the chondrogenic program, it provides an ideal system to test how SOX9 controls these genes.
Figure 7. Differential presence of SOX9-GLI sites in proliferating and hypertrophic phase-specific genes. (A) Position, orientation, and spacing of the conserved linked SOX9 (COL2C1 and COL2C2) and GLI binding sites within the inter- and intra-genic regions of genes preferentially expressed in proliferating (PC) or hypertrophic (HC) chondrocytes. Numbers between the sites indicate the length of nucleotide spacing. Conservation indicates the percentage of nucleotides and species conserved in the alignments spanning the SOX9 and GLI sites. The alignments were based on multiz30way in UCSC genome browser and were simplified to only include sequences from mouse (M), human (H), chimpanzee (P), canine (D), bovine (C), and opossum (O) genome. Genes tested but found negative include HC genes Bmp6, Cdkn1c, Loxl4, Spp1, and Vegfa as well as PC genes Acan, Bmp7, Col2a1, Col9a1, Col9a2, Col9a3, Col11a1, Col11a2, Fgfr3, Fos, Mia1, and Sox9. (B) Left panel: Venn diagram showing frequency of genes in the genome that contain perfectly conserved SOX9, GLI, or linked SOX9-GLI sites within the inter- and intra-genic regions. SOX9 and GLI sites co-exist but are not linked in the majority of genes. Right panel: Number of HC, PC and all genes in the mouse genome with conserved SOX9-GLI sites and p-values from hypergeometric test (HT, for the over-representation of positive HC and PC genes compared to the genome) and one-tailed Fisher’s exact test (FET, for the difference in number of positive genes between HC and PC group). The test was based on all genes in the mouse genome (32,120 genes). See also Figure S3 for alignment data of the linked sites.

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ning the SOX9 and GLI sites was calculated based on the number of perfectly matched nucleotides among all the aligned species (mouse, human, chimpanzee, canine, bovine, and opossum). From the 32,120 genes in the mouse genome, the number of genes containing conserved linked SOX9-GLI sites was found and used as the reference frequency of such genes in the genome. Whether the frequency of the presence of conserved SOX9-GLI sites in HC or PC genes exceeded this reference frequency was assessed by the hypergeometric distribution. The difference in the frequency of the presence of SOX9-GLI sites in the HC and PC genes was assessed by the Fisher’s exact test.

Cell culture and transfection

Hypertrophic chondrocyte cell line MCTs (gift of Ve´ronique Lefebvre [62]) was transfected with pCol10Flag-E, pSG-Sox9 (gift of Peter Koopman), or pSG5 expression vector using Fugene 6 (Roche). Expression of the exogenous collagen X from pCol10Flag-E in MCTs cells was examined by immunohistochemistry using anti-Flag M2 antibody (Sigma). CCL (gift of James Kimura [63]), MCT3T3-E1, and COS-1 cells were cultured in DMEM (Invitrogen) containing 10% FCS (Wisent) at 37°C under 5% CO2. MCTs cells were normally cultured at 32°C for expansion. Prior to assays, MCTs cells were cultured at 37°C for 1 day to induce growth arrest [62].

DNase I footprinting

A 300 bp DNA fragment within element A, corresponding to −4240 to −3935 bp of the mouse Col10a1, was used as probe. [γ-32P]ATP-labeled probes were incubated with nuclear extracts from CCL, MCTs, MCT3T3-E1, or COS-1 cells in the presence of BDAP1 and BDAP2 antibodies (Sigma) for immunoprecipitation.
of poly(dA·dT) at room temperature, followed by DNase I digestion and denaturing PAGE.

Electromobility shift assays

COS-1 nuclear extracts over-expressing SOX9 and RUNX2 were pre-incubated with poly(dI·dC) or poly(dG·dC) at room temperature, followed by reaction with [γ-32P]ATP-labeled probes with or without the presence of nucleotide competitors, or antibodies for SOX9 (gift of Peter Koopman [64]) and OSP2/ RUNX2 (gift of Gerard Karsenty [65]), then subjected to non-denaturing PAGE at room temperature. The sequences of oligonucleotide COL2C1 and OSE2 were as previously described [10,65].

Col10flag transgenic mice

The mouse Col10al element A, a 640 bp-fragment located between −4.2 and −3.6 kb, was cloned at the 5′ end of the pCol10flag, previously known as PCbnX [18] consisting of −2070 to +7176 bp mouse Col10al genomic sequence to generate pCol10flag-E. The SOX9, TG-rich motif, and GLI binding sites in pCol10Flag-E were mutated to generate the single site mutants – respectively pCol10flag-EA1, pCol10flag-EA2, and pCol10flag-EA3. All of these 3 motifs in pCol10flag-E were mutated to generate pCol10flag-EA4.

Col10al-Cre/Z/Sox9 compound mutants

A 4.8 kb fragment of mouse genomic DNA (from 82 bp upstream of the start of transcription of Sox9 to 1119 bp downstream of the polyadenylation sequences), including the Sox9 coding region, its two introns and 1.1 kb of 3′ flanking DNA, together with an IRES2-EGFP (Clontech) sequence inserted between the Sox9 stop codon and the polyadenylation site (at +3237 bp), was cloned downstream of the IoxP-flanked ßgeo/3xpA of the pCall2 vector (gift of Andras Nagy [66]) to create the pZ/Sox9 expression vector. pZ/Sox9 was transfected into 129/SvEv-derived L4 embryonic stem (ES) cells by electroporation and ES clones containing a single copy of the transgene were injected into blastocysts followed by crossing of the resulting chimeras with C57BL/6N mice to generate the mouse line Col10al-Cre/Z/Sox9. A mouse line carrying a single copy of pZ/Sox9 was generated which was then crossed with Col10al-Cre mice [21] to obtain compound mutants.

Chromatin immunoprecipitation

CCL cell lysates or 13.5 dpc mouse limb tissue lysates were cross-linked followed by lysis and sonication to yield 200–500 bp DNA fragments then immunoprecipitation with antibodies against acetylated histone H3/H4 (Ac-H3, Ac-H4) (Upstate), HDAC4 (Abcam), GLI1, GLI2, GLI3 (all from Santa Cruz), SOX9 (gift of Robin Lovell-Badge [67]), TRAP230/MED12 (gift of Robert Roeder [25]), or TRPS1 (gift of Yasuteru Murakagi [26]). The target elements in Col10al, Col2al, or Crygb genes were amplified by real-time or standard PCR.

Gene expression analysis

In-situ hybridization was performed as previously described [9]. The probes used were pRK26 for Col10al [68], pWF21 for the Flag sequence [18], p88 for full-length Sox9 (gift of Peter Koopman), pBS-Cbfa1-S (for full-length Runx2 (gift of Gerard Karsenty), pWF98 for full-length Egfp, pBS-H-Cre-frag for Cre (gift of Andrew Groves), and pN61 for Col2al [9]. Gene expression in cell culture was analyzed by RT-PCR.

For additional details of all experiments, see the Text S1.

Supporting Information

Figure S1 Additional gene/protein expression data and EMSA. (A) Expression of Col2al and Col10al in CCL, MCTs, and MC3T3-E1 was analyzed by RT-PCR. Controls were no RNA (−) or RNA from mouse E17.5 embryo (+). Expression of Col10al was found only in MCTs. (B) Western blot analysis showed that both SOX9 and RUNX2 were highly expressed in CCL but weakly in MCTs. (C) Expression of SOX9 in nuclear (lane 3) and cytoplasmic (lane 4) fractions of pDNA-Sox9 transfected COS-1 cells was compared with CCL (lane 1) and untransfected COS-1 (lane 2) nuclear extracts by Western blotting. (D) Expression of RUNX2 in nuclear (lane 3) and cytoplasmic (lane 4) fractions of pDNA-Cbfal transfected COS-1 cells was verified by Western blotting. (E) A diagram showing the sequences of oligonucleotides which contained wild-type, mutant SOX9 and/or TG-rich motif for EMSA. Only the mutated nucleotides were shown in the sequence of competitors. (F) Intact DNA-binding property of SOX9 in expressing nuclear extract was tested by its interaction with the COL2A1 enhancer probe (COL2C1) in EMSA, in which the retarded band was challenged with SOX9 antibody (lane 4), unlabeled COL2C1 (lane 5–6), or Col10al element A C101G oligonucleotides (lane 7–8). The triangles represent increasing concentration of competitors from 10× (lane 5,7) to 100× (lane 6,8) excess.

(TIF)

Figure S2 In vivo transcriptional activity of Col10al element A. (A) Expression pattern of Col10flag-E in E15.5 transgenic fetus (mid-sagittal plane) examined by in-situ hybridization using digoxigenin-labeled probes. Flag expression was detected in the hypertrophic chondrocytes of cervical pedicle (a) and developing vertebrae (b). Weak expression was detected in the trabecular bone of palate (c). No expression was found in the nucleus pulposus (np) of the intervertebral disc (b), immature costal cartilage (g, cc), brain (b), hair papillae (i), lung (m), aorta (a), or myocardium (o). Expression of Col10al is shown for comparison (d–f, j–l, p–r). No expression was found in erythrocytes or nucleated blood cells (i) in n and q. (B) In Col10flag transgenic fetus, strong expression of Flag was found in the ossifying zone of pedicle (a, circled) and the trabecular bone of palate (b, circled). Weak signal was detected in the prehypertrophic zone of the pedicle cartilage (a, arrow). No expression was identified in tissue other than cartilage and bone. Expression of Col10al is shown for comparison (c–d).

(TIF)

Figure S3 Alignment data from SOX9-GLI site analysis. Multispecies alignment of regions spanning the linked SOX9-GLI sites are shown along with chromosomal location (Chr), identical nucleotides (.), insertion or deletion (-), and unaligned positions (=).

(PDF)

Text S1 Additional details of experiments.

(DOC)

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Author Contributions
Conceived and designed the experiments: KSEC. Performed the experiments: VYLL BG KSEC. Analyzed the data: VYLL BG KSEC. Wrote the paper: VYLL KSEC.

References


