Sox9 Transcriptionally Represses Spp1 to Prevent Matrix Mineralization in Maturing Heart Valves and Chondrocytes

Jacqueline D. Peacock, Danielle J. Huk, Hasini N. Ediriweera, Joy Lincoln

Chondrocytes. PLoS ONE 6(10): e26769. doi:10.1371/journal.pone.0026769

Abstract

Sox9 is an SRY-related transcription factor required for expression of cartilaginous genes in the developing skeletal system and heart valve structures. In contrast to positively regulating cartilaginous matrix, Sox9 also negatively regulates matrix mineralization associated with bone formation. While the transcriptional activation of Sox9 target genes during chondrogenesis has been characterized, the mechanisms by which Sox9 represses osteogenic processes are not so clear. Using ChIP-on-chip and luciferase assays we show that Sox9 binds and represses transactivation of the osteogenic glycoprotein Spp1. In addition, Sox9 knockdown in post natal mouse heart valve explants and rib chondrocyte cultures promotes Spp1 expression and matrix mineralization, while attenuating expression of cartilage genes Type II Collagen and Cartilage Link Protein. Further, we show that Spp1 is required for matrix mineralization induced by Sox9 knockdown. These studies provide insights into the molecular mechanisms by which Sox9 prevents pathologic matrix mineralization in tissues that must remain cartilaginous.

Introduction

The vertebrate skeletal system is primarily composed of cartilage and bone, formed during embryonic development. Most bones develop from replacement of cartilage through a process known as endochondral ossification [1,2]. Cartilage formation is initiated with condensation of undifferentiated mesenchyme progenitor cells followed by proliferation and differentiation into immature chondrocytes. As development progresses, chondrocytes begin to secrete extracellular matrix (ECM) typical of cartilage including type II collagen (Col2a1), before passing through a transition stage to become hypertrophic [3]. In addition to increasing in volume, hypertrophic chondrocytes secrete ECM that is later mineralized. These cells then die, leaving behind a calcified matrix that is eventually broken down, allowing invasion of blood vessels and infiltration of bone-resorbing (osteoclasts) and bone-forming (osteoblasts) cells. Many key regulators have been shown to be required for cartilage and bone forming processes, including the transcription factor Sox9.

Sox9 is an SRY-related hydroxymethylguaneryl (HMG) box member of the Sox family and has been shown to regulate many developmental events [4]. In the developing cartilage, Sox9 is expressed in immature chondrocytes and its function is required for mesenchyme cell condensation, proliferation and differentiation [1,4]. This positive role of Sox9 during early stages of chondrogenesis is largely attributed to its ability to bind and transactivate SRY consensus sites within cartilage ECM genes; these include Col2a1, Col9a2, Col11a2, Col27a1 and the proteoglycans Aggrecan and Cartilage Link Protein (CLP) [5,6,7,8,9,10]. In vivo, mice with reduced Sox9 function fail to develop cartilage [11,12,13] and mimic the human skeletal disorder, Campomelic Dysplasia [13,14,15], further supporting a positive role for Sox9 in promoting formation of cartilaginous connective tissue.

Following chondrocyte maturation, Sox9 expression is downregulated and levels remain low during stages of terminal differentiation and matrix mineralization [4]. Enforced expression in mature chondrocytes results in delayed endochondral ossification [16], suggesting that Sox9 has a repressive role in bone formation. Consistently, Sox9 haploinsufficiency leads to premature mineralization in many skeletal tissues [13]. Despite these observations, the mechanisms of Sox9 function in bone are not fully understood. In contrast to Sox9, expression of the transcription factor Runx2 (cbfa1) is high in mature chondrocytes and osteoblasts [4,17,18,19]. It has previously been shown that Sox9

Competing Interests: The authors have declared that no competing interests exist.

Copyright: © 2011 Peacock et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

Published: October 26, 2011

Editor: Donald Gullberg, University of Bergen, Norway

Received: June 23, 2011; Accepted: October 3, 2011; Published: October 26, 2011

Citation: Peacock JD, Huk DJ, Ediriweera HN, Lincoln J (2011) Sox9 Transcriptionally Represses Spp1 to Prevent Matrix Mineralization in Maturing Heart Valves and Chondrocytes. PLoS ONE 6(10): e26769. doi:10.1371/journal.pone.0026769

Funding: This work was supported by The American Heart Association (09PRE2050088) (http://www.heart.org/HEARTORG), The Florida Biomedical Research Program, The James and Esther King Award (07KN-07) (http://www.floridabiomed.com), and the National Institutes of Health/National Heart, Lung and Blood Institute (R01HL091878) (http://www.nhlbi.nih.gov). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

PLoS ONE | www.plosone.org 1 October 2011 | Volume 6 | Issue 10 | e26769
binds RUNX2 to prevent transactivation of RUNX2 target genes, including the glycoprotein, Spp1 [20], thereby repressing bone formation. An additional mechanism of Sox9-mediated repression of osteogenic processes includes findings by Hattori et al., [16] showing that Sox9 negatively regulates Vegf to inhibit cartilage vascularization and subsequent mineralization. Together these previous studies have revealed pivotal roles for Sox9 in regulating formation of cartilage and bone connective tissues in the skeletal system.

In addition to the skeletal system, we, and others have shown that Sox9 is also expressed in the developing heart [21,22]. More specifically, Sox9 is highly expressed in a population of mesenchymal cells within the endocardial cushions that later give rise to mature valve structures [23,24]. During early stages of endocardial cushion formation, Sox9 is required to expand the pool of proliferating mesenchyme precursor cells [21]. Following endocardial cushion formation, Sox9 expression is maintained in valve primordia and studies in mice suggest that similar to functions in the skeletal system, Sox9 promotes cartilaginous matrix phenotypes [23]. Remodeling heart valves from embryonic Col2a1cre;Sox9fl/fl mice express significantly reduced levels of Col2a1 and CLP [21], while valves from viable Col2a1cre;Sox9fl/fl adult mice are calcified and express high levels of osteogenic genes including Runx2 and Spp1 [21,25]. This valvular phenotype in Sox9 mutant mice is consistent with human calcific valve disease, frequently described as a pathological ‘bone-like’ process [26]. However, the molecular mechanism by which Sox9 prevents pathological matrix mineralization in normal cartilaginous heart valve connective tissue has not been examined.

In this study, we use a genome-wide microarray technique to screen for interactions with novel target gene DNA enriched by Sox9 chromatin immunoprecipitation (ChIP) in primary neonate limb tissue. ChIP-on-chip analysis revealed Sox9 binding to over 450 promoters, including a region approximately 5000 bp upstream of the osteogenic glycoprotein, Spp1. At the functional level, luciferase assays show that Sox9 significantly represses Spp1 transactivation through interaction with an SRY binding site. Loss of Sox9 function in primary heart valve explants and chondrocyte cultures increases Spp1 transcript levels, while expression of chondrogenic matrix genes including Col2a1 and CLP are decreased. Conversely, Sox9 gain of function promotes Col2a1 and CLP expression. Finally, we show that Spp1 function is required for matrix mineralization induced by Sox9 knockdown in these culture systems. Taken together, these data suggest that in maturing heart valves and chondrocytes, Sox9 negatively regulates matrix mineralization through repressive regulation of Spp1.

**Results**

**Identification of Sox9 binding to Spp1 by ChIP-on-chip analysis**

Our previous work has shown that several molecular phenotypes and signaling pathways are common to the skeletal system and heart valves. Similarly, Sox9 positively promotes cartilage and negatively regulates matrix mineralization in both these systems [13,21,23,25]. While the mechanisms of Sox9 during chondrogenic processes have been well described, its molecular function in mineralized connective tissues is less well understood. To address this we performed ChIP-on-chip analysis using pooled Sox9 antibodies to enrich DNA/Sox9 complexes in chromatin isolated from post natal mouse limb tissue, a time point when endochondral ossification is active. Compared to IgG controls, over 450 promoter regions showed enrichment of Sox9 binding at levels comparable, or greater than the known target gene, CLP [8] (Table S1). From this array of findings we focused our study on the observed enrichment of Sox9 binding approximately 5000 bp upstream of the transcriptional start site of Spp1 (Osteopontin), a known regulator of osteoblast differentiation and mineralized matrix deposition in vitro [27,28].

Using Genomatix MatInspector software, we identified three canonical SRY binding sites within the 5000 bp candidate enhancer region of Spp1, denoted SRY#1 (−5065 bp), SRY#2 (−4224 bp) and SRY#3 (−1599 bp) (Figure 1A). In order to determine which SRY binding site(s) Sox9 binds to, and validate ChIP-onchip observations, we performed three independent ChIP experiments and tested for enrichment following Sox9 IP within these regions containing the putative SRY sites using specific primers. In these additional ChIP experiments, expected enrichment of Sox9 binding over IgG controls was observed at a known functional SRY binding site within the CLP promoter [8] (Figure 1B). In addition, enriched Sox9 binding was also observed within the Spp1 enhancer region containing SRY#1 (−5065 bp) (Figure 1). In contrast, significant enrichment was not observed within regions containing SRY#2 (−4224 bp) or SRY#3 (−1599 bp). These studies suggest that Sox9 molecularly interacts with an SRY binding site within the 5’ enhancer of Spp1.

**Sox9 represses Spp1 promoter activity**

To determine if the observed binding of Sox9 to the Spp1 enhancer region is functionally important, luciferase assays were performed in HEK293 cells that express low levels of Sox9 and Spp1, and MC-3T3 cells; a pre-osteoblastic cell line previously used to examine transcriptional regulation of Spp1 [29]. In this experiment, HEK293 and MC-3T3 cells were co-transfected with either empty pcDNA or pcDNA-Sox9 in the presence of pGL3-control, consisting of the 1599 bp 5’ Spp1 enhancer region containing SRY#1 in front of the published 826 bp Spp1 promoter region [30]. The normalized luciferase activity in cells co-transfected with...
pGL3-spp1 and empty pcDNA (pcDNA) was set at 100% and considered ‘baseline’ of pGL3-spp1 activity in the two cell lines (Figure 2A, 2B, top black bar). In contrast to pcDNA, co-transfection with pcDNA-Sox9 consistently repressed pGL3-spp1 activity by 63% ± 8% in HEK293 cells, and 60.3% ± 12.6% in MC-3T3 cells (Figure 2A, 2B, top grey bar). To examine the specificity of Sox9 mediating this repression through SRY-site1, site-directed mutagenesis was used to replace the Sox9/SRY core binding sequence (A/T A/T CAA A/T) site from TACAGA to TAGCAGG (pGL3-spp1mut) [31]. The repressive effect of pcDNA-Sox9 on pGL3-spp1 activity was not observed using the pGL3-spp1mut construct, suggesting that Sox9-mediated repression of Spp1 is through SRY-site1. Worthy of mention, pcDNA-Sox9 had no effect on activity of pGL3-empty or the spp1 enhancer region (pGL3-spp1/En) alone. However, pGL3-spp1p activity was significantly increased in MC-3T3 but not HEK293 cells, independent of SRY binding sites and exogenous Sox9 expression levels (pcDNA vs. pcDNA-Sox9). As this spp1 promoter region has previously been shown to be regulated by a functional Runx2 binding site, luciferase assays were repeated in MC-3T3 cells using a pGL3-spp1p mutant construct in which the Runx2 binding site had been mutated from ACCAGA to AGGAGA (pGL3-spp1pmut) [30]. Activation of the spp1 promoter was significantly diminished following mutation of Runx2 binding sites. This Runx2-mediated activation of the spp1 promoter region is consistent with high levels of endogenous Runx2 in MC-3T3, but not HEK293 cells (Figure 2C). In summary, findings from these luciferase assays show that Sox9 moderately represses Spp1 promoter activity.

Expression patterns of Sox9 and Osteopontin are mutually exclusive in postnatal heart valves and limbs

Previous studies by our group and others have shown that Sox9 and Spp1 play important roles in developing heart valves and limbs [12, 21, 25, 27]. Further, findings in this study suggest that Sox9 molecularly interacts with (Figure 1) and negatively regulates (Figure 2) Spp1. To support these observations, the temporal and spatial expression patterns of Sox9 and Spp1 were examined in heart valves and limbs using qPCR and immunohistochemistry (Figure 3). In both tissues, Sox9 and Spp1 are most highly expressed at E12.5 with a gradual decline to lower levels of expression by postnatal and adult (3-month-old) stages (Figure 3A, 3C). As expected for a negative regulator, Sox9 expression is consistently higher than Spp1 at all time points (Figure 3A, 3C). Spatially, immunohistochemistry reveals that in both postnatal heart valves and limbs, Sox9 and Osteopontin (the protein product of Spp1) expression does not overlap, consistent with the notion that Sox9 functions to repress Osteopontin expression. In the heart, Sox9 is highly detectable within valvular structures (tricuspid valve shown), while Osteopontin expression is restricted to the subendocardial region and myocardial ECM (Figure 3B); a region in which Sox9 expression was not detected. In the limbs, Sox9 expression is consistent with developing chondrocytes [11] (Figure 3B), while Osteopontin expression is excluded from this expression domain [32]. These expression studies suggest that spatially, expression of Sox9 and Osteopontin are mutually exclusive in postnatal heart valves and limbs.

Sox9 loss of function in chondrocyte cultures and heart valve explants promotes Spp1 and represses cartilage gene expression

To determine the effects of Sox9 function on Spp1 expression, primary cultures were derived from postnatal mouse rib chondrocytes or mitral valve explants and treated with adenovirus to target Sox9 function. Gene knockdown was achieved by infecting primary cultures isolated from Sox9<sup>+/−</sup> mice with adenovirus (AdV) producing Cre recombinase (AdV-Cre), while infection with AdV containing full length murine Sox9 (AdV-Sox9), served as an overexpression model [25]. As a control, cultures were infected with an equal titer of GFP-producing adenovirus (AdV-GFP) that did not alter Sox9 expression (data not shown). Compared to endogenous levels (AdV-GFP), Sox9 expression was increased (104-fold ± 32.13, 20-fold ± 8.7) and decreased (0.59-fold ± 0.07, 0.27-fold ± 0.08) at transcript and protein levels following AdV-Sox9 and AdV-Cre treatments respectively in heart valve explants (Figure 4A) and chondrocyte cultures (Figure 4B). In addition to reducing Sox9, AdV-Cre significantly increased Spp1 expression in both culture systems and reduced Col2a1 expression in chondrocytes (Figure 4B). CLP and Col2a1 expression levels were not altered in treated heart valve explants despite our previous studies showing downregulation in valves from embryonic Col2a1cre;Sox9<sup>+/−</sup> mice. This discrepancy is likely due to differences in endogenous CLP and Col2a1 expression levels between the in vivo and in vitro systems. In contrast to AdV-Cre, AdV-Sox9 treatment in heart valve explants significantly increased expression of CLP and Col2a1 (Figure 4A), and similarly in chondrocyte cultures, Col2a1 was increased (Figure 4B). Although trends toward decreased Spp1 expression were observed following AdV-Sox9 infection, significant changes were not achieved. This could be due to the low basal levels of Spp1 in these culture systems prior to adenoviral infection (Figure 3A, 3C, data not shown). Collectively, these Sox9 targeting experiments show that Sox9 function plays pivotal roles in regulating cartilage and osteogenic gene expression in cultured heart valves and chondrocytes.

Spp1 is required for Sox9-mediated calcification of heart valve and chondrocyte connective tissue

Previous studies in heart valves and limbs have shown that reduced Sox9 function promotes premature matrix mineralization [21, 25]. To determine if Spp1 is required for this Sox9-mediated process, heart valve explants and cultured rib chondrocytes isolated from postnatal Sox9<sup>+/−</sup> mice were subject to infection with AdV-Cre, and/or an adenovirus containing a short hairpin (sh) RNA sequence to target Spp1 knockdown (AdV-shSpp1). Compared to AdV-GFP controls, AdV-Cre and AdV-shSpp1 infection led to successful targeted knockdown of Sox9 and Spp1 respectively (Figure 5A, 5B). In addition, and consistent with our previous findings [25], AdV-Cre infection increased von Kossa reactivity in both heart valve explants (Figure 5C, 5E) and cultured chondrocytes (Figure 5D, 5F). However, the ability of AdV-Cre to promote von Kossa reactivity was significantly reduced in cultures pre-treated with AdV-shSpp1 (Figure 5C-5F). This suggests that Spp1 function is important for Sox9-mediated calcification in heart valves and chondrocytes.

Discussion

The replacement of cartilage by mineralized tissue is imperative for development of long bones, ribs and vertebrae [1]. However, it is equally important that endochondral ossification does not occur in tissues that must remain cartilaginous, including hyaline cartilage and heart valves. Aberrations in this process can lead to ectopic matrix mineralization associated with heterotopic ossification of the skeletal system and calcification of heart valve tissue. Therefore it is important that a correct balance of cartilage and bone is established in the developing embryo and maintained in the adult. The transcription factor Sox9 has been shown to play...
Figure 2. Luciferase assays of Sox9 function on Spp1 activity. HEK293 (A) and MC-3T3 (B) cells were co-transfected with 200 ng of the luciferase reporter construct pGL3 containing the Spp1 promoter region (no SRY binding sites) (pGL3-spp1), the 1599 bp 5’ Spp1 enhancer region containing SRY#1 (pGL3-spp1En), or both the enhancer and promoter regions (pGL3-spp1), along with 200 ng of empty pcDNA (grey bars) or pcDNA containing full length murine Sox9 (pcDNA-Sox9, black bars). In addition, cells were co-transfected with 20 ng of pGL4 for normalization. Luciferase activity of pGL3-spp1 with empty pcDNA was set to 100%. Note repression of pGL3-spp1 activity (63%±8% (A) and 60.3%±12.6% (B) in the presence of pcDNA-Sox9. Cells were also co-transfected with pcDNA or pcDNA-Sox9 in the presence of pGL3-spp1 in which the SRY#1 had been mutated from TACAAG to TAGCAGG (pGL3-spp1mut). Note that pcDNA-Sox9 could not significantly repress pGL3-spp1mut unlike pGL3-spp1. *Student’s t-test: p<0.05 compared to pcDNA. (C) qPCR-derived melting curves to show amplification of endogenous 18s and Runx2 expression levels in HEK293 and MC-3T3 cells.

doi:10.1371/journal.pone.0026769.g002
pivotal roles in both these processes. While the positive role of Sox9 in cartilage formation has been well characterized [5,6,7,8,9,10], its repressive function in osteogenic processes is less well understood. This study used a genome-wide approach along with supporting molecular assays and revealed a novel mechanism in which Sox9 interacts and negatively regulates the osteoblast-associated glycoprotein, Spp1. Our findings provide mechanistic insights into the pathological processes of matrix mineralization associated with human disease.

Calcific disease of the heart valves and vasculature has recently been described as an active, osteogenic-like process [26]. Despite our data suggesting that reduced Sox9 function promotes matrix mineralization via relieved repression of Spp1, Osteopontin and Sox9 are both highly expressed in human valves excised from patients with end-stage calcific aortic valve disease [25,33,34,35,36,37,38]. However, as Osteopontin can be secreted by both osteoblasts and osteoclasts, it is not clear if high levels in calcified valves are associated with high affinity for hydroxyapatite to promote calcification [39,40], or degradation of the mineralized matrix [41,42,43]. As our data suggests that Spp1 function is required for matrix mineralization induced by Sox9 knockdown, it is suggested that high levels promote calcific processes in this system and reflect the osteogenic phenotype of pathological lesions [26]. However, while the sufficiency of Spp1 to promote matrix mineralization in osteoblastic primary cells has previously been reported [28], further work is required to examine similar mechanisms in heart valves. In contrast to Osteopontin, it is not so clear why Sox9 is overexpressed in late stages of calcific aortic valve disease. As our previous work has shown that reduced Sox9 function promotes calcification [25], it is speculated that increased Sox9 is a compensatory response to attenuate disease progression. Based upon findings in this study and others, this is likely at the level of repressing osteogenic signaling, and/or ‘re-activating’ developmental pathways to promote valve cell proliferation and differentiation processes, as previously observed in diseased valves [21,44].

The observed repression of Spp1 by Sox9 complements previous studies [16,20] and provides an additional mechanism to explain the calcification phenotypes observed in Sox9 mutant mice [13,25]. However, further work is required to fully understand how Sox9 differentially regulates bone and cartilage target genes in the same system. One consideration is through the recruitment of transcriptional co-repressors and co-activators respectively. In cartilage, PGC-1α and CREB-binding protein associated with p300 form transcriptional complexes with Sox9 on the enhancer regions of chondrogenic genes to promote expression [45,46]. However, it is not yet clear if these ‘activating’ complexes are absent on osteogenic target genes, or replaced by repressive machinery. In addition to considering loss of co-activators and recruitment of repressors, studies have also shown that Sox9 can evoke inhibitory responses on biological process through post-transcriptional modification. This includes negative regulation by
human patients or mouse models of valve calcification. Nonetheless, our findings provide further support to show overlapping regulatory pathways between these two connective tissue systems and identify a previously unappreciated mechanism to prevent ossification in tissues that must remain cartilaginous for life-long function.

**Methods**

**Chromatin Immunoprecipitation**

**ChIP-on-chip array.** Sox9 chromatin immunoprecipitation (ChIP) was performed from postnatal wild type mouse limb chromatin according to manufacturer’s instructions (EZ-ChIP, Millipore) adapted for use with primary tissue lysates as follows. One neonate mouse forelimb per immunoprecipitation (IP) was diced and incubated in hypotonic lysis buffer for 30 minutes on ice and then vortexed for 10 s. Nuclear-enriched fraction was pelleted by centrifugation and resuspended in PBS containing 0.5% formaldehyde (Sigma) for 10 minutes. Chromatin shearing was achieved with 5, 10-second pulses, with a 10 second minimum refractionary period using a benchtop sonicator (Misonix). IPs were performed using a total of 5 µg of pooled polyclonal rabbit Sox9 antibodies (4 µg SC-17341 (Santa Cruz) and 1 µg anti-Sox9 antibody (a kind gift from Dr. Michael Wegner), with 5 µg of normal rabbit IgG used in parallel control experiments. Each ChIP was validated by polymerase chain reaction (PCR) to confirm enrichment of a known Sox9-bound promoter region in the CLP promoter [8].

Purified DNA from Sox9, and IgG control ChIP experiments was hybridized to a GeneChipMouse Promoter 1.0R Array (Affymetrix). Data analysis was performed with Tiling Analysis Software and Integrated Genome Browser (Affymetrix).

Enrichment of target genes was determined by setting a graph threshold for signal intensity (value>30) and minimum run (100 base pairs) in the Integrated Genome Browser (Affymetrix) based on a known Sox9 binding site in the CLP promoter.

**ChIP-on-chip validation.** Sox9 binding to the Spp1 5’ region observed in the ChIP-on-chip analysis was validated by quantitative real-time PCR in three independent experiments with postnatal mouse limb lysates. Primers were designed to amplify 100–200 bp genomic DNA sequences surrounding the known SRY binding site within the promoter region of CLP: 5’-GAAAGGAGTTGAGTTTATATAT, 3’-AGCACCACCTTTTGGT GGT [8], in addition to SRY#1, SRY#2 and SRY#3 binding sites on Spp1 as detected by the GeneChipMouse Promoter1.0R Array. SRY#1: 5’-CAATCTATCTCATTAGTGGCT; SRY#2: 5’-TGTATGCCATCTCATTAGTGGCT; SRY#3: 5’-GAGTACCTTGCAGTGTTG

**Luciferase Assays**

The pGL3-spp1p construct was obtained from Dr. Paul Shore and contains a published 829 bp Spp1 promoter sequence [30]. To generate pGL3-spp1En, a 1599 bp 5’ region of Spp1 (Accession number AY220127) containing SRY#1 was amplified from genomic mouse tail DNA by PCR using the following primers

**PI3K/AKT** [47], DNA methylaion [48], as well as competition binding with other signaling proteins for SRY sites [49]. Based upon the reported complexity of Sox9 regulation, it is likely that defining molecular codes that determine Sox9 as an activator or repressor in connective tissue system will not be clear-cut. However, understanding more thoroughly how Sox9 mediates antagonism between cartilage and bone signaling pathways would have significant impact in understanding developmental and disease processes.

In summary, this current study highlights a novel mechanism by which Sox9 acts as a transcriptional repressor of Spp1 to prevent matrix mineralization in heart valves and chondrocytes, in vitro. Despite these two connective tissue systems being functionally diverse, there is ever-increasing evidence to show that normal heart valve ECM shares molecular characteristics and signaling pathways with chondrogenesis [21,23,25]. Similarly, pathological calcification of heart valve ECM has recently been referred to as a bone-like process, although parallel ‘endochondral ossification’ events in valve interstitial cells have not been yet reported in

**Figure 4. Effects on bone and cartilage gene expression in heart valve and chondrocyte cultures following Sox9 manipulation.** Mitral valve explants (A) and chondrocyte cultures from postnatal Sox9fl/fl mice (B) were infected for 4 hours with 2.5×10^10 PFU/10 cm² adenovirus targeting GFP (AdV-GFP, Cre (AdV-Cre) (grey bars), or full length murine Sox9 (AdV-Sox9) (black bars) to target knockdown controls. Note logarithmic scale. *Student’s t-test*.
into pGL4 using introduced Sac1 and Kpn1 (Promega) sites. pGL3-spp1 was produced by ligating the 1599 bp spp1 enhancer region into the pGL3-spp1p plasmid using Kpn1 and Sac1 sites, 5' of the spp1 promoter sequence. Site-directed mutagenesis was used to target mutation of the SRY#1 consensus sequence using the following internal mutagenesis primers: 5'TGTCTATGGCATTTAAGCGTCTTAAATGCCATAGA and 3'AAATTCTTTTACCTCCCTGCTTAAATGCCATAGA. This mutated fragment was similarly ligated 5' of the spp1 promoter sequence in pGL3-spp1p, to generate pGL3-spp1mut.

Luciferase assays were performed in HEK 293 and MC-3T3 cells (ATCC) plated at 2×10⁵ or 2×10⁴ per well of a 24-well plate respectively, 16–20 hours prior to transfection with Lipofectamine reagent (Invitrogen) according to manufacturer’s instructions. 200 ng of pGL3-spp1, pGL3-spp1mut or pGL3 (200 ng/well), and pcDNA or pcDNA-Sox9 (200 ng/well), were co-transfected into each well, along with 20 ng of pGL4 (Renilla luciferase, Promega). All transfections were performed in 0.5 mL OptiMem for 4 hours before the addition of 0.5 mL DMEM (Sigma) supplemented with 4 mM L-Glutamine and 10% FBS. Cell lysates were collected 24 hours following transfection according to the manufacturer’s instructions for dual luciferase assays (Promega). Data is represented as an average percent of luciferase activity of the pGL3-spp1 co-transfected with pcDNA (set at 100%) and normalized to pGL4 Renilla signal (n = 3–8).

Figure 5. The requirement of Spp1 for Sox9-mediated matrix mineralization. Primary heart valve explants (A, C, E) and chondrocyte cultures (B, D, F) were infected with AdV-GFP, AdV-Cre or AdV-shSpp1 for 4 hours. In addition after 24 hours of infection, AdV-shSpp1 treated cultures were also treated with AdV-Cre. After 72 hours, RNA was isolated and qPCR performed to show expected Sox9 and Spp1 gene knockdown following AdV-Cre and AdV-shSpp1 infection respectively, compared to AdV-GFP. Alternatively, treated cultures were fixed and stained with von Kossa and alcin blue to detect mineralization and proteoglycan secretion respectively. (E, F) Quantitation of von Kossa reactivity normalized to area (alcian blue positive) in treated cultures compared to AdV-GFP controls. *Student’s t-test <0.05 compared to AdV-GFP.

doi:10.1371/journal.pone.0026769.g005
Immunohistochemistry

Postnatal mouse limbs and heart valves from E12.5, E18.5, and 3 month old adult mice were processed, fixed, through 10, 20, and 30% sucrose solutions before embedding in OCT freezing compound (Tissue-Tek) and sectioned at 10μm-thick. Frozen sections were post-fixed in 100% acetone for 10 minutes at −20°C and rinsed in 1xPBS prior to immunofluorescent staining as described [25] using antibodies against Sox9, a kind gift from Dr. Michael Wegner at 1:1000 dilution, and Osteopontin (Santa Cruz sc-10593) at 1:100 dilution. Following primary antibody incubation, sections were washed in 1xPBS and incubated with respective anti-rabbit and anti-goat Alexa 488 or 568 secondary antibodies at 1:400/1xPBS dilution for 1 hour at room temperature, washed, and incubated with DAPI (blue) for identification of nuclei before mounting in Vectashield (Vector Laboratories).

Primary Culture Systems

Heart valve explants. Mitral valve explants were isolated from postnatal Sox9+/− mice and cultured as previously described [25]. Valve explant cultures were infected immediately after dissection with 1×10^10 PFU/filter AdV-shSpp1 produced by Vector Biolabs using a commercially available shSpp1 targeting shRNA (174_0576-H-5, Open Biosystems) or control AdV-GFP [25]. Adenoviral infections were conducted in serum free medium for 4 hours before incubation in regular culture medium for 20 additional hours. 24 hours following the initial infections (AdV-shSpp1 or AdV-GFP), valve explants were treated with AdV-Cre (Vector Labs) or control Ad-GFP at 1×10^10 PFU in serum free medium for 4 hours before incubation in regular culture medium for an additional 68 hours. This provided valve explants with loss of Shp1 expression prior to Sox9 knockdown, or infection with controls including Shp1 knockdown only, Sox9 knockdown only, or AdV-GFP. After 72 hours of infection, treated explants were removed for histological analysis [25] or RNA was extracted using standard Trizol protocols (Invitrogen) and cDNA was synthesized from 200 ng RNA using the qScriptcDNA Synthesis Kit (Quanta) for real-time PCR analysis.

Chondrocyte cultures. Primary chondrocytes were prepared from rib cartilage of postnatal day 1–3 Sox9+/− mice, according to an established protocol designed to maintain chondrocyte differentiation in monolayer culture [50]. Following 18 hours of culture, primary chondrocytes were infected with AdV-GFP or AdV-shSpp1 at 2.5×10^10 PFU/10 cm^2 in serum free medium for 4 hours before incubation in regular culture medium (DMEM supplemented with 2 mM L-Glutamine and 10% FBS) for an additional 20 hours. 24 hours following the initial infections (AdV-shSpp1 or AdV-GFP), chondrocytes were treated with AdV-Cre (Vector Labs) or control AdV-GFP at 2.5×10^10 PFU/10 cm^2 in serum free medium for 4 hours and then incubated in regular culture medium for an additional 68 hours. After 72 hours of infection, treated chondrocytes were fixed in 4% paraformaldehyde for histological analysis or RNA was extracted using standard Trizol protocols (Invitrogen) and cDNA was synthesized from 200 ng RNA using the qScriptcDNA Synthesis Kit (Quanta) for real-time PCR analysis. All animal procedures were approved by The University of Miami Institutional Animal Care and Use Committee (protocol # 09-171) and performed in accordance with institutional guidelines.

Primary Cultures: Polymerase Chain Reaction

Quantitative real-time PCR was performed using a Step One Plus Real Time PCR system (Applied Biosystems) with the following TaqMan assays (Applied Biosystems): Human 18s and mouse Sox9, Spp1, CLP and Col2a1. Reactions were performed using 10μl PerfeCTaTM FastMix (Quanta), 1μl TaqMan assay, 1μl cDNA, and 8μl deionized water per reaction. Real-time PCR for ChIP validation was performed using 10μl PerfeCTaTM SYBR Green FastMix (Quanta) with 0.5μl of each primer (at 20 pmol/μl), 1μl cDNA and 9μl deionized water per 20μl reaction. All reactions were performed with a 20 second activation step at 95°C followed by 40 cycles with 1 second denaturation at 95°C and a 20 second annealing/polymerization step at 60°C.

Western Blotting

Protein lysates from treated chondrocyte cultures (1 well of a 6-well plate) were collected and lysed in 1× buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X–100, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, supplemented with complete EDTA free protease inhibitor cocktail). 30μg of total protein was run on a 12% SDS PAGE gel and transferred to nitrocellulose membranes. Membranes were blocked in 5% fat free dried milk for 1 hr and probed with antibodies against Sox9 (1:1000, 1 hr, Santa Cruz) and α-tubulin (1:10,000, 1 hr, Chemicon/Millipore), followed by incubation with anti-rabbit-horseradish peroxidase (HRP)-conjugated secondary antibody (1:10,000, 1 hr, Cell Signaling). Membranes were then washed three times in 1×TBST for 10 minutes. Western blots were developed using Super Signal West Femto Substrate (Pierce) and BioMax MR film (Eastman Kodak).

Primary Cultures: Histology

Following culture and viral infection as described (Section 4.4), one mouse valve explant per filter was mounted and von Kossa staining was performed as described previously [25]. Chondrocyte cultures were stained following a similar protocol; cells were rinsed in PBS, fixed in 4% paraformaldehyde, washed 3 times in deionized water, and incubated in 5% silver nitrate solution for 1 hour under direct light (Schott Modulamp) in a reflective chamber. Chondrocytes were then washed in water, differentiated in 5% sodium thiosulphate pentahydrate for five minutes, and rinsed and counterstained for 2 minutes in 1% Alcian blue in 20% acetic acid. Quantification of von Kossa reactive area was performed using Image Pro Plus software and calculated as a percentage of von Kossa positive area (black) over total area indicated by alcian blue staining.

Supporting Information

Table S1 Data analysis of ChIP-on-chip using Tiling Analysis Software and Integrated Genome Browser (Affymetrix). Enrichment of indicated target genes was determined by setting a graph threshold for signal intensity (value≥30) and minimum run (100 base pairs) in the Integrated Genome Browser (Affymetrix) based on a known Sox9 binding site in the CLP promoter.

(XLSX)

Acknowledgments

We thank Agata Levay, Damien Barnette and Harriet Hammond for technical assistance and Drs. Paul Shore and Michael Wegner for providing us with experimental reagents.

Author Contributions

Conceived and designed the experiments: JL JDP DJH. Performed the experiments: JDP HE DJH. Analyzed the data: JL JDP DJH. Wrote the paper: JL DJH.
References

1. Lefebvre V, Smits P (2005) Transcriptional control of chondrocyte fate and differentiation. Birth Defects Res C Embryo Today 75: 209–212.
2. Provost S, Schipani E (2005) Molecular mechanisms of endochondral bone development. Biochem Biophys Res Commun 328: 650–665.
3. Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M (2008) Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol 40: 46–62.

4. Akiyama H (2008) Control of chondrogenesis by the transcription factor Sox9. Mod Rheumatol 18: 213–219.
5. Jenkins E, Moss JB, Pace JM, Bridgewater LC (2005) The new collagen gene COL2A1 contains SOX9-responsive enhancer elements. Matrix Biol 24: 177–184.
6. Genzer MA, Bridgewater LC (2007) A Col2a1 enhancer element activated by two interdependent SOX9 dimers. Nucleic Acids Res 35: 1178–1186.
7. Bridgewater LC, Lefebvre V, de Crombrugghe B (1998) Chondrocyte-specific enhancer elements in the Col1a2 gene resemble the Col2a1 tissue-specific enhancer. J Biol Chem 273: 14986–15006.

8. Kou I, Isegawa S (2004) SOX9-dependent and -independent transcriptional regulation of human cartilage link protein. J Biol Chem 279: 59492–59498.
9. Sekiya I, Tsugi K, Koopman P, Watanabe H, Yamada Y, et al. (2000) SOX9 enhances aggrecan gene promoter/enhancer activity and is up-regulated by retinoic acid in a cartilage-derived cell line. TCG. J Biol Chem 275: 10735–10744.
10. Ng IJ, Wheatley S, Muscat GE, Conway-Campbell J, Bowles JE, et al. (1997) SOX9 binds DNA, activates transcription, and coexpresses with type II collagen during chondrogenesis in the mouse. Dev Biol 185: 155–165.
11. Akiyama H, Chaboissier MC, Martin JP, Schedl A, de Crombrugghe B (2002) The transcription factor Sox9 has essential roles in successive steps of the chondrocyte differentiation pathway and is required for expression of Sox5 and Sox6. Genes Dev 16: 2013–2028.
12. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrugghe B (1999) Sox9 is required for cartilage formation. Nat Genet 22: 85–89.
13. Bi W, Huang W, Whitworth DJ, Deng JM, Zhang Z, et al. (2001) Haploinsufficiency of Sox9 results in defective cartilage primordia and premature skeletal mineralization. Proc Natl Acad Sci U S A 98: 6690–6703.
14. Foster JW, Dominguez-Steglich M, Guidos S, Kwok C, Weller PA, et al. (1994) Campomelic dysplasia and autosomal sex reversal by mutations in an SRY-related gene. Nature 372: 525–530.
15. Wagner T, Wirth J, Meyer J, Zabel B, Hold M, et al. (1994) Autosomal sex reversal and campomelic dysplasia are caused by mutations in and around the SRY-related gene Sox9. Cell 79: 1111–1120.
16. Hatten M, Muller C, Gehbard S, Bauer E, Pausch A, et al. (2000) Sox9 is a major negative regulator of cartilage vascularization, bone marrow formation, and endochondral ossification. Development 127: 901–911.
17. Komori T (2010) Regulation of bone development and extracellular matrix support of chicken and mouse embryos. Dev Dyn 230: 239–250.
18. Komori T, Hattori T, Muller C, Gebhard S, Bauer E, et al. (2010) SOX9 is a transcriptional repressor of Cbfa1 in the developing kidney and is essential for bone development. Development 137: 901–911.
19. Jenkins E, Moss JB, Pace JM, Bridgewater LC (2005) The new collagen gene COL2A1 contains SOX9-responsive enhancer elements. Matrix Biol 24: 10735–10744.

20. Mackie EJ, Ahmed YA, Tatarczuch L, Chen KS, Mirams M (2008) Endochondral ossification: how cartilage is converted into bone in the developing skeleton. Int J Biochem Cell Biol 40: 46–62.