Dynamic epigenetic mechanisms regulate age-dependent SOX9 expression in mouse articular cartilage

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ABSTRACT

While the developmental role of the SOX9 transcription factor in chondrocyte differentiation and cartilage formation is well documented, age-dependent SOX9 expression in articular chondrocytes (ACs) and its regulatory mechanisms remain unclear. This study aimed to explore epigenetic regulatory mechanisms for age-related changes in SOX9 expression in ACs of mice, spanning from the developmental stage to 18 months of age. *Sox9* mRNA and protein were highly expressed in ACs during joint development but significantly decreased after 2 months of age. Histopathological features of osteoarthritis were not observed in examined hip and shoulder joints by 18 months of age. Epigenetic studies revealed that DNA methylation levels were increased at specific CpG islands of the *Sox9* gene at 6 and 12 months; treatment of cultured ACs from 6-month-old mice with 5-Azacytidine (an inhibitor of DNA methylation) elevated the level of *Sox9* expression in ACs by lowering DNA methylation levels in the *Sox9* promoter region. Histone 3 lysine 4 dimethylation (H3K4me2, a histone modification for transcriptional activation) in the *Sox9* promoter region was decreased with age, which was associated with the age-dependent decrease in SOX9 expression in ACs. Knockdown of lysine-specific demethylase-1 up-regulated SOX9 expression in ACs of adult mice through increased recruitment of H3K4me2 in the *Sox9* promoter region. Our results suggest that SOX9 expression in mouse ACs is significantly decreased after the completion of joint development. These age-dependent changes in SOX9 expression are dynamically regulated by DNA methylation and histone methylation.

Keywords: SOX9; epigenetics; chondrocyte; articular cartilage; osteoarthritis

Abbreviations: ACs, articular chondrocytes; H3K4me2, histone 3 lysine 4 dimethylation; OA, osteoarthritis; WT, wild-type; IHC, immunohistochemistry; HE, haematoxylin and eosin; qPCR, quantitative real-time RT-PCR; P1, first passage of cultured cells; MeDIP, Methylated DNA immuno-

precipitation; 5-AzaC, 5-azacytidine; ChIP, chromatin immunoprecipitation; TSS, transcription start site; DNMT, DNA methyltransferase; LSD1, lysine-specific demethylase-1; NFAT, nuclear factor of activated T cells.

1. Introduction

The role of the transcription factor SOX9 in embryonic development was first demonstrated by the identification of heterozygous mutation in the *SOX9* gene in autosomal sex reversal and campomelic dysplasia, which affects skeletal formation and testis development (Foster et al. , 1994, Wagner et al. , 1994). During embryogenesis, the *Sox9* gene is expressed in all chondroprogenitor cells and in chondrocytes, but its expression is shut off in hypertrophic chondrocytes (Bi et al. , 1999, Wright et al. , 1995). Transgenic mice misexpressing *Sox9* in hypertrophic chondrocytes display impaired cartilage vascularization, bone marrow formation, and endochondral ossification. Terminal differentiation of hypertrophic chondrocytes is impaired by overexpressing *Sox9* in CAG*Sox9*; *Col2a1*-Cre and CAG-*Sox9*; *Col10a1*-Cre mice (Hattori et al. , 2010, Kim et al. , 2011). Inactivation of the *Sox9* gene in mouse limb buds before mesenchymal condensations results in a complete absence of cartilage formation. Mouse embryos in which *Sox9* is deleted after mesenchymal condensations exhibit chondro-dysplasia and defective joint formation (Akiyama et al. , 2002, Bi, Deng, 1999). SOX9 is required for expression of SOX5 and SOX6, two other SOX genes involved in chondrogenesis (Akiyama, Chaboissier, 2002, Liu and Lefebvre, 2015). Tamoxifen-induced cartilage-specific *Sox9* depletion in 1 to 2-week-old *Agc1CreERT2*; *Sox9*^{*fllox/fllox*} mice results in lethality, presumably due to collapse of the trachea or failure of rib cage expansion (Henry et al. , 2012). These studies have established the notion that SOX9 is required for chondrocyte differentiation and cartilage formation

but inhibits terminal differentiation of chondrocytes and endochondral ossification during skeletal development.

The molecular mechanisms by which SOX9 regulates chondrocyte differentiation and function have been extensively studied. SOX9 activates *Col2a1* gene transcription by binding a 48-base pair enhancer and co-expresses with type-II collagen during chondrogenesis in mice (Ng et al. , 1997, Zhou et al. , 1998). A *Col9a1* enhancer element is activated by two interdependent SOX9 dimers (Genzer and Bridgewater, 2007). SOX9 also enhances *Aggrecan (Acan)* gene promoter/enhancer activity in a mouse cartilage-derived cell line (Sekiya et al. , 2000) and positively regulates the expression of mRNA for cartilage-derived retinoic acid-sensitive protein (Xie et al. , 1999).

The expression and function of SOX9 in normal adult articular cartilage and osteoarthritis (OA) cartilage is not fully understood. SOX9 expression in human OA cartilage has been controversial. An early study reported no significant difference in quantity and subcellular localization of SOX9 protein between normal and OA cartilage, and SOX9 expression does not correlate with type-II collagen expression in adult articular cartilage (Aigner et al. , 2003). However, other studies recounted that SOX9 expression is reduced in human OA cartilage (Brew et al. , 2010, Lee and Im, 2011, Orfanidou et al. , 2009). Moreover, a recent study identified that SOX9 was a regulator of ADAMTSs-induced cartilage degeneration at the early stage of human OA (Zhu et al. , 2009). In animal studies, Salminen *et al.* examined postnatal (10 days to 9 months) expression of *Sox9* mRNA in the knee joint articular chondrocytes (ACs) of normal and *Del1* mice (a mouse model of spontaneous OA) carrying a deletion mutation in the *Proa1 (II)* collagen transgene. Based on the band size of *Sox9* mRNA on the gels of RNase protection assays, the authors estimated that *Sox9* mRNA expression is highest in growth cartilage but is decreased in mature cartilage of both normal and *Del1* mice (Salminen et al. , 2001).

These previous studies have raised an important question: What is the mechanism for the regulation of the age-dependent SOX9 expression if SOX9 level in ACs is significantly reduced in adulthood? The present study aimed to test our hypothesis that the SOX9 expression level is spontaneously decreased in ACs of adult mice due to developmental switch controlled by epigenetic regulatory mechanisms.

2. Materials and method

2.1. Animal procedures

Wild-type (WT) BALB/c mice were used in this study for various analyses of joint tissues and cells at embryonic day 16.5 (E16.5), and 1, 2, 6, 12, and 18 months of age. All animal procedures were approved by the Institutional Animal Care and Use Committee at the University of Kansas Medical Center.

2.2. Collection of articular cartilage

Samples of essentially pure articular cartilage were collected from femoral heads of mice at 1 to 18 months of age for isolation of RNA and chondrocytes by using a microdissection procedure as previously described (Rodova et al. , 2011). This is because femoral head articular cartilage is much thicker and most readily removed compared to articular cartilage in any other joints. Articular cartilage from both femoral and humeral heads was used for isolation of DNA and chromatin for epigenetic analyses because humeral head cartilage is thicker and more readily removed compared to articular cartilage in the elbow, knee, and ankle joints. At E16.5, the cartilage tissue collected from articular regions might contain a small amount of non-articular cartilage tissue since the articular cartilage structure is not well defined at this early stage. The morphology of collected articular cartilage and the

ability of isolated ACs to express proteoglycans were confirmed previously by safranin-O and Alcian blue staining (Rodova, Lu, 2011).

2.3. Histopathology and immunohistochemistry (IHC)

The hip and shoulder joints were harvested and processed for histopathology and IHC in order to assess the association of morphological changes with molecular alterations in articular cartilage from femoral and humeral heads. The joint tissue samples were fixed in 2% paraformaldehyde. Tissue samples processed for haematoxylin and eosin (HE) or safranin-O staining were decalcified in 25% formic acid and embedded in paraffin. Tissue samples for IHC were decalcified in 10% EDTA to detect the expression of SOX9 protein using a rabbit polyclonal antibody against SOX9 (Santa Cruz Biotechnology, Dallas, USA, sc-20095). At least five mice per gender were examined at each time point.

2.4. Gene expression analysis by quantitative real-time RT-PCR (qPCR)

Total RNA was isolated from freshly prepared and pulverized femoral head articular cartilage or cultured primary ACs using TRIzol reagent (Invitrogen, Carlsbad, USA). A High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, USA) was used to synthesize cDNAs. qPCR was performed in triplicate using a 7500 qPCR system (Applied Biosystems) with specific primers (Table 1) and SYBR Green reagents. The expression level of *Gapdh* from each sample was used as an internal control. Expression levels of genes of interest were quantified using $2^{-\Delta\Delta C_t}$ relative quantification methods, as described previously (Livak and Schmittgen, 2001, Wang et al. , 2009).

2.5. Isolation and culture of mouse articular chondrocytes

Primary ACs were isolated from pooled femoral head articular cartilage samples using collagenase D (1.5 mg/ml, Roche Diagnostics, Indianapolis, USA) in DMEM/Ham's F-12 (Cellgro, Manassas, USA) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin in a humidified incubator with 5% CO₂ at 37°C. Due to the uneven growth rate and cell density of primary chondrocytes (designated as P0), chondrocytes passaged once (P1) were used for various treatments. Cells were seeded in 12-well tissue culture plates at 1×10^5 cells/well for specific experiments.

2.6. DNA methylation assay

Genomic DNA was purified from femoral and humeral head articular cartilage and sonicated. Methylated DNA immune-precipitation (MeDIP) assay was conducted using a CpG MethylQuest DNA Isolation Kit (Millipore, Billerica, USA). qPCR analysis was performed to quantify the methylation level at the CpG islands. The MeDIP enrichment of methylated DNA was presented as a value (in percentage) relative to the total DNA in input samples. MeDIP specificity was verified using control HeLa DNA with positive and negative primers provided by the Kit. P1 chondrocytes from 6-month-old mice were used for 5-azacytidine (5-AzaC, in DMSO) treatment. Cells were seeded in 12-well tissue culture plates at 1×10^5 cells/well and cultured for 120 hours in daily changed DMEM/F12 medium in the presence of 20 μ M 5-AzaC (Sigma-Aldrich, St. Louis, USA). P1 chondrocyte cultures without 5-AzaC (DMSO only) treatment were used as controls.

2.7. Chromatin immunoprecipitation (ChIP) assays

Chromatin was obtained from femoral and humeral head articular cartilage. Chromatin immunoprecipitation (ChIP) assays were performed using a Magna ChIP A/G kit (Millipore). ChIP grade antibodies against mouse dimethylated histone 3 lysine 4 (H3K4me2, Millipore), H3K9me2 (Millipore), H3K27me3 (Millipore), DNA methyltransferase (DNMT) 1 (abcam), 3a (abcam), 3b

(Abnova) and histone lysine-specific demethylase-1 (LSD1, abcam) were used for ChIP assay. Normal mouse IgG was used as a negative control to confirm ChIP specificity. Specific antibody- or IgG-precipitated samples were quantified by qPCR using specific primers around the transcription start site (TSS) of the *Sox9* gene (Table 1).

2.8. siRNA-mediated knockdown of histone lysine-specific demethylase-1 (*Lsd1*)

A pooled target-specific 19-25 nucleotides siRNAs designed to knock down the expression of mouse *Lsd1* (*Kdm1a*, *Aof2*, *Bhc110*) was purchased from Santa Cruz (sc-60971). Control siRNA (sc-36869, Santa Cruz) was used as a negative control. Transfection of siRNA was performed as previously described (Urita et al. , 2011). Subconfluent P1 chondrocytes from 18-month-old mice (with lowest *Sox9* expression) were transfected with *Lsd1*/control siRNA using Lipofectamine RNAiMAX transfection reagent (Invitrogen). After 72 hours of incubation with the siRNA and transfection reagent total RNA, protein and chromatin were isolated from the cultures for gene expression analysis, Western blot and ChIP assay, respectively.

2.9. Western blot

Total protein was isolated from cultured ACs and quantified by Bradford method (Bio-Rad) in RIPA buffer (Cell Signaling). 10 µg of total protein was applied to SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Millipore). After being blocked in 5% dry milk at 4°C overnight, membrane was incubated with specific antibodies against mouse *Lsd1* (C69G12, Cell Signaling) or mouse *Sox9* (sc-20095, Santa Cruz) at room temperature for 2 hours. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (sc-2004, Santa Cruz) was used as the secondary antibody. Monoclonal anti-β-Actin (A1978, Sigma) was used as a loading control. The protein bands

were detected in Pierce 1-Step Ultra TMB Blotting Solution (Prod#37574, Thermo Scientific) and photographed with a computerized gel documentation system (FOTO/Analyst Express, FOTODYNE).

2.10. Statistical analysis

Data were presented as mean $\pm$ SD. The difference between means from two different groups was determined by paired or unpaired 2-tail Student's *t*-tests. The difference between means for three or more groups was determined by ANOVA. A *p* value < 0.05 was considered statistically significant.

3. Results

3.1. Significant reduction of SOX9 expression in articular cartilage of adult mice

Sox9 mRNA was highly expressed in the early-stage developing articular cartilage (cartilage near the developing joint surface) at E16.5; its expression was slightly lower but still at a high level at 1 month of age, at which time articular cartilage structure is well defined but is still in the growth stage (late-stage developing articular cartilage). *Sox9* mRNA expression level was significantly reduced (by 57% compared to E16.5) at 2 months and remained at a low level thereafter with 81% reduction at 18 months (**Fig. 1A**). Moreover, the expression of *Sox9* mRNA in articular cartilage was significantly higher at 1 month than those at 2, 6, 12, 18 months (**Fig. 1B**). IHC revealed a remarkable decrease in the number of SOX9-expressing ACs in articular cartilage (particularly in the middle and deep zones) of mice at 6 months or older. By 18 months, only scattered SOX9-expressing ACs were seen in the superficial zone with essentially no SOX9-expressing ACs in the middle and deep zones of articular cartilage (**Fig. 1C**). Histopathological analyses demonstrated focal loss of safranin-O staining in articular cartilage of 18-month-old mice, indicating decreased synthesis and/or increased degradation of proteoglycans in articular cartilage (**Fig. 1C**). However, no osteoarthritic structural changes (e.g., articular surface fibrillation, osteophytes, subchondral bone change) were observed in the hip and

shoulder joints by 18 months of age. In literature, the process of joint development and sexual maturity in male mice is complete around 2 months of age while female mice are mature around 1.5 months. Therefore, 2- to 6-month-old mice are considered to be young adults, 12-month-old mice to be middle-aged and 15-month or older mice to be aged (Jacoby et al. , 2002, Kozłowska et al. , 2007, Nishikawa et al. , 2007). The data suggest that SOX9 expression in mouse ACs is significantly decreased after the completion of joint development. However, the ability of ACs to synthesize proteoglycans is not compromised in young adult and middle-aged mice with a significant reduction of SOX9 expression.

In addition, we examined the expression level of two other SOX members, SOX5 and SOX6, which are essential for cartilage formation (Smits et al. , 2001). As shown in **Fig. 1D**, expression levels of *Sox5* and *Sox6* in articular cartilage were decreased at 12 and 18 months of age with no significant changes at 1-6 months, compared to their levels at E16.5. The results suggest that there is no compensatory up-regulation of *Sox5* and *Sox6* when the SOX9 expression is decreased in adult articular cartilage.

3.2. Age-related alteration in DNA methylation at the Sox9 promoter

To explore potential epigenetic regulation of the age-dependent SOX9 expression in articular cartilage, we isolated genomic DNA from femoral and humeral head articular cartilage (**Fig. 2A**) and examined the DNA methylation status of the *Sox9* promoter. Five CpG islands were identified using the MethyPrimer program (<http://www.urogene.org/methprimer/index1.html>) within 4,000 base pairs (bp) around the *Sox9* transcription start site (TSS) (**Fig. 2B**). The sequences of the 5 CpG islands were highly conserved between human and mouse. Following MeDIP, 6 CpG regions (CpG island 3 was divided into CpG3A and CpG3B) were evaluated by qPCR using specific primers (**Table 1**). The methylation levels of all 6 CpG regions were low at E16.5 and 2 months but significantly increased at 6 and 12 months (**Fig. 2C**). Treatment of ACs from 6-month-old mice with 5-AzaC, an inhibitor of

DNA methylation (Jones and Taylor, 1980), decreased the DNA methylation levels at the CpG islands (**Fig. 2D**) and resulted in an up-regulated expression of *Sox9* mRNA and some of the SOX9 target genes, such as *Acan* and *Col11a1* (**Fig. 2E**).

To further investigate the candidate DNA methyltransferase(s) responsible for the age-dependent change of DNA methylation at the *Sox9* promoter region, ChIP experiments were performed using the chromatin isolated from articular cartilage tissue of 2-month and 6-month-old mice with the ChIP-grade antibodies against mouse DNMT1, 3a and 3b. No significant signals were detected from any of these ChIPed samples, suggesting that the levels of the endogenous DNMT(s) recruited to the *Sox9* promoter in articular cartilage tissue at these specific time points were too low to be detected by ChIP assay.

3.3. Age-dependent changes in histone methylation at the Sox9 promoter

Next, we examined the status of histone H3 methylation in the *Sox9* promoter region by ChIP assay and qPCR analysis using 3 specific primers (Pr1-3) for the sequences around the TSS (**Fig. 3A**). The level of histone 3 lysine 4 dimethylation (H3K4me₂, a histone modification linked to transcriptional activation) was highest at E16.5 and significantly reduced from 2 to 18 months (**Fig. 3B**). In contrast, histone 3 lysine 9 dimethylation (H3K9me₂, a histone modification linked to transcriptional repression) was lowest at E16.5 and significantly increased at 2, 6, and 18 months at specific primer regions (**Fig. 3C**). Moreover, the level of histone 3 lysine 27 trimethylation (H3K27me₃, another marker of transcriptional repression) was also significantly increased at 2 months (**Fig. 3D**).

LSD1 is a key lysine-specific demethylase (Shi et al. , 2004) which has been shown to regulate H3K4me₂ and H3K9me₂ in chondrocytes (El Mansouri et al. , 2014, Rodova, Lu, 2011). Although the expression of *Lsd1* in articular cartilage was not changed with age (**Fig. 4A**), the recruitment of LSD1

to the *Sox9* promoter was significantly increased in the articular cartilage of 6-month-old mice compared to recruitment level of LSD1 at E16.5 when SOX9 was highly expressed (**Fig. 4B**). Knockdown of *Lsd1* (encoding LSD1) in cultured ACs isolated from the femoral heads of 18-month-old mice (**Fig. 4C**) increased the expression of *Sox9* mRNA and protein (**Fig. 4D**), concomitant with increased H3K4me2 levels (**Fig. 4E**) and unchanged H3K9me2 levels in the *Sox9* promoter region (**Fig. 4F**). However, no significant increase in expression of *Sox9* target genes, such as *Acan* and *Col2a1*, was observed (data not shown).

These epigenetic analyses suggest that both DNA methylation and histone methylation are involved in the regulation of age-dependent SOX9 expression in mouse articular cartilage because either inhibition of DNA methylation or knockdown of *Lsd1* is sufficient to increase SOX9 expression. However, histone methylation, particularly H3K4me2, appears to play a more important role in the age-dependent SOX9 expression, as the H3K4me2 level in the *Sox9* promoter region is more closely correlated with the *Sox9* expression level at each age point.

4. Discussion

The present study has quantified for the first time the age-dependent reduction of *Sox9* mRNA expression in ACs of normal BALB/c mice from the embryonic to aged stage by qPCR, which is a more accurate measurement than the previously reported non-quantitative RNase protection assay of *Sox9* expression (Salminen, Vuorio, 2001). More importantly, we have explored the dynamic epigenetic mechanisms for the age-dependent changes in *Sox9* expression. Our qPCR analyses have revealed that the *Sox9* mRNA level in the ACs of mice is reduced by 57% (compared to E16.5) at 2 months, 79% at 12 months, and 81% at 18 months of age. An age-dependent reduction of SOX9 protein expression in ACs is also detected by IHC. By 18 months of age, IHC demonstrates only

scattered SOX9-expressing ACs in the superficial zone and essentially no SOX9-expressing ACs in the middle and deep zones of articular cartilage. However, none of the examined mice joints display osteoarthritic structural changes in the hip and shoulder joints by 18 months, although a decrease in safranin-O stained proteoglycans is detected in the articular cartilage after 15 months. These findings suggest that a significant reduction of SOX9 expression in the ACs of adult mice does not trigger the development of OA. This is consistent with a recent study by others indicating that postnatal inactivation of *Sox9* in mouse cartilage resulted in a reduction of proteoglycan content in the articular cartilage without histopathological signs of OA by 18 months of age (Henry, Liang, 2012).

As Sox family members, SOX5 and SOX6 have a high degree of sequence identity with each other but no homology with SOX9 except for the HMG-box. SOX5 and SOX6 are co-expressed with SOX9 during chondrogenic differentiation (Lefebvre and de Crombrughe, 1998). However, unlike SOX9, SOX5 and SOX6 do not contain a transcriptional activation domain, so SOX5 and SOX6 cooperate with SOX9 to activate the *Col2a1* and *Acan* genes (Han and Lefebvre, 2008, Lefebvre et al. , 1998). In this study, we examined the expression of *Sox5* and *Sox6* in ACs of different age points and found the expression of *Sox5* and *Sox6* was decreased only at 12 and 18 months of age. This result indicates that although SOX5 and SOX6 are required to fulfill the function of SOX9 in chondrogenesis (Akiyama, 2008), the age-dependent decrease of SOX9 expression does not lead to a compensatory up-regulation of *Sox5* and *Sox6* in ACs.

SOX9 is essential for cartilage formation during skeletal development and has been used as a chondrogenic marker for *in vitro* chondrogenesis studies (Bi, Deng, 1999, Dehne et al. , 2009, Kumar and Lassar, 2014, Zhu, Tang, 2009). However, the use of SOX9 as a potential therapeutic target for OA has generated controversial results. Cucchiaroni et al. reported that *SOX9* gene transfer in cultured human OA chondrocytes increased the expression levels of proteoglycans and type-II collagen

(Cucchiari et al. , 2007), whereas others suggested that overexpression of SOX9 was unable to restore the chondrocyte phenotype in dedifferentiated osteoarthritic chondrocytes (Kypriotou et al. , 2003). These previous and current studies suggest that articular chondrocyte activity switches from active chondrogenesis to maintenance of metabolic balance between anabolic and catabolic events after the completion of joint development. Accordingly, the physiological demand for SOX9 is diminished in adult articular cartilage.

It is possible that a low expression level of SOX9 in adult articular cartilage is sufficient to regulate the synthesis of type II collagen and aggrecan in healthy ACs. Therefore, a significant decrease in SOX9 expression in ACs may not compromise the function of adult articular cartilage or cause OA-like changes. Another possibility would be that other regulatory factors may play a role in homeostasis of adult articular cartilage, in concert with SOX9. Recent studies revealed that specific members of the nuclear factor of activated T cells (NFAT/Nfat) family of transcription factors may play important roles in the homeostasis of adult articular cartilage. NFAT1 (NFATc2) expression level in developing articular cartilage is very low but significantly increases at 2 months of age when skeletal development is essentially completed. Mice with global loss of NFAT1 exhibit normal skeletal development but display dysfunction of ACs and OA-like changes in adults, suggesting that NFAT1 is not required for development of the skeleton including articular cartilage but is required for the homeostasis of adult articular cartilage (Rodova, Lu, 2011, Wang, Gardner, 2009). Furthermore, mice lacking both NFAT1 and NFAT2 (NFATc1) in cartilage display more severe OA-like changes with joint subluxation (Beier, 2014, Greenblatt et al. , 2013).

In order to investigate the potential regulatory mechanisms for the age-dependent SOX9 expression in ACs of mice, age-related epigenetic changes in the *Sox9* promoter region were analyzed in this study. This is the first report on age-dependent epigenetic changes in the *Sox9* promoter in ACs of

mice, although epigenetic status of the human *SOX9* promoter in end-stage OA cartilage cells has been reported (Kim et al. , 2013). In this study, we found a significant increase in DNA methylation in the *Sox9* promoter region at 6 and 12 months, but not at other age points. Treatment of cultured ACs from 6-month-old mice with 5-AzaC, a DNMT inhibitor, decreased the DNA methylation levels at CpG islands in the *Sox9* promoter region and subsequently increased *Sox9* mRNA expression. The data suggest that DNA methylation is involved in the regulation of SOX9 expression at specific age points. This is consistent with the previous reports that in differentiated mammalian somatic cells, non-expressed genes may be silenced by DNA methylation in the promoter region, where the cytosine residues in the sequence 5'-CG-3' (CpG motifs) are methylated (Attwood et al. , 2002, Bird and Wolffe, 1999).

Wnt/ β -catenin signaling pathway plays an important role in homeostasis of chondrocytes in both development and adulthood. Thus, Wnt signaling may be involved in the pathogenesis of OA (Zhu et al. , 2008, Zhu, Tang, 2009). Recently, Kumar et al. reported that Wnt signals can induce DNA methylation over *Sox9* promoter which involved the DNMT3a in the study of chondrogenic potential of chicken limb bud mesenchymal cells (Kumar and Lassar, 2014). In this study, we failed to identify which DNMT(s) is responsible for DNA methylation at *Sox9* promoter region. Several possible reasons may be accounted for our results. First, the level of endogenous DNMT(s) recruited to the *Sox9* promoter in articular cartilage tissue (an *in vivo* condition) may not be high enough to be detected by ChIP assay, while Kumer et al. detected the recruitment of DNMT3a to the *Sox9* promoter using the cells overexpressing DNMTs with DNMTs expression vectors. Second, we might miss the right time as we just test two time points (2 months and 6 months). Third, other unknown DNMTs and/or DNA demethylases may be involved in the regulation of DNA methylation in articular cartilage.

The current study has revealed that the age-dependent decrease in SOX9 expression is closely associated with decreased H3K4me2 (a histone modification linked to transcriptional activation) and increased H3K9me2 and H3K27me3 (histone modifications linked to transcriptional repression). Knockdown of *Lsd1*, a key histone demethylase gene (El Mansouri, Nebbaki, 2014, Rodova, Lu, 2011, Shi, Lan, 2004), increased *Sox9* mRNA expression in cultured ACs from 18-month-old mice by enhancing the recruitment of H3K4me2 in the *Sox9* promoter region, suggesting that H3K4me2 is a key regulatory mechanism for the reduction of SOX9 expression in adult articular cartilage. *Lsd1* knockdown significantly up-regulated *Sox9* expression in cultured ACs from 18-month-old mice, but expression of SOX9 target genes was not significantly altered. This may be due to the overall reduction of transcriptional response in aged ACs, as we did observe an up-regulation of *Sox9* target genes when the *Sox9* expression was increased in ACs of 6 months old mice after 5-AzaC treatment. The proposed epigenetic mechanisms for the age-dependent changes in SOX9 expression are illustrated in **Fig. 5**.

Our results are consistent with previous reports that histone modifications are mostly short-term changes and therefore are reversible in specific cells (Berger, 2007, Li et al. , 2009, Reik, 2007). Although the involvement of other histone modifications such as acetylation and sumoylation cannot be fully eliminated, the results suggest that histone methylation plays an essential role in regulation of age-dependent SOX9 expression in mouse ACs because the H3K4me2 level in the *Sox9* promoter region is closely correlated with the *Sox9* expression level at each age point and knockdown of *Lsd1* is sufficient to increase SOX9 expression in the ACs.

In summary, SOX9 expression in mouse articular cartilage decreases significantly after the completion of skeletal development, which is regulated by dynamic DNA methylation and histone methylation. The spontaneous reduction of SOX9 expression in the ACs of adult mice does not trigger

development of OA. These novel findings suggest that the physiological demand for SOX9 expression is diminished in adult articular cartilage and that the low level of SOX9, in concert with other factors, may be sufficient to maintain the homeostasis of healthy ACs of adult mice.

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FIGURE LEGENDS

FIGURE 1. Reduction of *Sox9* expression in articular cartilage with no osteoarthritic changes in adult mice. **(A)** Age-dependent expression of *Sox9* mRNA in femoral head articular cartilage determined by qPCR (m = months of age). The expression levels at E16.5 were normalized to 1. **(B)** Age-dependent expression of *Sox9* mRNA in femoral head articular cartilage determined by qPCR. The expression levels at 1 month of age were normalized to 1. n = 3 pooled RNA samples, each prepared from the articular cartilage of six femoral heads for both A and B. ** $p < 0.01$, *** $p < 0.001$. **(C) *SOX9* IHC micrographs:** Age-related SOX9 protein localization in hip articular cartilage detected by immunohistochemistry (IHC, counterstained with haematoxylin) shows the highest SOX9 expression in the developing articular cartilage near the hip joint surface (red arrowheads) at E16.5 and a substantial decrease in the number of SOX9-expressing chondrocytes in articular cartilage of the hip joints of adult mice. ***Safranin-O* micrographs:** Immature chondrocytes with weak Safranin-O staining (for proteoglycans, red) in hip articular cartilage is seen in the superficial zone of articular cartilage at 1 month and a general loss of Safranin-O staining in hip articular cartilage is observed at 18 months. No OA-like structural changes are seen in the hip joints at any age points. All photomicrographs are representative of at least five mice per gender at each time point. acet = acetabulum, fh = femoral head, scale bar = 100 μ m. **(D)** Age-dependent expression of *Sox5* and *Sox6* mRNA in femoral head articular cartilage determined by qPCR. n = 3 pooled RNA samples, each prepared from the articular cartilage of six femoral heads. The expression level of each gene at E16.5 was normalized to 1. * $p < 0.05$, *** $p < 0.001$.

FIGURE 2. Age-dependent DNA methylation status in the *Sox9* promoter region in mouse articular cartilage tissue. (A) Fragmentation of genomic DNA prepared from articular cartilage by sonication. (B) Five CpG islands within 4,000 base pairs (bp) around the *Sox9* transcription start site (TSS). A total of 6 pairs of primers were designed for qPCR analysis of MeDIP. (C) MeDIP assays show an increase in DNA methylation in all 5 CpG islands at 6 months and in CpG island 4 at 12 months (m = months of age). (D) 5-AzaC treatment of cultured ACs from 6-month-old mice decreases DNA methylation level in CpG islands of the *Sox9* promoter. (E) 5-AzaC treatment increases the expression of mRNA for *SOX9* and some of its target gene in cultured ACs. The MeDIP enrichment of methylated DNA is presented as a value (in percentage) relative to the total DNA in input samples. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGURE 3. Age-dependent histone methylation status in the *Sox9* promoter region in mouse articular cartilage tissue. (A) Genomic structure of the *Sox9* gene with the location of three qPCR primer sets (*Sox9* TSS Pr1-3) used for qPCR analysis of ChIP assay. (B) ChIP assays with qPCR analyses demonstrate an age-dependent reduction of H3K4me2 in the *Sox9* promoter region. (C) ChIP-qPCR assays show an age-dependent increase in H3K9me2 in the *Sox9* promoter region. (D) ChIP-qPCR assays show an increase in H3K27me3 in the *Sox9* promoter region at 2 months. Upper panels of (B), (C) and (D) show representative agarose gel images of qPCR products amplified from the three different primers (Pr1-3) at ages. Lower panels of (B), (C) and (D) show the levels of qPCR amplification from the three primers at different ages relative to E16.5. The levels of H3K4me2, H3K9me2, and H3K27me3 of the E16.5 group have been normalized to 1.0. $n = 3$ independent experiments, and each experimental sample was prepared from articular cartilage of two mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

FIGURE 4. siRNA-mediated knockdown of *Lsd1* in cultured ACs from 18-month-old mice. (A) No significant changes in *Lsd1* mRNA expression are detected in mouse articular cartilage with age. (B) LSD1 recruitment to *Sox9* promoter is increased while the expression of *Sox9* is decreased. (C) qPCR analysis (left panel) and Western blots (right panel) confirm that the expression of *Lsd1* is significantly suppressed by *Lsd1* siRNA. (D) Up-regulation of *Sox9* mRNA (left panel) and protein (right panel) expression after knockdown of *Lsd1*. Knockdown of *Lsd1* increases the H3K4me2 level (E) but has no effect on H3K9me2 level (F) in the *Sox9* promoter region. The histone methylation level of the siRNA control group has been normalized to 1.0. n = 3 independent experiments, and each experimental sample was prepared from articular cartilage of two mice. Ctrl: control. * $p < 0.05$, *** $p < 0.001$.

FIGURE 5. A schematic diagram shows that age-related dynamics of epigenetic modifications results in age-dependent expression of SOX9 in mouse articular cartilage. At E16.5, H3K4me2 (a histone code for transcriptional activation) is dominant in the *Sox9* promoter region, leading to high expression of SOX9. From 2 months onwards, the recruitment of H3K4me2 in the *Sox9* promoter region is decreased, whereas the recruitment of H3K9me2 and H3K27me3 (histone codes for transcriptional repression) is increased. At 6 months, the level of DNA methylation in the *Sox9* promoter region is also increased, resulting in a further decrease in SOX9 expression.

Fig. 1

Fig. 2

Fig. 3

Fig. 4

Figure 5

Table 1. Specific primers used for quantitative real-time PCR

Method and Gene	Forward	Reverse	GenBank Accession No.
Gene expression			
<i>Sox9</i>	TCTGGAGGCTGCTGAACGA	TCCGTTCTTCACCGACTTCCT	NM-011448
<i>Sox5</i>	TCTGAGGAATTCAGCCAGTTTC	GCCATCGACTTCATTGTGTT	NM-011444
<i>Sox6</i>	AGGCGAGAGGCGGTAGGA	AGTCACTGGTAAATCGCCTTTGA	NM-011445
<i>Lsd1</i>	GCAGCCTGTTTCCCAGACA	CTGCAATGTGCGATTCCTGAT	NM-133872
<i>GAPDH</i>	AGGTTGTCTCCTGCGACTTCA	CCAGGAAATGAGCTTGACAAAG	NM-008084
DNA methylation immunoprecipitation (MeDIP)			
<i>CpG1</i>	CGGCTTTGGTTTTTCATTGAT	AGATTAACGCGCTCTGGTTC	
<i>CpG2</i>	GGGTTGTGGAGGGTCCTAGT	TGTGAACCGATGTGTGTGTG	
<i>CpG3A</i>	TGATTGGCCCGAGGTATCTA	GCTCCACAGAAGTTTCCAG	
<i>CpG3B</i>	CTGAAGAAGGAGAGCGAGGA	GTCCAGTCGTAGCCCTTCAG	
<i>CpG4</i>	GGATCAACAGAGCGAGCTTC	CTGCTCTAAACGCCTTTGCT	
<i>CpG5</i>	GAGGAAGTCGGTGAAGAACG	CAGCGCCTTGAAGATAGCAT	
Chromatin immunoprecipitation (ChIP)			
<i>Sox9-TSS-Pr1*</i>	CCAAAATCCGGTCCAATCAG	CCAATCACGGCTAGCTCTTGA	NM-133872
<i>Sox9-TSS-Pr2</i>	GCCCAGCTCCGCTTTGA	CGCTGGCGTTTACAGCAA	NM-133872
<i>Sox9-TSS-Pr3</i>	CGGGCTCGCGTATGAATCT	CTCCTGCTCGTCGGTCATCT	NM-133872

*TSS: transcription start site, Pr: primer