Published in final edited form as:

J Bone Miner Res. 2011 August ; 26(8): 1974–1986. doi:10.1002/jbmr.397.

Nfat1 Regulates Adult Articular Chondrocyte Function through Its Age-Dependent Expression Mediated by Epigenetic Histone Methylation

Marianna Rodova^{1,*}, Qinghua Lu^{1,*}, Ye Li², Brent G Woodbury¹, Jamie D Crist¹, Brian M Gardner¹, John G Yost¹, Xiao-bo Zhong², H Clarke Anderson³, and Jinxi Wang^{1,4}

¹Harrington Laboratory for Molecular Orthopedics, Department of Orthopedic Surgery, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160, USA.

²Department of Pharmacology, Toxicology, and Therapeutics, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160, USA.

³Department of Pathology and Laboratory Medicine, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160, USA.

⁴Department of Biochemistry and Molecular Biology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas 66160, USA.

Abstract

The development of disease-modifying pharmacologic therapy for osteoarthritis (OA) currently faces major obstacles, largely because the regulatory mechanisms for the function of adult articular chondrocytes remain unclear. We previously demonstrated that lack of Nfat1, one of the NFAT (nuclear factor of activated T cells) transcription factors, causes OA-like changes in adult mice. This study aimed to identify whether Nfat1 specifically regulates adult articular chondrocyte function and its age-dependent regulatory mechanism using both Nfat1-deficient and wild-type mice. Deletion of Nfat1 did not induce OA-like articular chondrocyte dysfunction (e.g., overexpression of proinflammatory cytokines and matrixdegrading proteinases) until the adult stage. RNAi-mediated Nfat1 knockdown caused dysfunction of wild-type adult articular chondrocytes. Nfat1 expression in wild-type articular chondrocytes was low in the embryonic, but high in the adult stage. Chromatin immunoprecipitation assays demonstrated that an increase in Nfat1 expression in articular chondrocytes was associated with increased H3K4me2 (a histone modification linked to transcriptional activation); while a decrease in Nfat1 expression in articular chondrocytes was correlated with increased H3K9me2 (a histone modification linked to transcriptional repression). Knockdown of lysine-specific demethylase-1 (Lsd1) in embryonic articular chondrocytes up-regulated Nfat1 expression concomitant with increased H3K4me2 at the *Nfat1* promoter. Knockdown of Jmjc-containing histone demethylase-2a (Jhdm2a) in 6-month articular chondrocytes down-regulated Nfat1 expression concomitant with increased H3K9me2 at the *Nfat1* promoter. These results suggest that Nfat1 is an essential transcriptional regulator of chondrocyte homeostasis in adult articular cartilage. Age-dependent Nfat1 expression in articular chondrocytes is regulated by dynamic histone methylation, one of the epigenetic mechanisms that regulate gene transcription.

Address correspondence to: Jinxi Wang, Department of Orthopedic Surgery, University of Kansas Medical Center, MS 3017, 3901 Rainbow Boulevard, Kansas City, Kansas 66160, USA. Phone: 913-588-0870; Fax: 913-588-0070. jwang@kumc.edu.

*These authors contributed equally to this work

Keywords

Articular chondrocyte; Nfat1; gene expression; histone modifications; epigenetics

Introduction

Osteoarthritis (OA), which involves the dysfunction of adult articular chondrocytes, is the most common form of joint disease and the major cause of chronic disability in middle-aged and older populations. Although multiple factors have been proposed to be involved in the development of OA, the regulatory mechanisms for the function of adult articular chondrocytes after joint development are still poorly understood. Currently, no proven disease-modifying OA pharmacologic therapy is available mainly because the biological mechanisms of OA pathogenesis remain unclear. The development of preventive and early-stage interventions for OA is likely to depend upon a better understanding of the regulatory mechanisms for the function of adult articular chondrocytes.

Joint development involves the formation of hyaline articular cartilage, subchondral bone, and other joint-specific tissues. Mouse articular cartilage and joints are identifiable at 13.5–16.5 days post-coitum (E13.5–16.5) and continue to grow and remodel until puberty (the age of ~4–5 weeks for females and ~2 weeks later for males) (1–3). Transcription factor Sox9 is critical for the differentiation of mesenchymal progenitor cells into chondrocytes during cartilage morphogenesis (1). Joint formation is defective in Sox9-deficient mouse embryos (4). Two other members of the Sox family, L-Sox5 and Sox6 may also be essential for cartilage formation (5). The Erg (Ets-related gene) transcriptional activator may play a role in directing the fate of chondroblasts toward an articular chondrocyte phenotype or a growth plate phenotype (3). Transforming growth factor beta (TGF- β), bone morphogenetic protein (BMP) and its antagonist, Noggin, and fibroblast growth factor (FGF) regulate cartilage formation. Indian hedgehog (Ihh) is a key regulator of endochondral bone formation. Transcription factor Cbfa1/Runx2 and vascular endothelial growth factor (VEGF) enhance endochondral ossification (6–8). Wnt/ β -catenin signaling may inhibit chondrogenic differentiation and promote osteoblast differentiation (9).

Previous studies have proposed that anabolic activity of articular chondrocytes may be maintained by insulin-like growth factor-1 (IGF-1), TGF- β , and BMP (8,10,11). Interleukin-1 β (IL-1 β), tumor necrosis factor- α (TNF α), and nitric oxide inhibit anabolic, but promote catabolic, activity of chondrocytes (12–14). Osteoarthritic cartilage usually displays an imbalance between anabolic and catabolic events in favor of matrix catabolism, which leads to cartilage degradation. Another aberrant behavior of osteoarthritic chondrocytes is abnormal expression of collagen-10 and other factors favoring chondrocyte terminal differentiation and/or endochondral ossification such as Runx2 and Wnt/ β -catenin signaling molecules (15–19). Smad3 deficiency accelerates chondrocyte maturation and leads to OA (20). Interestingly, no significant difference in quantity and subcellular localization of Sox9 protein was observed between osteoarthritic and healthy control human articular cartilage. Furthermore, Sox9 expression does not correlate with collagen-2 expression in adult articular chondrocytes (10). Thus, key transcription factors for maintaining the balance between anabolic and catabolic activity of adult articular chondrocytes remain to be identified.

Nfat1 (Nfatc2/Nfatp), a member of the nuclear factor of activated T cells (NFAT) transcription factor family, was originally identified as a regulator of the expression of cytokine genes during the immune response (21). Early studies also suggested that adult Nfat1-deficient mice displayed neoplastic or tumor-like cartilage cell proliferation in

articular cartilage and peri-articular tissues (22); however, gene mutation analyses indicated that *Nfat1* was not a tumor suppressor (23). Our recent extensive histopathological studies revealed that the phenotypes of adult *Nfat1*-deficient mouse joints fit the diagnostic characteristics of OA, including articular cartilage degradation with chondrocyte clustering, chondro-osteoecyte formation, and thickening of subchondral bone (24). All of these changes are similar to those of human OA (25,26). These previous studies described that skeletal development of the *Nfat1*-deficient mice was essentially normal; however, no data were presented to elucidate the effect of *Nfat1* deficiency on articular cartilage formation and articular chondrocyte function at the cellular and molecular levels in the embryonic and newborn stages. It remains unclear whether *Nfat1* deficiency affects the function of articular chondrocytes specifically in adults or in both the developmental and adult stages. If *Nfat1* does specifically regulate the function of adult articular chondrocytes, what are the regulatory mechanisms underlying age-dependent effects of *Nfat1* on articular chondrocyte function?

In this study, we report that deletion of *Nfat1* does not cause detectable dysfunction of articular chondrocytes until the young adult stage (2–4 months of age). *Nfat1* specifically regulates adult articular chondrocyte function through its age-dependent expression mediated by dynamic histone methylation, one of the epigenetic mechanisms that regulate gene transcription (27,28).

Materials and Methods

Animals

The method for generation of *Nfat1*^{-/-} mice was previously described (21). The breeder pairs of *Nfat1*-deficient (*Nfat1*^{-/-}) mice are a kind gift of Dr. Laurie Glimcher (Harvard University School of Public Health). The *Nfat1*^{-/-} and wild-type (WT) mice used in this study were bred and maintained in the Laboratory Animal Resources facility at the University of Kansas Medical Center (KUMC). All animal procedures were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) at KUMC in compliance with all federal and state laws and regulations. *Nfat1*^{-/-} and WT mouse embryos at E16.5, postnatal day 1, and 1, 2, 3, 4, and 6 months old mice were used for various analyses. At least five *Nfat1*^{-/-} and WT mice were evaluated at each time point. We chose E16.5 mouse embryos to study the joint formation and function of embryonic articular chondroblasts/chondrocytes because most cartilage tissues including articular cartilage are well formed at this stage (1,2,29).

Histochemistry and immunohistochemistry (IHC)

Mouse joint tissue samples were fixed in 2% paraformaldehyde, decalcified in 25% formic acid, and embedded in either paraffin or JB-4 plastic medium (Polysciences). Safranin-O and fast green stains were utilized to identify cartilage cells and matrices. 10% EDTA-decalcified tissue sections were used for immunohistochemical staining. To observe both immunoreaction and cellular morphology, some tissue sections were stained by the avidin-biotin peroxidase complex methods. AEC or DAB chromagen was used for color detection (30). Microscopic structure and cellular details of cartilaginous appendicular joints were examined at the embryonic stage (E16.5) through 6 months of age.

Skeletal tissue staining

After euthanasia, newborn mice were skinned, eviscerated, and fixed in 95% ethanol. The animals were then stained with Alcian blue for identification of cartilaginous tissue, digested with 2% KOH solution to remove soft tissues, and stained in Alizarin-red solution for identification of mineralized regions.

Collection of articular cartilage

Femoral head articular cartilage was used for isolation of RNA and cartilage cells since femoral head articular cartilage is much thicker and more readily removed compared to articular cartilage in other joints. In addition, the early OA-like phenotype (e.g. loss of proteoglycans) first occurs in the femoral heads of *Nfat1*^{-/-} mice. Femoral head articular cartilage samples were collected sterily under a surgical microscope. The microdissecting procedures include: 1) exposure and dislocation of the hip joint, 2) peeling the helmet-like articular cartilage from the underlying subchondral bone, 3) cutting articular cartilage into 4–5 vertical slices, and 4) removing the residual ligament teres, synovial tissue, and a small amount of bony/cartilaginous tissue that belongs to the secondary ossification center or the residual growth plate. Essentially pure articular cartilage samples were obtained by this method from 1–6 months old mice since the secondary ossification center is well formed by 1 month of age. At E16.5 and postnatal 1 day, the cartilage tissue collected from articular regions might contain a small amount of non-articular cartilage tissue from the developing secondary ossification center since the boundary between articular cartilage and the underlying secondary ossification center is not well defined at these early stages. Articular cartilage samples obtained through the microdissecting procedures were cut into small particles (~1.0 mm in diameter) for isolation of RNA or articular chondrocytes. The morphology of collected articular cartilage and the ability of isolated articular chondrocytes to express proteoglycans were confirmed by Safranin-O and Alcian blue stains (Supplemental Figure 1).

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

Freshly dissected articular cartilage from femoral heads and cultured cells derived from WT and *Nfat1*^{-/-} mice was collected in "RNA^{later}" solution (Ambion) at 4°C, homogenized in TRIzol reagent (Invitrogen), and treated with an RNeasy Mini Kit (Qiagen) to remove contamination of proteoglycans and a DNA Digestion Kit (Ambion) to remove DNA. The number of articular chondrocytes derived from a single mouse was not enough to obtain a sufficient amount of RNA. Thus, total RNA was isolated from pooled articular cartilage tissue samples harvested from at least 6 femoral heads. One µg of total RNA and a "RETRO^{Script}" kit (Ambion) were used for reverse transcription reactions to yield cDNA. "Primer Express 3.0" software (Applied Biosystems) was used to design the primers of target genes. Specific primers used in this study are presented in Table 1. qPCR reactions were performed in triplicate in 96-well plates using a 7500 Real-Time PCR system and "SYBR Green" reagents (Applied Biosystems) under standard conditions recommended by the manufacturer. Primers for rodent *Gapdh* (glyceraldehyde-3-phosphate dehydrogenase, Applied Biosystems) were used as housekeeping controls. Expression levels of genes of interest were quantified using the 2^{-ΔΔCt} relative quantification method as previously described (24,31).

Lentiviral RNA interference (RNAi) constructs

Lentiviral RNAi constructs were used to stably suppress the expression of the genes of interest in cultured mouse primary articular chondrocytes. "BLOCK-iTTM RNAi Designer" program (Invitrogen) was used to design *Nfat1*-specific RNAi sequences and scrambled sequences for negative controls. RNAi constructs were made by synthesizing oligonucleotides encoding 19–25 bp shRNA that specifically target mouse *Nfat1*. The H1 RNA polymerase III promoter pGreenPuro shRNA Cloning and Expression Lentivector (System Biosciences) was utilized to deliver *Nfat1*-specific short hairpin RNA (shRNA). BamH1 and EcoR1 restriction sites were added on the ends of the sequence to enable directional cloning. The oligonucleotides were annealed, phosphorylated by polynucleotide kinase (Promega), and then ligated into pSIH-CMV/FerL-cG-H1F using the unique BamH1 and EcoR1 sites located just downstream of the H1 promoter. Competent DH5α strains of *E.*

coli (Invitrogen) were transformed and clones were screened by PCR with primers flanking the insert. The final constructs were verified by sequencing.

Three lentiviral vectors (lentivectors) each containing an Nfat1-specific RNAi target sequence were tested in cultured primary articular chondrocytes, of which two sequences (5'-GACTATCTGAACCCTATCG-3'; 5'-CAGCGGAGTCCAAGGTTGTGTTTCAT-3') were found functional with at least 50% silencing efficiency of Nfat1 mRNA when used independently. The silencing efficiency reached more than 80% when a pool of two functional Nfat1-specific lentivectors was applied. Mouse lysine-specific histone demethylase-1 (Lsd1) shRNA lentiviral particles (Santa Cruz, sc-60971-V) containing a pool of three Lsd1-specific constructs were used to knock down Lsd1 expression. Mouse Jmjc-containing histone demethylase-2a (Jhdm2a), also known as lysine-specific demethylase-3a (Kdm3a) or Jumonji domain-containing 1a (Jmjd1a) shRNA lentiviral particles (Santa Cruz, sc-146322-V) containing a pool of 3 Jhdm2a-specific constructs was used to knock down Jhdm2a expression. Control shRNA lentiviral particles (Santa Cruz, sc-108080) encoding a scrambled shRNA sequence that does not lead to specific degradation of any mRNA was used as a negative control.

Cell culture and RNAi-mediated gene silencing

Primary articular chondrocytes were isolated from pooled femoral head articular cartilage samples from E16.5, 3 months, and 6 months old WT mice with collagenase D (1.5 mg/ml, Roche) in DMEM. Cells were plated at 2×10^5 cells per 23-mm diameter culture well in DMEM supplemented with 2 mM L-glutamine, 10% heat-inactivated fetal calf serum, and 1% penicillin/streptomycin, then placed into a humidified incubator with 5% CO₂ at 37°C. When cultivated cells reached ~70% of confluence, the cultures were transfected with specific target RNAi delivered by lentivectors according to previously described general methods (32) and specific instructions for the use of Lsd1/Jhdm2a shRNA lentiviral particles provided by Santa Cruz Biotechnology. To discount the changes caused by transfection delivery reagents, vectors containing non-specific scrambled sequences were used as controls for RNAi experiments. Packaging plasmid psPAX2 and envelope plasmid pMD2.G were obtained from Addgene. 293T cells (ATCC) were used for the production of viral particles. Cells treated with these specific RNAi or negative control constructs were used for ChIP assays, RNA isolation and qPCR, and Western blotting to validate the silencing efficiency of target genes/proteins.

Western blot

Total protein was isolated from the organic phase following TRIzol reagent treatment and RNA isolation from cells/tissues according to the manufacturer's instructions (Invitrogen). Small aliquots were applied to SDS-PAGE and then transferred to a polyvinylidene difluoride membrane (Hybond P, Amersham Biosciences). After transfer, the membranes were blocked using 5% dry milk in PBS with 0.3% Tween 20. For Western blotting, specific antibodies against mouse Nfat1 (Santa Cruz), mouse Lsd1 (Abcam), and mouse Jhdm2a (Abcam) were used as primary antibodies, followed by 1:2,000 horseradish peroxidase (HRP)-conjugated anti-mouse/rabbit IgG (Santa Cruz) as the secondary antibody. For the loading control, a 1:10,000 dilution of peptide-affinity purified goat polyclonal antibody against Gapdh (IMGEX) was used as the primary antibody, and a 1:5,000 dilution of anti-goat HRP-conjugated antibody (Santa Cruz) was used as the secondary antibody. A bound secondary antibody was detected using chemiluminescent HRP substrate (Millipore).

Chromatin immunoprecipitation (ChIP) assay for histone modifications

ChIP assays were performed based on the methods for quantitative measurement of protein-DNA interactions (33). Articular chondrocytes were isolated from femoral head articular

cartilage of WT mice at ages E16.5, 2 months, and 6 months with collagenase D (1.5 mg/ml, Roche). Each chromatin sample was prepared from 10–12 million chondrocytes, which were collected from pooled femoral head articular cartilage samples or RNAi-treated cell cultures. The cells were incubated with 1% formaldehyde at room temperature for 10 min to crosslink proteins to cognate-binding DNA sequences. The cross-linking reaction was quenched by adding 0.125 M glycine for 5 min. The cells were then washed twice with PBS and subjected to chromatin preparation according to the ChIP assay kit procedure (Upstate Biotechnology). ChIP was carried out using antibodies against mouse histone 3 lysine 4 dimethylation (H3K4me2, Millipore 07–030), histone 3 lysine 9 dimethylation (H3K9me2, Millipore 17–648), and histone 3 lysine 27 trimethylation (H3K27me3, Millipore 07–449) according to the manufacturers' protocols. An anti-mouse RNA polymerase II (Pol-II) antibody (Millipore 05-623B) was used as a positive control and an antibody against mouse IgG (Millipore, 12–371B) was used as a negative control to confirm the specificity of ChIP reactions. These antibodies were used to co-immunoprecipitate specific modifications of the histone protein and the bound DNA. Upon reverse crosslinking (deproteinization) by proteinase K, co-immunoprecipitated DNA was released and subjected to qPCR analysis using the primers designed from the sequences in the *Nfat1* promoter region to determine the level of an immunoprecipitated histone modification linked to *Nfat1*-specific DNA sequence in living cells. Age-related mouse *Gapdh* (a housekeeping gene)-specific histone modification levels were measured as controls using the same antibodies and assay methods for *Nfat1* gene. Thirty-five qPCR cycles were used for quantitative ChIP analyses. The PCR products were electrophoresed on a 3% Nu Sieve agarose gel stained with ethidium bromide.

Statistical analysis

Data were expressed as means $\pm$ S.D. from at least three independent experiments for each experimental condition. Statistical analyses were performed with the assistance of a computer software program (SPSS, version 19.0, IBM). The difference between means from two different groups was analyzed by Student's *t*-test (two-tailed); the difference between means for three or more groups was assessed by one-way ANOVA followed by a post-hoc test (Tukey). $p < 0.05$ was considered statistically significant.

Results

Nfat1^{-/-} articular chondrocytes do not display OA-like cellular dysfunction until the adult stage

No abnormal cartilage differentiation or joint formation was observed in *Nfat1*^{-/-} mouse embryos at E16.5 and early postnatal (1 day and 1 month) mice, which is the period of rapid growth of the skeletal system (Figure 1A–C). Loss of Safranin-O staining for proteoglycans was first observed in articular cartilage of *Nfat1*^{-/-} mice at the age of 2 months. This early sign of articular cartilage degradation was more clearly seen in tissue sections of *Nfat1*^{-/-} femoral head articular cartilage obtained through microdissection (Figure 1D). By 4–6 months of age, articular surface fibrillation and chondrocyte clustering (a typical feature of OA) were observed in *Nfat1*^{-/-} articular cartilage (Figure 1E). The late-stage OA-like changes, including chondro-osteophyte formation, destruction of articular surfaces, and exposure of thickened subchondral bone were demonstrated in detail in our previous publication (24).

To determine age-dependent effects of *Nfat1* deficiency on the function of articular chondrocytes, we examined expression levels of specific genes in articular cartilage from age E16.5 through 6 months by qPCR. Anabolic activity of articular chondrocytes was examined by measuring the expression levels of major chondrocyte marker genes *Acan*

(encoding aggrecan), *Col2a1*, *Col9a1*, and *Col11a1* (encoding collagen-2, -9 and -11) in articular cartilage. *Col10a1* (encoding collagen-10) was tested as a marker for hypertrophic chondrocytes. Catabolic activity was examined by measuring the expression levels of mRNA for cartilage-degrading proteinases such as Mmps (matrix metalloproteinases)-1 α , -3, -9, -13 and Adamts (a disintegrin and metalloproteinase with thrombospondin motifs)-4 and -5 (34,35), as well as proposed proinflammatory cytokines IL-1 β , IL-6, IL-17 α , IL-18, and TNF α (12,13). qPCR analyses showed no significant differences in expression levels of major chondrocyte marker genes and genes for catabolic proteinases/cytokines between *Nfat1*^{-/-} and WT articular cartilage at E16.5 and postnatal 1 day. At 2 months, the expression of *Acan*, *Col2a1*, and *Col11a1* decreased significantly in *Nfat1*^{-/-} articular chondrocytes. At 6 months, the expression of *Col10a1* and some of the chondrocyte marker genes (*Col2a1* and *Col11a1*) was significantly up-regulated in *Nfat1*^{-/-} articular chondrocytes, indicating reparative changes at this stage (Figure 1F). Expression levels of mRNA for proinflammatory cytokines IL-1 β and IL-17 α as well as matrix-degrading proteinases Mmp1a, Mmp13, and Adamts5 were up-regulated to varying degrees at 2–6 months. *Timp3* (tissue inhibitor of metalloproteinase-3) expression was significantly decreased in *Nfat1*^{-/-} articular cartilage at 2 months (Figure 1G).

Knockdown of Nfat1 causes severe dysfunction of adult articular chondrocytes

To examine whether OA-like dysfunction of adult articular chondrocytes is directly affected by Nfat1 deficiency or is a secondary response to abnormal joint mechanics caused by chondro-osteophytes, we performed RNAi-mediated stable knockdown of Nfat1 expression in cultured primary articular chondrocytes isolated from 3-month-old WT femoral heads. Using a pool of two lentiviral constructs each containing an Nfat1-specific RNAi sequence, Nfat1 expression was significantly suppressed at both the mRNA level (Figure 2A) and the protein level (Figure 2B). Figure 2A demonstrated that the Nfat1 RNAi sequences used in this study specifically suppressed the expression of Nfat1, but not other Nfat members. The expression levels of major chondrocyte marker genes for aggrecan, collagen-2, -9, -10, and 11, as well as proposed proinflammatory cytokines (IL-1 β , IL-6, IL-17 α , IL-18, and TNF α) and matrix-degrading proteinases (Mmps-1 α , -3, -9, and -13 and Adamts-4 and -5) in Nfat1 RNAi-treated cartilage cells were evaluated by qPCR analyses. Knockdown of Nfat1 significantly down-regulated the expression of mRNA for aggrecan, collagen-2, and collagen-11 and up-regulated the expression of mRNA for collagen-10, IL-1b, IL-17a, Mmp13, and Adamts5 (Figure 2C). These results suggest that OA-like dysfunction of adult articular chondrocytes is directly affected by Nfat1 deficiency.

Age-dependent expression of Nfat1 in articular chondrocytes

To explore the mechanisms by which OA-like dysfunction of articular chondrocytes initially appears in the adult stage but not in the developmental stage of *Nfat1*^{-/-} mice, we examined the expression pattern of Nfat1 from embryonic age E16.5 to 6 months by qPCR and IHC. *Nfat1* mRNA expression in articular chondrocytes was lowest at E16.5, gradually elevated postnatally, and then maintained at a high level at the adult stage (2–6 months) that we examined (Figure 3A). Immunohistochemical analyses demonstrated that intracellular localization of Nfat1 protein was essentially undetectable in articular chondrocytes at E16.5 but highly expressed at the adult stage (Figure 3B). Nfat1 protein expression was not detectable in *Nfat1*^{-/-} articular chondrocytes at all stages, confirming the specificity of the Nfat1 antibody used in this study (Figure 3C).

These results suggest that age-related function of Nfat1 is associated with its age-dependent expression in articular chondrocytes. A low Nfat1 expression in articular chondrocytes at the developmental stage suggests that Nfat1 is not required for the development of articular cartilage. Thus, deletion of Nfat1 does not cause significant dysfunction of articular

chondrocytes during development. In contrast, high expression of *Nfat1* in adult articular chondrocytes suggests that *Nfat1* is required for maintaining the function of adult articular chondrocytes. Lack of *Nfat1* in adults causes severe dysfunction of articular chondrocytes.

Age-dependent histone methylation in the promoter region of the *Nfat1* gene

We next investigated potential regulatory mechanisms for age-dependent *Nfat1* expression. DNA methylation and histone modification are the two best-studied epigenetic regulatory mechanisms of gene transcription and cellular function in eukaryotic cells (27,28,36). DNA methylation patterns are stable, which confer long-term epigenetic modifications. In contrast, histone modifications are mostly short-term changes and therefore are reversible (36–38). Since the expression pattern of *Nfat1* appeared to be reversible changes, we examined age-dependent histone modifications in the promoter region of the *Nfat1* gene by ChIP assays.

ChIP assays were performed using chromatin prepared from articular chondrocytes isolated from femoral head articular cartilage of E16.5, 2-month, and 6-month WT mice and antibodies against H3K4me2 (a histone code associated with transcriptional activation), as well as H3K9me2 and H3K27me3 (histone codes associated with transcriptional repression) (33,36,39,40). The binding level of co-immunoprecipitated DNA associated with histone methylation in each assay was quantified by qPCR analyses using three different primers (P1–3) designed from the sequences around the transcription start site (TSS) of the *Nfat1* gene. A primer pair designed from the untranscribed genomic region located on mouse chromosome 17 (Untr17) was used as a negative control (Figure 4A). Untr17 was previously used as a negative control primer pair for qPCR analysis of mouse Pet-1 ChIP assay (41). Our sequence analysis indicated that mouse Untr17 sequence is located in a 1.5 Mb gene deserts (devoid of genes). qPCR analyses of the ChIP samples using P1–3 revealed an age-related increase in H3K4me2 (Figure 4B) and an age-related decrease in H3K9me2 (Figure 4C), but no age-related change in H3K27me3 (data not shown). In contrast, qPCR using Untr17 control primer pair showed no detectable changes of H3K4me2 or H3K9me2 in this genomic DNA region (Figure 4B–C, **P-ctl**), confirming the specificity of the amplified PCR products for *Nfat1*-specific DNA sequences that are co-immunoprecipitated with H3K4me2 or H3K9me2.

Nfat1-specific Pol-II binding levels were correlated with H3K4me2 levels at different ages, while *Gapdh*-specific histone methylation levels showed no significant changes with age (data not shown), which further confirmed the specificity of the age-related histone methylation at the *Nfat1* promoter. These results suggest that age-dependent expression of *Nfat1* is associated with dynamic changes in specific histone methylation.

Knockdown of *Lsd1* in E16.5 articular chondrocytes results in an upregulation of *Nfat1* expression concomitant with increased H3K4me2 at the *Nfat1* promoter

To determine whether age-dependent *Nfat1* expression in articular chondrocytes is regulated by epigenetic histone modifications, we performed RNAi-mediated loss-of-function experiments to test if knockdown of a key enzyme for H3K4 demethylation in chondrocytes affects *Nfat1* expression. Previous studies identified *Lsd1* as a key histone demethylase for H3K4me2 (42) and this study revealed that *Nfat1* expression in E16.5 articular chondrocytes was very low, both in *in vivo* and in primary cultures. Thus, we tested whether knockdown of *Lsd1* in E16.5 articular chondrocytes can up-regulate *Nfat1* expression through increased H3K4me2 in the *Nfat1* promoter region. The results demonstrated that *Lsd1* RNAi efficiently reduced *Lsd1* expression in cultured E16.5 primary articular chondrocytes, as judged by qPCR (Figure 5A, **left panel**) and Western blotting (Figure 5A, **right panel**). Concomitant with the decrease in *Lsd1* expression, we observed increased H3K4me2 levels

around the TSS of the *Nfat1* promoter by ChIP assays (Figure 5B) and an increase (derepression) in *Nfat1* expression by qPCR (Figure 5C) in *Lsd1* RNAi-treated E16.5 articular chondrocytes. The data suggest that the transcription of *Nfat1* gene in E16.5 articular chondrocytes is negatively regulated by *Lsd1* through demethylation of H3K4me2 at the *Nfat1* promoter (Figure 5D).

Knockdown of Jhdm2a in 6-month articular chondrocytes causes decreased Nfat1 expression concomitant with increased H3K9me2 at the Nfat1 promoter

We next tested if knockdown of a key enzyme for H3K9 demethylation affects *Nfat1* expression in articular chondrocytes. Previous studies identified Jhdm2a as a specific histone demethylase for H3K9me2 (43) and this study demonstrated that *Nfat1* was highly expressed in 6-month articular chondrocytes, both in *in vivo* and in primary cultures. Thus, we examined whether knockdown of Jhdm2a in 6-month articular chondrocytes can down-regulate *Nfat1* expression through increased H3K9me2. The results confirmed that Jhdm2a RNAi efficiently reduced Jhdm2a expression in cultured 6-month primary articular chondrocytes, as judged by qPCR (Figure 6A, **left panel**) and Western blotting (Figure 6A, **right panel**). Concomitant with the decrease in Jhdm2a expression, we observed increased H3K9me2 levels around the TSS of the *Nfat1* promoter by ChIP assays (Figure 6B) and a decrease in *Nfat1* expression by qPCR analyses (Figure 6C) in Jhdm2a RNAi-treated 6-month articular chondrocytes. The data suggest that the transcription of *Nfat1* gene in 6-month articular chondrocytes is positively regulated by Jhdm2a through demethylation of H3K9me2 at the *Nfat1* promoter (Figure 6D).

Taken together, these results demonstrate that age-dependent *Nfat1* expression in articular chondrocytes is regulated by dynamic histone methylation, one of the epigenetic mechanisms in eukaryotic cells.

Discussion

This study has presented for the first time the age-dependent effect of *Nfat1* on the function of mouse articular chondrocytes. Deletion of *Nfat1* does not affect joint formation or articular chondrocyte function during the developmental stage; however, it does cause severe dysfunction of adult articular chondrocytes. RNAi-mediated knockdown of *Nfat1* expression causes OA-like dysfunction of cultured WT adult articular chondrocytes, suggesting that *Nfat1* deficiency can directly affect the function of adult articular chondrocytes without abnormal mechanical influence or metabolic interactions between articular cartilage and other joint tissues. Our data reveal that deletion or knockdown of *Nfat1* triggers overexpression of collagen-10 and multiple proinflammatory cytokines and matrix-degrading proteinases in adult articular chondrocytes (Figure 1F and 1G). *Nfat1* appears to be a key transcription factor that represses the expression of specific catabolic molecules and chondrocyte hypertrophy in adult articular cartilage. Thus, *Nfat1* deficiency results in overexpression of specific catabolic molecules and chondrocyte hypertrophy, leading to OA-like articular cartilage degradation.

To our knowledge, *Nfat1* is the first transcription factor to date identified as a regulator of articular chondrocyte function in adult, but not in developing mice. This is in contrast to the function of *Sox9*, which is critical for cartilage formation during skeletal development (1,4); however, *Sox9* expression does not correlate with collagen-2 expression in adult articular chondrocytes and no significant difference in *Sox9* expression is observed between osteoarthritic and healthy control articular cartilage (10). Secondly, deletion of transcription factor *Runx2/Cbfa1* results in a complete lack of bone formation during development due to maturational arrest of chondrocytes and osteoblasts (44). Thirdly, β -catenin, a key molecular node of transcriptional regulation of the canonical Wnt pathway, plays an important role in

homeostasis of chondrocytes in both development and adulthood. Inactivation of β -catenin affects chondrocyte differentiation during development (9). Both gain- and loss-of-function of β -catenin in articular cartilage leads to OA-like phenotypes in adult mice (18,45).

Changes in gene expression caused by mechanisms other than changes in the underlying DNA sequences are referred to as “epigenetics.” DNA methylation and histone modification are the two best-studied epigenetic regulatory mechanisms. Abnormal expression of specific matrix-degrading enzymes by human OA chondrocytes has been reported to be associated with demethylation of specific CpG sites in the promoter regions of these genes (46). Inflammatory cytokines (TNF α and oncostatin M) may change the DNA methylation status at key CpG sites, resulting in long-term induction of IL-1 β in cultured articular chondrocytes (47). The fundamental unit of chromatin, the nucleosome core particle, consists of super helical DNA wrapped around a histone octamer core. Post-translational modifications (e.g., methylation and acetylation of lysines) in the N-terminal tails of histone proteins that usually occur around the TSS of a gene may change the DNA-histone interactions and accessibility of specific regulatory proteins to DNA, thereby regulating gene transcription (33,39,40). Depending on what types of modifications at which amino acids, histone modifications may result in activation or repression of gene transcription (27,28). Histone modifications are flexible and reversible (36–38), consistent with the age-dependent expression pattern of *Nfat1*. This study reveals that the age-related regulatory effect of *Nfat1* on the function of articular chondrocytes is associated with its age-dependent expression, which is regulated by age-related dynamic changes in histone methylation around the TSS of *Nfat1* gene. During the developmental stage, *Nfat1* is transiently held in a repressed state by specific histone modifications (a low level of H3K4me2 and a high level of H3K9me2), suggesting that *Nfat1* is not required at this stage. In the adult stage, this process is reversed to meet the needs for *Nfat1* expression that is required for maintenance of the adult chondrocyte phenotype.

Nfat1 deficiency-induced OA-like changes include articular cartilage degradation with slow progression of articular destruction, abnormal cartilage formation in the synovium/periosteum resulting in the formation of chondro-osteophytes, and exposure of thickened subchondral bone (24). All of these pathological changes in *Nfat1*-deficient joints resemble human OA (25,26). The earliest pathological change in *Nfat1*^{-/-} joints is the reduced expression of chondrocyte markers and overexpression of specific proinflammatory cytokines (e.g., IL-1 β) at 2 months of age. However, the overall expression levels of collagen-10 and some chondrocyte markers (*Col2a1* and *Col11a1*) increases at 6 months of age with increased proteoglycan staining in the mid-deep zones of *Nfat1*^{-/-} articular cartilage. These changes suggest that *Nfat1* deficiency initially provokes articular cartilage degradation with reduced expression of chondrocyte markers, which triggers a secondary reparative reaction with a regional increase in expression of chondrocyte markers in the *Nfat1*^{-/-} articular cartilage at later stages. This is consistent with increased synthesis of collagen-2 and collagen-10 in human OA cartilage harvested at relatively late stages of disease (16,19), as well as an early report which showed increased expression of both collagen-2 and collagen-10 in articular cartilage of older *Nfatp*^{-/-} mice (22).

Nfat1^{-/-} joints display both articular cartilage degradation and bony changes, including osteophyte formation and thickening of subchondral bone (24). Thus, we cannot eliminate the possibility that *Nfat1* deficiency also primarily affects the function of cells in synovium, peri-articular periosteum, and subchondral bone, causing abnormal chondrocyte differentiation and endochondral ossification in these tissues, leading to chondro-osteophyte formation and thickening of subchondral bone. Further work is needed to elucidate whether pathological endochondral ossification observed in the peri-articular tissues of *Nfat1*^{-/-}

mouse joints are the primary changes, or secondary reactions to the dysfunctional articular cartilage.

The molecular and cellular mechanisms for initiation and progression of OA are still poorly understood. Current treatments for OA are largely palliative, and many cases eventually require replacement of the osteoarthritic joints with prostheses. No proven pharmacologic therapy is currently available to prevent the initiation or reverse the progression of OA. In addition, efforts to develop methods for the surgical and biological repair of damaged articular cartilage face major obstacles due to de-differentiation and dysfunction of newly formed chondrocytes in the repair tissue. This study has identified that Nfat1 is a key transcription factor, which may act together with other factors, for maintaining the physiological function of adult articular chondrocytes. Thus, Nfat1 deficiency is a risk factor for dysfunction of adult articular chondrocytes and development of OA.

In conclusion, the present study demonstrates that Nfat1 specifically regulates the function of adult articular chondrocytes through its age-dependent expression, which is mediated by dynamic histone modifications. These findings have revealed a novel mechanism for transcriptional regulation of adult articular chondrocyte function, which may provide new insights into the etiopathogenesis of OA and the development of effective therapeutic strategies for the treatment of OA and traumatic articular cartilage defects.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported in part by the Mary Alice & Paul R. Harrington Distinguished Professorship Endowment and US National Institutes of Health grants AR052088, DE05262, and 5P20 RR021940. The authors thank Dr. Maurizio Pacifici (Thomas Jefferson University, Philadelphia, USA) for his thoughtful suggestions and comments on the joint development studies, as well as his critical reading of this manuscript. We also thank James Bernard and M. Kareem Shaath for their assistance in statistical analyses, graphic design, and editing.

References

1. Bi W, Deng JM, Zhang Z, Behringer RR, de Crombrughe B. Sox9 is required for cartilage formation. *Nat Genet.* 1999; 22:85–89. [PubMed: 10319868]
2. Jacoby, RO.; Fox, JG.; Davisson, M. Biology and Diseases of Mice. In: Fox, JG.; Anderson, LC.; Loew, FM.; Quimby, FW., editors. *Laboratory Animal Medicine*. 2nd ed. Vol. vol. San Diego: Academic Press; 2002. p. 35-120.
3. Iwamoto M, Tamamura Y, Koyama E, Komori T, Takeshita N, Williams JA, Nakamura T, Enomoto-Iwamoto M, Pacifici M. Transcription factor ERG and joint and articular cartilage formation during mouse limb and spine skeletogenesis. *Dev Biol.* 2007; 305:40–51. [PubMed: 17336282]
4. Akiyama H, Chaboissier MC, Martin JF, Schedl A, de Crombrughe B. 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.* 2002; 16:2813–2828. [PubMed: 12414734]
5. Smits P, Li P, Mandel J, Zhang Z, Deng JM, Behringer RR, de Crombrughe B, Lefebvre V. The transcription factors L-Sox5 and Sox6 are essential for cartilage formation. *Dev Cell.* 2001; 1:277–290. [PubMed: 11702786]
6. Kronenberg HM. Developmental regulation of the growth plate. *Nature.* 2003; 423:332–336. [PubMed: 12748651]
7. Zelzer E, Glotzer DJ, Hartmann C, Thomas D, Fukai N, Soker S, Olsen BR. Tissue specific regulation of VEGF expression during bone development requires Cbfa1/Runx2. *Mech Dev.* 2001; 106:97–106. [PubMed: 11472838]

8. Reddi AH. Role of morphogenetic proteins in skeletal tissue engineering and regeneration. *Nature Biotech.* 1998; 16:247–252.
9. Day TF, Guo X, Garrett-Beal L, Yang Y. Wnt/beta-catenin signaling in mesenchymal progenitors controls osteoblast and chondrocyte differentiation during vertebrate skeletogenesis. *Dev Cell.* 2005; 8:739–750. [PubMed: 15866164]
10. Aigner T, Gebhard PM, Schmid E, Bau B, Harley V, Poschl E. SOX9 expression does not correlate with type II collagen expression in adult articular chondrocytes. *Matrix Biology.* 2003; 22:363–372. [PubMed: 12935820]
11. Fortier LA, Mohammed HO, Lust G, Nixon AJ. Insulin-like growth factor-I enhances cell-based repair of articular cartilage. *J Bone Joint Surg Br.* 2002; 84:276–288. [PubMed: 11922373]
12. Goldring SR, Goldring MB. The role of cytokines in cartilage matrix degeneration in osteoarthritis. *Clin Orthop Relat Res.* 2004; 427(Suppl):S27–S36. [PubMed: 15480070]
13. van den Berg, WB.; van der Kraan, PM.; van Beuningen, HM. Synovial mediators of cartilage damage and repair in osteoarthritis. In: Brandt, KD.; Doherty, M.; Lohmander, LS., editors. *Osteoarthritis.* 2nd ed. Vol. vol. Oxford: Oxford University Press; 2003. p. 147-155.
14. Lotz M. The role of nitric oxide in articular cartilage damage. *Rheum Dis Clin North Am.* 1999; 25:269–282. [PubMed: 10356417]
15. Kirsch T, Swoboda B, Nah H. Activation of annexin II and V expression, terminal differentiation, mineralization and apoptosis in human osteoarthritic cartilage. *Osteoarthritis Cartilage.* 2000; 8:294–302. [PubMed: 10903884]
16. Sandell, LJ.; Heinegard, D.; Hering, TM. Cell Biology, Biochemistry, and Molecular Biology of Articular Cartilage in Osteoarthritis. In: Moskowitz, RW.; Altman, RD.; Hochberg, MC.; Buckwalter, JA.; Goldberg, VM., editors. *Osteoarthritis: Diagnosis and Medical/Surgical Management.* 4th ed. Vol. vol. Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins; 2007. p. 73-106.
17. Kawaguchi H. Regulation of osteoarthritis development by Wnt-beta-catenin signaling through the endochondral ossification process. *J Bone Miner Res.* 2009; 24:8–11. [PubMed: 19016582]
18. Zhu M, Tang D, Wu Q, Hao S, Chen M, Xie C, Rosier RN, O'Keefe RJ, Zuscik M, Chen D. Activation of beta-catenin signaling in articular chondrocytes leads to osteoarthritis-like phenotype in adult beta-catenin conditional activation mice. *J Bone Miner Res.* 2009; 24:12–21. [PubMed: 18767925]
19. Poole, AR.; Guilak, F.; Abramson, SB. Etiopathogenesis of Osteoarthritis. In: Moskowitz, RW.; Altman, RD.; Hochberg, MC.; Buckwalter, JA.; Goldberg, VM., editors. *Osteoarthritis: Diagnosis and Medical/Surgical Management.* Vol. vol. Philadelphia: Wolters Kluwer/Lippincott Williams & Wilkins; 2007. p. 27-49.
20. Li TF, Darowish M, Zuscik MJ, Chen D, Schwarz EM, Rosier RN, Drissi H, O'Keefe RJ. Smad3-deficient chondrocytes have enhanced BMP signaling and accelerated differentiation. *J Bone Miner Res.* 2006; 21:4–16. [PubMed: 16355269]
21. Hodge M, Ranger A, Charles de la Brousse F, Hoey T, Grusby M, Glimcher L. Hyperproliferation and dysregulation of IL-4 expression in NF-ATp-deficient mice. *Immunity.* 1996; 4:397–405. [PubMed: 8612134]
22. Ranger AM, Gerstenfeld LC, Wang J, Kon T, Bae H, Gravalles EM, Glimcher MJ, Glimcher LH. The nuclear factor of activated T cells (NFAT) transcription factor NFATp (NFATc2) is a repressor of chondrogenesis. *J Exp Med.* 2000; 191:9–21. [PubMed: 10620601]
23. Aoyama T, Nagayama S, Okamoto T, Hosaka T, Nakamata T, Nishijo K, Tsuboyama T, Nakayama T, Nakamura T, Toguchida J. Mutation analyses of the NFAT1 gene in chondrosarcomas and enchondromas. *Cancer Lett.* 2002; 186:49–57. [PubMed: 12183075]
24. Wang J, Gardner BM, Lu Q, Rodova M, Woodbury BG, Yost JG, Roby KF, Pinson DM, Tawfik O, Anderson HC. Transcription factor NFAT1 deficiency causes osteoarthritis through dysfunction of adult articular chondrocytes. *J Pathol.* 2009; 219:163–172. [PubMed: 19526482]
25. Pritzker KP, Gay S, Jimenez SA, Ostergaard K, Pelletier JP, Revell PA, Salter D, van den Berg WB. Osteoarthritis cartilage histopathology: grading and staging. *Osteoarthritis Cartilage.* 2006; 14:13–29. [PubMed: 16242352]

26. Samuels J, Krasnokutsky S, Abramson SB. Osteoarthritis: a tale of three tissues. *Bull NYU Hosp Jt Dis.* 2008; 66:244–250. [PubMed: 18937640]
27. Kiefer JC. Epigenetics in development. *Dev Dyn.* 2007; 236:1144–1156. [PubMed: 17304537]
28. Kouzarides T. Chromatin modifications and their function. *Cell.* 2007; 128:693–705. [PubMed: 17320507]
29. Stasko SE, Wagner GF. Possible roles for stanniocalcin during early skeletal patterning and joint formation in the mouse. *J Endocrinol.* 2001; 171:237–248. [PubMed: 11691643]
30. Wang J, Zhou HY, Salih E, Xu L, Wunderlich L, Gu X, JG H, Torres M, Glimcher MJ. Site-specific in vivo calcification and osteogenesis stimulated by bone sialoprotein. *Calcif Tissue Int.* 2006; 79:179–189. [PubMed: 16969594]
31. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2- $\Delta\Delta C_t$ Method. *Methods.* 2001; 25:402–408. [PubMed: 11846609]
32. Llano M, Gaznick N, Poeschla EM. Rapid, controlled and intensive lentiviral vector-based RNAi. *Methods Mol Biol.* 2009; 485:257–270. [PubMed: 19020831]
33. Im H, Grass JA, Johnson KD, Boyer ME, Wu J, Bresnick EH. Measurement of protein-DNA interactions in vivo by chromatin immunoprecipitation. *Methods Mol Biol.* 2004; 284:129–146. [PubMed: 15173613]
34. Glasson SS, Askew R, Sheppard B, Carito B, Blanchet T, Ma HL, Flannery CR, Peluso D, Kanki K, Yang Z, Majumdar MK, Morris EA. Deletion of active ADAMTS5 prevents cartilage degradation in a murine model of osteoarthritis. *Nature.* 2005; 434:644–648. [PubMed: 15800624]
35. Burrage P, Mix K, Brinckerhoff C. Matrix metalloproteinases: role in arthritis. *Front Biosci.* 2006; 11:529–543. [PubMed: 16146751]
36. Li Y, Cui Y, Hart SN, Klaassen CD, Zhong XB. Dynamic patterns of histone methylation are associated with ontogenic expression of the Cyp3a genes during mouse liver maturation. *Mol Pharmacol.* 2009; 75:1171–1179. [PubMed: 19188337]
37. Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature.* 2007; 447:425–432. [PubMed: 17522676]
38. Berger SL. The complex language of chromatin regulation during transcription. *Nature.* 2007; 447:407–412. [PubMed: 17522673]
39. Margueron R, Trojer P, Reinberg D. The key to development: interpreting the histone code? *Curr Opin Genet Dev.* 2005; 15:163–176. [PubMed: 15797199]
40. Schones DE, Cui K, Cuddapah S, Roh TY, Barski A, Wang Z, Wei G, Zhao K. Dynamic regulation of nucleosome positioning in the human genome. *Cell.* 2008; 132:887–898. [PubMed: 18329373]
41. Liu C, Maejima T, Wyler SC, Casadesus G, Herlitze S, Deneris ES. Pet-1 is required across different stages of life to regulate serotonergic function. *Nat Neurosci.* 2010; 13:1190–1198. [PubMed: 20818386]
42. Shi Y, Lan F, Matson C, Mulligan P, Whetstone JR, Cole PA, Casero RA, Shi Y. Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell.* 2004; 119:941–953. [PubMed: 15620353]
43. Yamane K, Toumazou C, Tsukada Y, Erdjument-Bromage H, Tempst P, Wong J, Zhang Y. JHDM2A, a JmJC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell.* 2006; 125:483–495. [PubMed: 16603237]
44. Takeda S, Bonnamy J, Owen MJ, Ducy M, Karsenty G. Continuous expression of Cbfa1 in nonhypertrophic chondrocytes uncovers its ability to induce hypertrophic chondrocyte differentiation and partially rescues Cbfa1-deficient mice. *Genes Dev.* 2001; 15:467–481. [PubMed: 11230154]
45. Zhu M, Chen M, Zuscik M, Wu Q, Wang YJ, Rosier RN, O'Keefe RJ, Chen D. Inhibition of beta-catenin signaling in articular chondrocytes results in articular cartilage destruction. *Arthritis Rheum.* 2008; 58:2053–2064. [PubMed: 18576323]
46. Roach HI, Yamada N, Cheung KS, Tilley S, Clarke NM, Oreffo RO, Kokubun S, Bronner F. Association between the abnormal expression of matrix-degrading enzymes by human osteoarthritic chondrocytes and demethylation of specific CpG sites in the promoter regions. *Arthritis & Rheumatism.* 2005; 52:3110–3124. [PubMed: 16200590]

47. Hashimoto K, Oreffo RO, Gibson MB, Goldring MB, Roach HI. DNA demethylation at specific CpG sites in the IL1B promoter in response to inflammatory cytokines in human articular chondrocytes. *Arthritis Rheum.* 2009; 60:3303–3313. [PubMed: 19877066]
48. Bour-Jordan H, Grogan JL, Tang Q, Auger JA, Locksley RM, Bluestone JA. CTLA-4 regulates the requirement for cytokine-induced signals in T(H)2 lineage commitment. *Nat Immunol.* 2003; 4:182–188. [PubMed: 12524538]

Figure 1. *Nfat1*^{-/-} articular chondrocytes do not display OA-like cellular dysfunction until the young adult stage. Photomicrographs were stained with Safranin-O and fast green. Scale bar = 200 μ m for all photomicrographs. (A) At the E16.5 embryonic stage, developing *Nfat1*^{-/-} hip, knee, and shoulder joints display no morphological abnormalities compared to WT joints. Cartilage is stained in red with Safranin-O. (B) At postnatal 1 day, skeletal tissues stained with Alcian blue and Alizarin red show no morphological abnormalities in *Nfat1*^{-/-} mice. (C) At 1 month of age (1m), both WT and *Nfat1*^{-/-} hip joints display normal development. Cellular and extracellular matrix (ECM) morphology of WT and *Nfat1*^{-/-} articular cartilage with Safranin-O staining (red) is presented in magnified square on right. (D) Photomicrographs of articular cartilage harvested from femoral heads of WT and *Nfat1*^{-/-} mice at age 2 months (2m) show loss of Safranin-O staining (red) in the superficial zone (arrow heads) of *Nfat1*^{-/-} articular cartilage. The boxed regions are magnified to show cellular and ECM morphology of WT and *Nfat1*^{-/-} articular cartilage. The cartilage tissue above the dotted line is articular cartilage used for isolation of RNA and cartilage cells, and

the tissue underneath the dotted line is cartilaginous/bony tissue which is removed and discarded during microdissection. **(E)** Articular cartilage harvested from the femoral head of a 4-month (4m) WT mouse shows normal articular surface and chondrocyte morphology with rich Safranin-O staining (red). In contrast, a 4-month *Nfat1*^{-/-} femoral head shows articular surface fibrillation (arrow heads) with focal loss of Safranin-O staining and chondrocyte clustering (arrows), a typical feature of OA. **(F–G)** qPCR analyses demonstrate temporal changes in expression of mRNA for chondrocyte markers **(F)** as well as specific proteinases and cytokines **(G)** in *Nfat1*^{-/-} articular chondrocytes. The expression level of each WT group has been normalized to 1.0. 1d = postnatal 1 day. n = 3 pooled RNA samples, each prepared from the articular cartilage of six femoral heads. * $p < 0.05$; ** $p < 0.01$.

Figure 2.

Knockdown of *Nfat1* using a pool of two *Nfat1* RNAi lentiviral constructs causes dysfunction of cultivated adult (3-month-old) primary articular chondrocytes isolated from WT femoral head articular cartilage. (A) qPCR analyses show that *Nfat1* RNAi significantly reduces the expression of mRNA for *Nfat1*, but not *Nfat2-4*, confirming the specificity of *Nfat1* RNAi sequences used in this study. $n = 3$ pooled RNA samples, *** $p < 0.001$. (B) Western blots using *Nfat1* antibody (Ab), with Gapdh Ab as a loading control, confirm that knockdown of *Nfat1* expression by RNAi substantially reduces the synthesis of *Nfat1* protein in articular chondrocytes. Note, two bands are detected in the scrambled control blot, which represent phosphorylated and dephosphorylated *Nfat1* protein, consistent with previously published data by other investigators (21,48). Negative control represents a control blot using a protein sample from the scrambled control group but without using primary Ab against *Nfat1*. (C) qPCR analyses indicate that *Nfat1* RNAi significantly alters the expression levels of *Acan*, *Col2a1*, and *Col11a1*, as well as specific inflammatory cytokines and proteinases. $n = 3$ pooled RNA samples, * $p < 0.05$, ** $p < 0.01$.

Figure 3.

Age-dependent expression of *Nfat1* in articular chondrocytes. **(A)** Temporal changes of *Nfat1* mRNA in articular chondrocytes of WT mice determined by qPCR. $n = 3$ pooled RNA samples; each prepared from the articular cartilage of six femoral heads. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. **(B)** Immunohistochemistry (IHC) using a specific antibody against mouse *Nfat1* (Santa Cruz) with hematoxylin counterstaining demonstrates that intracellular or intranuclear *Nfat1* protein expression in WT articular chondrocytes was undetectable at E16.5 (the box covers the developing joint space of the hip), but highly expressed (brown) at 6 months (6m). **(C)** IHC using a specific *Nfat1* antibody indicates an absence of *Nfat1* protein expression in *Nfat1*^{-/-} chondrocytes within the degradative femoral head articular cartilage of a 6-month old *Nfat1*^{-/-} mouse, which display pathological features of OA including articular surface fibrillation (arrowheads) and fissuring (arrow). Scale bar = 200 μm for **B–C**.

Figure 4.

ChIP assays demonstrate age-dependent histone modifications specific to the *Nfat1* gene. (A) Genomic structure of the *Nfat1* gene, location, and sequences of three different qPCR primers (P1–3) designed from the sequences around the transcription start site (TSS, +1) of the *Nfat1* gene, as well as negative control primer sequences (P-ctl) designed from the promoter region without any histone modifications. (B–C) qPCR analyses of co-immunoprecipitated *Nfat1* DNA level, which reflects the immunoprecipitation level of histone modifications interacting with *Nfat1* DNA during the ChIP reaction, show an age-dependent increase in H3K4me2 recruitment (B) and an age-related decrease in H3K9me2

recruitment around the TSS of *Nfat1* DNA (C). Top panels of (B) and (C) show qPCR products amplified from the four different primers (P1–3 and P-ctl) at three different ages. Bottom panels of (B) and (C) show the levels of qPCR amplification from all four different primers at 2 months (2m) and 6 months (6m) relative to E16.5. The binding level of the E16.5 group has been normalized to “1.0” in each panel. n = 3; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 5.

Knockdown of Lsd1 using Lsd1 shRNA lentiviral particles (Santa Cruz) in E16.5 articular chondrocytes results in an upregulation of *Nfat1* expression through increased H3K4me2 at the *Nfat1* promoter. (A) qPCR analyses (left panel) and Western blots (right panel) confirm the efficiency of Lsd1 knockdown in Lsd1 RNAi-treated E16.5 articular chondrocytes. “Neg ctrl” represents a negative control blot using a protein sample from the scrambled control group but without using primary antibody against Lsd1. (B) ChIP assays using an anti-H3K4me2 antibody with qPCR analyses of co-immunoprecipitated *Nfat1* DNA using three different primers (P1–3) designed from the sequences around the TSS of the *Nfat1* gene. Lsd1 RNAi up-regulates H3K4me2 level in the *Nfat1* promoter region, which is detected by P1–3 primers, but not by Untr17 negative control primers (data not shown). (C) qPCR shows that RNAi-mediated knockdown of Lsd1 up-regulates *Nfat1* expression in E16.5 articular chondrocytes. The qPCR level of each control group has been normalized to “1.0” in A–C. n = 3; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (D) A schema for Lsd1-mediated epigenetic regulation of *Nfat1* transcription.

Figure 6.

Knockdown of Jhdm2a using Jhdm2a shRNA lentiviral particles (Santa Cruz) in 6-month (6m) articular chondrocytes causes decreased Nfat1 expression through increased H3K9me2 at the Nfat1 promoter. **(A)** qPCR analyses (left panel) and Western blots (right panel) confirm the efficiency of Jhdm2a knockdown in Jhdm2a RNAi-treated 6m articular chondrocytes. “Neg ctrl” represents a negative control blot using a protein sample from the scrambled control group but without using primary antibody against Jhdm2a. **(B)** ChIP assays using an anti-H3K9me2 antibody with qPCR analyses of co-immunoprecipitated *Nfat1* DNA using three different primers (P1–3) designed from the sequences around the TSS of the *Nfat1* gene. Jhdm2a RNAi up-regulates H3K9me2 level in the *Nfat1* promoter region, which is detected by P1–3 primers, but not by Untr17 negative control primers (data not shown). **(C)** qPCR shows that RNAi-mediated knockdown of Jhdm2a down-regulates Nfat1 expression in 6m articular chondrocytes. The qPCR level of each control group has been normalized to “1.0” in A–C. $n = 3$; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. **(D)** A schema for Jhdm2a-mediated epigenetic regulation of *Nfat1* transcription.

Table 1

Specific primers used for quantitative real-time RT-PCR

Gene	Forward	Reverse	GeneBank accession no.
<i>Nfat1</i>	GTGCAGCTCCACGGCTACAT	GCGGCTTAAGGATCCTCTCA	NM_010899
<i>Mmp1a</i>	CCTCGTTGGACCAAAACACA	GCGATGGCATCTTCCACAA	NM_032006
<i>Mmp3</i>	TCCTGATGTTGGTGGCTTCA	CACACTCTGTCTTGGCAAATCC	NM_010809
<i>Mmp9</i>	GCCTCAAGTGGGACCATCAT	CTCGCGGCAAGTCTTCAGA	NM_010809
<i>Mmp13</i>	TCACCTGATTCTTGCGTGCTA	CAGATGGACCCCATGTTTGC	NM_008607
<i>Timp3</i>	CGACCCTTGGCCACTTAGTC	CTGCCGCTCTTTTCTTCAAAG	NM_011595
<i>Adamts4</i>	ACCCGGCAGGACCTGTGT	CCAGTTCATGAGCAGCAGTGA	NM_172845
<i>Adamts5</i>	GCTGCTGGTAGCATCGTTACTG	GAGTGTAGCGCGCATGCTT	NM_011782
<i>Acan</i>	TGGGATCTACCGCTGTGAAGT	CTCGTCCTTGTCACCATAGCAA	NM_007424
<i>Col2a1</i>	CGAGATCCCCTTCGGAGAGT	TGAGCCGCGAAGTTCTTTTC	NM_031163
<i>Col9a1</i>	TCTTAAGCGTCGTGCAAGATTTT	CTTGGGACACAGTTCACCTCCA	NM_007740
<i>Col10a1</i>	TTATGCTGAACGGTACCAAACG	TGGCGTATGGGATGAAGTATT	NM_009925
<i>Col11a1</i>	CACAAAACCCCTCGATAGAAGTG	CCTGTGATCAGGAAGTCTGAA	NM_007729
<i>IL1b</i>	GCTTCCTTGTCGAAGTGCTGA	TCAAAAGGTGGCATTTCACAGT	NM_008361
<i>IL6</i>	TCGGAGGCTTAATTACACATGTTC	TGCCATTGCACAACCTTTTCT	NM_031168
<i>IL17a</i>	TCTGTGTCTCTGATGCTGTTGCT	TCGCTGCTGCCTTCACTGT	NM_010552
<i>IL18</i>	CTCTTGCGTCAACTTCAAGGAA	GTGAAGTCGGCCAAAGTTGTC	NM_008360
<i>Tnfa</i>	AGGGATGAGAAGTCCCAAATG	GGCTTGTCACCTCGAATTTTGAGA	NM_013693
<i>Lsd1</i>	GCAGCCTGTTTCCCAGACA	CTGCAATGTGCGATTCTGAT	NM_133872
<i>Jhdm2a</i>	TGGAAACCATCATCCAAAAC	ACCACATGTCACAAAGTTGTCATG	NM_173001