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■ OSTEOARTHRITIS

NFAT1 protects articular cartilage against osteoarthritic degradation by directly regulating transcription of specific anabolic and catabolic genes

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Objectives

Adult mice lacking the transcription factor NFAT1 exhibit osteoarthritis (OA). The precise molecular mechanism for NFAT1 deficiency-induced osteoarthritic cartilage degradation remains to be clarified. This study aimed to investigate if NFAT1 protects articular cartilage (AC) against OA by directly regulating the transcription of specific catabolic and anabolic genes in articular chondrocytes.

Methods

Through a combined approach of gene expression analysis and web-based searching of NFAT1 binding sequences, 25 candidate target genes that displayed aberrant expression in $Nfat1^{-/-}$ AC at the initiation stage of OA, and possessed at least four NFAT1 binding sites in the promoter of each gene, were selected and tested for NFAT1 transcriptional activities by chromatin immunoprecipitation (ChIP) and promoter luciferase reporter assays using chondrocytes isolated from the AC of three- to four-month-old wild-type mice or $Nfat1^{-/-}$ mice with early OA phenotype.

Results

Chromatin immunoprecipitation assays revealed that NFAT1 bound directly to the promoter of 21 of the 25 tested genes encoding cartilage-matrix proteins, growth factors, inflammatory cytokines, matrix-degrading proteinases, and specific transcription factors. Promoter luciferase reporter assays of representative anabolic and catabolic genes demonstrated that NFAT1-DNA binding functionally regulated the luciferase activity of specific target genes in wild-type chondrocytes, but not in $Nfat1^{-/-}$ chondrocytes or in wild-type chondrocytes transfected with plasmids containing mutated NFAT1 binding sequences.

Conclusion

NFAT1 protects AC against degradation by directly regulating the transcription of target genes in articular chondrocytes. NFAT1 deficiency causes defective transcription of specific anabolic and catabolic genes in articular chondrocytes, leading to increased matrix catabolism and osteoarthritic cartilage degradation.

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Article focus

■ This study focuses on the molecular mechanism for transcription factor NFAT1 deficiency-induced cartilage degradation during the initiation stage of osteoarthritis (OA) in mice.

Key messages

■ By using a combined approach of quantitative gene expression, chromatin immuno-precipitation, and luciferase reporter assays, this study has identified 21 transcriptional targets of NFAT1 in articular chondrocytes. NFAT1 protects articular cartilage (AC) against degradation by directly regulating the transcription of its target genes. NFAT1 deficiency causes defective transcription of specific anabolic and catabolic genes in articular chondrocytes, triggering increased matrix catabolism

and cartilage degradation during the initiation stage of OA.

Strengths and limitations

- This study reports for the first time that NFAT1 binds directly to 21 target genes and regulates their promoter activity and transcription in articular chondrocytes, thereby maintaining the balance of anabolic and catabolic activities in AC. Therefore, NFAT1 could be a more promising target for OA therapy than other anti-OA drug candidates that target a single anabolic or catabolic molecule.
- A limitation of this study is that potential NFAT1 target genes in other joint tissues are not examined. Future investigations on the regulatory role of NFAT1 in other joint tissues, particularly the synovium, would further improve our understanding of the pathogenic mechanisms of OA.

Introduction

Nuclear factor of activated T cells (NFAT/*Nfat*) is a family of transcription factors originally identified in T cells. The family consists of five members: NFAT1 (NFATc2/NFATp), NFAT2 (NFATc/NFATc1), NFAT3 (NFATc4), NFAT4 (NFATc3/NFATx), and NFAT5. Except for NFAT5, which is essential for cellular response to hypertonic stress, the remaining four canonical members (NFAT1 to NFAT4) are activated by calcium-calcineurin signalling and play important roles in regulating gene transcription in lymphocytes and in progenitor cells of multiple lineages, including osteoblastic and osteoclastic lineage cells.[1-4]

Early studies identified NFAT1 as a major regulator of immune response, as *Nfat1*^{-/-} mice with a targeted deletion of the Rel homology binding domain in the *Nfat1* gene showed enhanced immune response and dysregulation of interleukin-4 (IL-4) expression.[5,6] Our recent studies revealed that *Nfat1*^{-/-} mice exhibited normal skeletal development but began to show articular chondrocyte dysfunction and early osteoarthritis (OA) changes as young adults.[7,8] The OA phenotypes of *Nfat1*^{-/-} mice were involved in articular cartilage (AC), synovium, and subchondral bone, similar to the changes seen in human OA.[9] More recently, Greenblatt et al[10] reported that mice lacking NFAT2 (NFATc1) in cartilage displayed no evidence of OA and were no more susceptible to post-traumatic OA than wild-type littermates after surgical destabilization of the medial meniscus. However, mice lacking both NFAT1 and NFAT2 in cartilage developed early joint instability as manifested by dislocation of the elbow at one week of age and subluxation of the tarsus at three weeks of age, followed by OA-like changes.[10] Moreover, *Nfat1*^{-/-} mice survived well postnatally, while *Nfat2*^{-/-} mouse embryos died during embryogenesis due to developmental defects in cardiac morphogenesis.[11,12] These findings suggest that NFAT1 deficiency does not cause obvious developmental defects, while NFAT2 deficiency results in severe developmental defects in both cardiac and skeletal systems.[13] The data further demonstrate that NFAT1 is more important than NFAT2 for cartilage homeostasis and prevention of OA in adult mice.

To explore the mechanisms by which *Nfat1*^{-/-} mice exhibited normal skeletal development but began to show early OA changes (e.g. articular surface roughening or fissuring) as young adults, we examined age-dependent NFAT1 expression. Expression of NFAT1 in murine articular chondrocytes was essentially undetectable at embryonic day 16.5 (E16.5) and postnatal day 1, but was highly expressed at the young adult stage and declined as the mice aged.[8,14] This finding suggests that NFAT1 is not required for the development of skeletal tissues including AC; thus, its deletion does not cause dysfunction of articular chondrocytes during development. In contrast, high expression of NFAT1 in articular chondrocytes of young adult mice suggests that NFAT1 is required for maintaining the chondrocyte function in adults; thus, lack of NFAT1 in adult mice causes severe dysfunction of articular chondrocytes and the subsequent OA with abnormal expression of a large number of genes in AC and synovium.[7,8] However, whether these genes are directly or indirectly regulated by NFAT1 in AC remains unclear.

The present study aimed to investigate whether NFAT1 directly binds to and regulates the transcription of specific catabolic and anabolic genes that are aberrantly expressed in NFAT1-deficient AC during the initiation stage of OA. We chose to address this research question by chromatin immunoprecipitation (ChIP) and promoter reporter luciferase assays instead of by genome-wide ChIP sequencing (ChIP-seq)[15,16] for the following reasons: 1) a very large number of genes have already been identified as being involved in OA pathogenesis in animal models and in humans,[8,17,18] but little is known as to which genes are directly bound and regulated by NFAT1; 2) ChIP-seq grade anti-NFAT1 antibodies are currently unavailable; and 3) although Giatromanolaki et al[19] obtained some ChIP-seq data by using a ChIP grade NFAT1 antibody from Abcam (Cambridge, Massachusetts) in cultured mouse T cells,[19] our preliminary ChIP-seq experiments using ChIP grade NFAT1 antibodies and mouse AC tissue did not result in conclusive results due to high background noise and low specificity.

Materials and Methods

Animals. Both sexes of two- to four-month-old *Nfat1*^{-/-} mice on BALB/c background and their wild-type (WT) BALB/c littermates were used for various analyses. The original breeder pairs of *Nfat1*^{-/-} mice were a gift from Dr Laurie Glimcher (Harvard University, Cambridge, Massachusetts). The methods for generation of the original *Nfat1*^{-/-} mice have been described previously.[6] All animal procedures were approved by the Institutional

Table I. qPCR primers for gene expression assay

Gene	Primer (forward)	Primer (reverse)	GenBank no.
Adamts4	ACCCGGCAGGACCTGTGT	CCAGTTCATGAGCAGCAGTGA	NM_172845
Bmp2	TGTTTGGCCTGAAGCAGAGA	GCCTGCGGTACAGATCTAGCA	NM_007553
Bmp3	CGACAAGGTGTCGGAGCATA	TCCGGGTAGCCTGGACTCTA	NM_001310677.2
Bmp3b	CAGAGCCATGCTGTGTTCCA	ACCGCATTCCGATTTTCATC	NM_145741.3
Bmp6	GCTGAAGTCCGCTCCACTCT	CCCATCCTCTTCGTCGTCAT	NM_007556.3
Bmp8a	TGGTCTGCTTGGACGACAAG	GCTGGCCCTGAAGAAGGTTA	NM_007558.3
Bmp9	CAGCCACTGCCAGAAGACTTC	CCTTGGGTGCAATGATCCA	AF188286
Bmp15	ACGATTGGAGCGAAAATGGT	ACGATTGGAGCGAAAATGGT	NM_009757
Gdf5	AACAGCAGCGTGAAGTTGGA	GGTCATCTTGCCCTTTGTCAA	NM_008109.3
Hif1a	TGGATTACCACAGCTGACCAGT	AGGTTTCTGCTGCCTTGTATGG	NM_010431.1
Il4	CGGATGCGACAAAAATCACTT	CCCTTCTCCTGTGACCTCGTT	NM_021283
Il10	GGACCAGCTGGACAACATACTG	ACCAGGTAAAACTGGATCATTTCC	NM_010548
Il13	TTGAGGAGCTGAGCAACATCAC	GCGGCCAGGTCCACACT	NM_008355
Il18	CTCTTGCGTCAACTTCAAGGAA	GTGAAGTCGGCCAAAGTTGTC	NM_008360.1
Nog	TGGTGGACCTCATCGAACATC	CAGCGTCTCGTTCAGATCCTT	NM_008711
Timp3	CGACCCTTGGCCACTTAGTC	CTGCCGCTCTTTTCTTCAAAG	NM_011595.2
Gapdh	AGGTTGTCTCCTGCGACTTCA	CCAGGAAATGAGCTTGACAAAG	NM_008084

Animal Care and Use Committee at the University of Kansas Medical Center in compliance with federal and state laws and regulations.

Histology and histochemistry. After euthanasia of the animals, the hip, knee, and shoulder joints of WT and *Nfat1*^{-/-} mice were harvested and fixed in 2% paraformaldehyde, decalcified in 25% formic acid, embedded in paraffin, and sectioned for various stains. Safranin-O and fast green stains were used to identify chondrocytes and cartilage matrices. Haematoxylin and eosin (H&E)-stained sections were used for evaluation of other joint structures and tissues. Ten mice per strain/gender were examined at two, three, and four months of age to confirm the penetrance of early OA phenotype in each gender of *Nfat1*^{-/-} mice.

Collection of articular cartilage. Because *Nfat1*^{-/-} mice began to show OA changes first in the hip joints where AC of the femoral heads was much thicker than AC in other joints, the femoral head AC was collected from WT and *Nfat1*^{-/-} mice by microdissection for isolation of RNA, chromatins, and chondrocytes. The purity of collected AC and the bioactivity of isolated articular chondrocytes have been confirmed and described in a previous study.[8]

Quantitative real-time reverse transcription-polymerase chain reaction (qPCR). Total RNA was isolated from femoral head AC using TRIzol reagent (Invitrogen, Waltham, Massachusetts). Complementary DNA (cDNA) was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, Massachusetts). Quantitative real-time reverse transcription-polymerase chain reactions were performed in triplicate using a 7500 Real-Time qPCR system and SYBR Green reagents (Applied Biosystems, Waltham, Massachusetts). Specific primers used for qPCR gene expression analyses are listed in Table I (see supplementary Table i for a full list of gene names). *Gapdh* expression levels were used as internal controls. Gene expression levels were relatively quantified using $2^{-\Delta\Delta Ct}$ methods as described previously.[7,20]

Web-based searching of putative NFAT1 binding DNA motifs in candidate genes. Mouse genome DNA sequences in the promoter region of the genes of interest were exported from Ensembl.[21] The putative NFAT1 binding consensus core DNA sequences (5'-TGAAAAT-3', 5'-GAAATTC-3', 5'-GGAAAAA-3', and 5'-GGAAA-3') were used to locate the NFAT1 binding sites in the promoter region of the candidate genes.[22,23]

Chromatin immunoprecipitation (ChIP) assay. Chromatins were prepared directly from femoral head articular cartilage tissue and crosslinked with 1% formaldehyde. Chromatin immunoprecipitation assays were performed according to the manual for the Magna ChIP A/G kit (Millipore, Burlington, Massachusetts) using a ChIP grade NFAT1 antibody (sc-13034; Santa Cruz Biotechnology, Dallas, Texas). An absolute qPCR method was used to quantify the amount of DNA purified from samples of NFAT1-ChIP and IgG-ChIP, and input with the specific primers in the promoter encompassing NFAT1 binding sites of the candidate target genes. Normal mouse IgG and a pair of primers without NFAT1 binding sequences served as negative controls to verify the ChIP specificity (Table II).

Gel electrophoresis and image analysis. Polymerase chain reaction products of ChIPed DNA samples were separated on 1.5% agarose gels in Tris-acetate-EDTA (TAE) buffer stained with ethidium bromide. Gel images were acquired using the FOTO/Analyst Apprentice System (Heartland, Wisconsin) and the intensity of the bands was quantified using ImageJ software from National Institutes of Health (Bethesda, Maryland).

Promoter luciferase reporter assay. Articular chondrocytes were isolated from pooled femoral head AC samples of WT or *Nfat1*^{-/-} mice using collagenase D (1.5 mg/ml; Roche Diagnostics, Indianapolis, Indiana) and cultivated in Dulbecco's Modified Eagle Medium (DMEM)/F12 medium in a humidified incubator with 5% CO_2 at

Table II. qPCR primers for ChIP assay

Gene	Primers (forward)	Primers (reverse)	NCBI[†] Gene ID
Acan	AAATCACTGTATGCTGCAACTAGC	GCCTGCCCTCTACAACACTC	11595
Adamts4	GCCTCTGGGGTAAAGGGTAG	GGGAGAATTCAGGTCCTTCC	240913
Adamts5	GACCGACTGGAGAGAACCAG	GCTCTGGCCTTGTAGACCTG	23794
Bmp2	GTGGGTAGCCAGTGGTGGTA	TGCTGAGCCAGGATTCTTTT	12156
Bmp7	CGCAGGGCCCTTGTATTG	CGAACCCTAGCTCCCTCCT	12162
Col2a1	ATGGGTTAGGGAGGCTGTG	GGGGACAGTTTTCCAGACCT	12824
Col9a1	CTCTGGTTAAATCCCCCACA	CTGAAGGAGGGACTGAATGG	12839
Col10a1	TCATTCCATCATGAACCAACA	TTAATAGCTTCGGCTGTGCAT	12813
Col11a1	TGCTTCTCCCTTTCATCCAG	TCACTGAGGGGCTTGTCTTG	12814
Ctnnb1	CCACTGGTAGGGCTTTGGTA	TCGGATTTCCGGAGAGTAAAA	12387
Gdf5	AAAACTGGAGCC=GCACAGG	TTTCTCCCGCACAACTGACT	14563
Hif1a	GACAGGGACACTCACTGAACC	GGCAAGGTGAACAACACCAT	15251
Igf1	TCTGTCTCCACCGATGTGTC	TCTCTCCAGGTGTGCTTTCC	16000
Il1b	TCCCCTAAGAATTCCCATCA	GCTGTGAAATTTTCCCTTGG	16176
Il4	GGGAGGGGTGTTTCATTTTC	GTTATTCTGGGCCAATCAGC	16189
Il6	CCCACCCTCCAACAAAGATT	GCTCCAGAGCAGAATGAGCTA	16193
Il10	GCAGAAGTTCATTCCGACCA	GGCTCCTCCTCCCTCTTCTA	16153
Il13	CTTTAGCGGCCACTGGATT	ACCCCTGGTCTCTGCTTTGT	16163
Il17a	CATGCAACCGTAATGACTTCAC	ACCGCAACTGTTTTTCCT	16171
Mmp13	CTGCTGCTTCTCCCCACTAT	GAGGGAAATGGAAAATGAAGG	17386
Nog	CCAAGCCCGAGAGAGAGAG	CATGTGCATGGAGCATTTTC	18121
Tgfb1	TGACACTCTCATCCGCAAAG	GGAAAAATTGGGACTTCGTG	21803
Timp1	GCTTCAGCCACAGGAAGTTT	CACAGTCATGCACAGTCCTTC	21857
Timp3	GCTGAGCTCTCAAGTTGTCCA	GGCTCCACAGAGAAACCTTG	21859
Tnfa	CCATGGAGAAGAAACCGAGA	CTCATTCAACCCTCGGAAAA	21926
NegPri*	TTTTCTCCTCCCAGGTCCTC	CTCTTAAGCACTGAGCCATC	11595

*The primer pair was designed in *Acan* gene body with no NFAT1 binding site.
†NCBI, National Center for Biotechnology Information, USA

37°C in monolayer. Because primary chondrocytes (P0) displayed an uneven growth rate and cell density, passage 1 (P1) of cultured articular chondrocytes was used for plasmid transfection and luciferase assay.

The DNA fragments in the promoter region of the genes of interest containing the putative NFAT1 binding sequences were cloned into the multiple cloning site of pGL3 firefly luciferase vector (Promega, Madison, Wisconsin). The DNA fragments containing mutated putative NFAT1 binding consensus core DNA sequences (GGAAA to GCATA) were synthesized and cloned into pGL3 vector by Genewiz (South Plainfield, New Jersey). Passage 1 chondrocytes were cultured in 24-well plates and used for transfection of luciferase reporter plasmids with Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, Massachusetts). Empty pGL3 vector was used as a negative control. Renilla luciferase expression plasmid (Promega) was co-transfected to normalize the transfection efficiency. Luciferase activity was read on a GloMax luminometer (Promega).

Statistics. Quantitative data are presented as mean and standard deviation from at least three independent repeats for each experimental condition. Microsoft Excel 2010 software (Seattle, Washington) was used for statistical analysis. The differences between two experimental groups were determined by Student's *t*-tests. The differences between three or more groups were assessed by analysis of variance (ANOVA) followed by a *post-hoc* test (Tukey). A p-value of less than 0.05 was considered statistically significant.

Results

Histopathological analysis of penetrance of early osteoarthritis phenotype. To determine when and where to collect AC samples with early OA changes for quantitative assays, we examined the penetrance of early OA phenotype in hip, knee, and shoulder joints (major synovial joints) of male and female *Nfat1*-/- mice at two to four months of age. Age-matched WT hip, knee, and shoulder joints were used as controls. At two months of age, a focal loss of proteoglycans (stained by Safranin-O) was first seen in female *Nfat1*-/- hip joints (in 4/10 mice) without structural changes. Early OA phenotype (e.g. loss of proteoglycans in the AC, articular surface fibrillations, chondrocyte clustering) was observed in female *Nfat1*-/- femoral head AC at three months in four out of ten mice, and more frequently at four months in eight out of ten mice. Early OA phenotype was less frequently observed in male *Nfat1*-/- mice (1/10 at three months and 4/10 at four months) and in none of the *Nfat1*-/- knees and shoulders at the same age points. Some *Nfat1*-/- hips displayed pathological cartilage formation in the joint margin and synovium at three to four months, but subchondral bone changes were not observed at this stage. OA-like changes were not seen in any WT joints. The representative photomicrographs of hip, knee, and shoulder joints
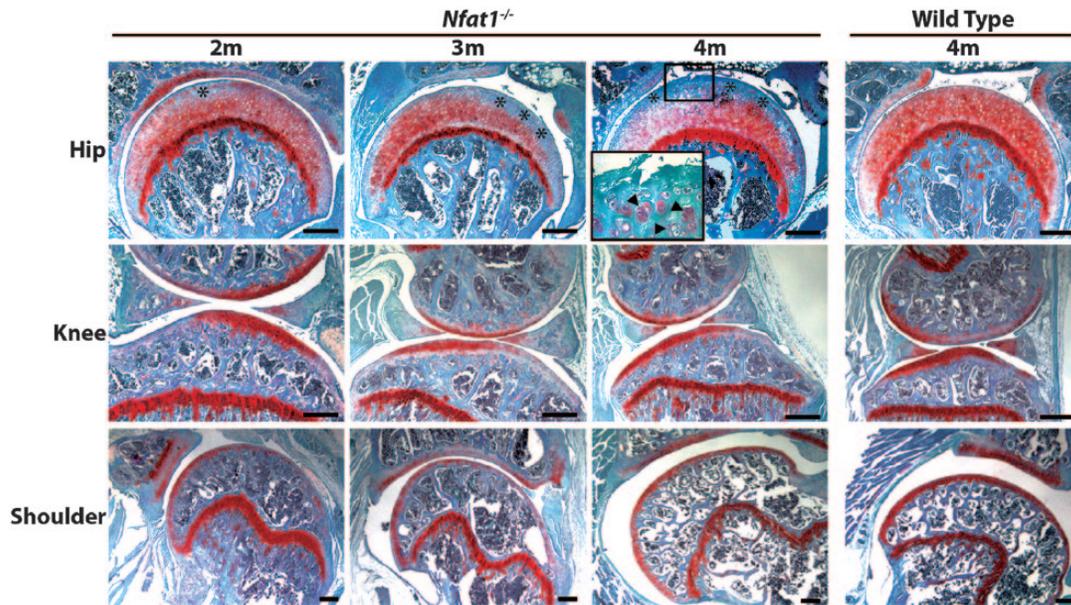
Fig. 1

Representative photomicrographs of hip, knee, and shoulder joints of two-, three-, and four-month-old female *Nfat1*^{-/-} mice and four-month-old female wild-type (WT) mice showing that the early osteoarthritis (OA) phenotype appears in the *Nfat1*^{-/-} hip (femoral head), but not in the *Nfat1*^{-/-} knees or shoulders. *indicates the area with loss of Safranin-O staining and arrowheads in the enlarged image from the rectangular box denote chondrocyte clustering (a typical feature of OA) in an *Nfat1*^{-/-} femoral head. Safranin-O and fast green stain, scale bar = 200 μm.

from two- to four-month-old female *Nfat1*^{-/-} mice and four-month-old female WT mice are presented in Figure 1. Since the early OA-like structural changes appear more frequently in female *Nfat1*^{-/-} femoral heads, femoral head cartilage (FH-cartilage) from female *Nfat1*^{-/-} and WT mice at three to four months were used for all quantitative assays in this study.

Selection of putative NFAT1 target genes. Our previous study revealed that some anabolic genes (e.g. *Acan, Col2a1, Col9a1, Col11a*, and *Igf1*) were downregulated and some catabolic genes (e.g. *Il1b, Il6, Il17a, Mmp1a, Mmp13*, and *Adamts5*) were upregulated in *Nfat1*^{-/-} FH-cartilage.[7] Tissue localizations of specific proteins, such as AGGRECAN, COL2A1, degraded AGGRECAN product (neo G1), and IL1-β in *Nfat1*^{-/-} FH-cartilage were also examined by Western blotting or immunohistochemistry.[7] In this study, we performed qPCR analysis to examine the expression of additional genes, which have been thought to be involved in skeletal tissue homeostasis or OA pathogenesis[24-26] but have not yet been tested in our previous report, in FH-cartilage of three- to four-month-old female *Nfat1*^{-/-} mice. Age-matched WT FH-cartilage samples were used as controls.

Expression levels of these additional genes in *Nfat1*^{-/-} FH-cartilage and the rates of change are presented in Figure 2. Some anabolic genes (e.g. *Bmp2, Bmp3b, Gdf5*, and *Timp3* (tissue inhibitor of metalloproteinases 3)) and anti-inflammatory cytokine genes (e.g. *Il4, Il10*, and *Il13*) were significantly decreased, while catabolic gene *Adamts4* was significantly increased in *Nfat1*^{-/-} FH-cartilage

at three to four months. Notably, the expression of *Hif1a*, a proposed anabolic gene important for chondrocyte viability, was significantly increased in *Nfat1*^{-/-} FH-cartilage (Fig 2a and 2b). This is consistent with previous reports showing increased expression levels of HIF1a in human OA cartilage samples.[9,19,27] On the other hand, HIF1a has recently been found to restrict the anabolic actions during bone formation in response to intermittent parathyroid hormone.[28] These studies suggest that HIF1a may play a dual role in skeletal tissue metabolism. Another interesting finding was that the expression of the *Nog* gene (encoding NOGGIN, BMP antagonist[29]) was decreased, while the expression of other BMP members (e.g. *Bmp2, Bmp3b*, and *Gdf5*) was also downregulated (Figs 2a and 2b). This could be explained as an effect of a negative feedback loop through which NOGGIN expression is either increased to abolish the bioactivity of BMPs when BMP expression is high, or decreased when BMP activity is reduced.[11,24] Together with the altered expression of 43 anabolic and catabolic genes in our previous report,[7,8] our data suggest that NFAT1 deficiency can cause aberrant expression of a large number of anabolic and catabolic genes, resulting in cartilage degradation and OA-like changes.

Next, we performed software-assisted web searches of the NFAT1 binding consensus core DNA sequences in the promoter region of putative NFAT1 target genes. As expected, we found a very large number of genes with NFAT1 binding sequences in their promoter regions. It was impossible to test all of them by ChIP assays. Thus,
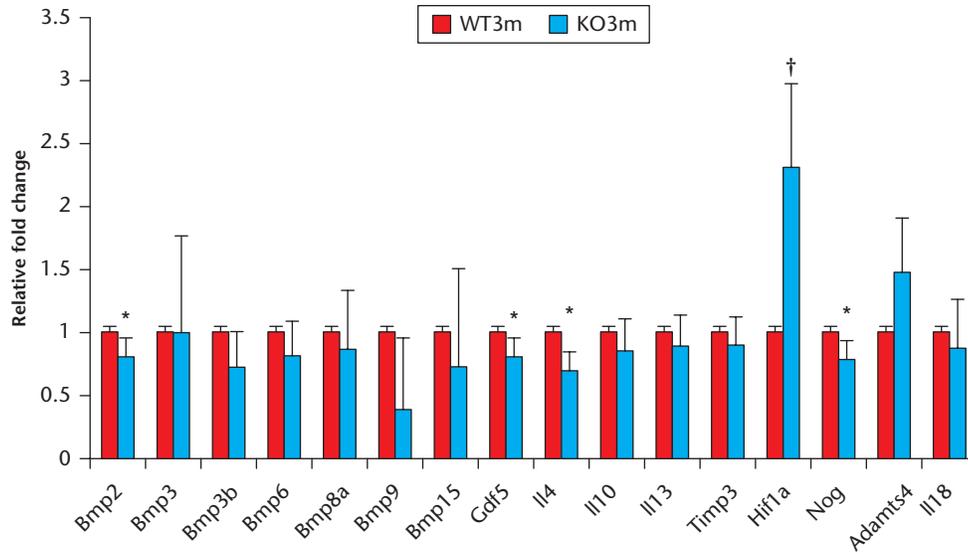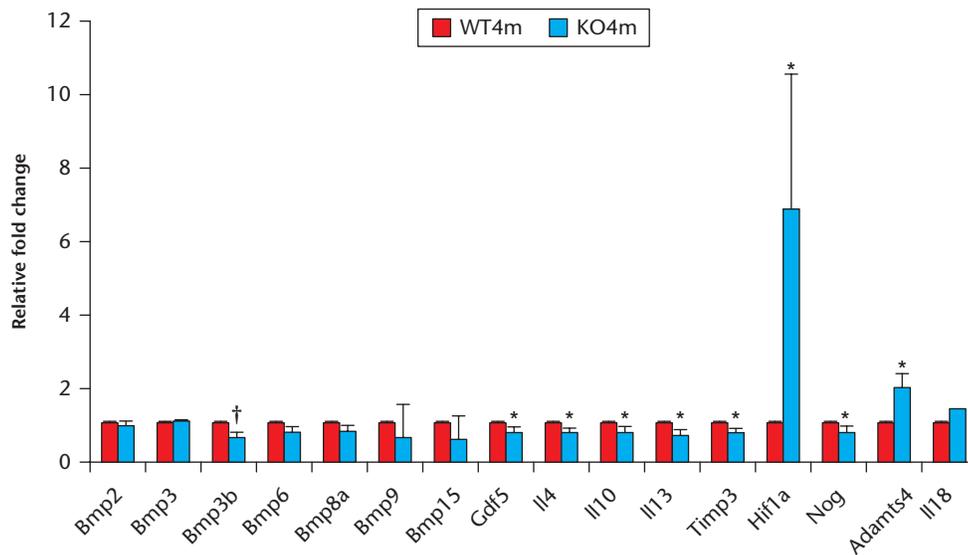
Fig. 2a

Fig. 2b

Quantitative polymerase chain reaction (qPCR) analyses demonstrate differential expression of mRNA for anabolic genes and catabolic genes in articular cartilage from a) three-month-old, and b) four-month-old wild-type (WT) or $Nfat1^{-/-}$ (KO) mice. The expression level of each WT group has been normalized to "1.0". n = 4 pooled RNA samples, each pooled sample prepared from the articular cartilage of six femoral heads (three mice). *p < 0.05 and †p < 0.01.

we selected 25 genes that met all three inclusion criteria: 1) having at least four NFAT1 binding sites in their promoters; 2) aberrantly expressed in the $Nfat1^{-/-}$ FH-cartilage during the initiation phase of OA as described in this article and in our previous publications; and 3) previously thought to be involved in AC homeostasis and OA pathogenesis in humans.[25,26] The NFAT1 binding sites of selected genes for ChIP assay, as well as their fold changes in mRNA expression, are presented in Table III.

NFAT1 directly binds to the promoter of its target genes. Transcription factors regulate gene expression by binding to specific DNA sequences in the promoter region of their target genes. We performed ChIP assays, followed by qPCR to quantify the binding enrichment of

NFAT1 to the promoter region of each candidate gene. As shown in Figure 3a to 3e, NFAT1 bound directly to the promoter region of the vast majority of the candidate genes (21/25) with different enrichment levels, which were significantly greater than the IgG control. The genes with positive ChIP binding can be generally classified into five categories: 1) cartilage matrix genes: *Acan, Col2a1, Col10a1*, and *Col11a1*; 2) growth factor genes: *Bmp7, Gdf5, Nog* (*Bmp* antagonist), *Igf1*, and *Tgfb1*; 3) pro- or anti-inflammatory cytokine genes: *Il1b, Il4, Il6, Il10, Il17a*, and *Tnfa*; 4) matrix-degrading proteinases and their inhibitor genes: *Adamts4, Adamts5, Mmp13*, and *Timp1*; and 5) transcription factor genes: *Ctnnb1* (encoding beta-catenin) and *Hif1a* (encoding hypoxia-inducible

Table III. Putative NFAT1 binding locations in the promoter region of its candidate genes in mice

Gene	Putative NFAT1 binding location[*]	Fold change (mRNA)[†]
Acan	-347, -505, -662, -774, -969, -1297, -1404	0.14
Adamts4	-168, -265, -301, -381, -774, -887, -1139, -1364	1.979
Adamts5	-122, -516, -552, -771, -854, -1099, -1132	2.467
Bmp2	-740, -831, -876, -1005, -1148, -1222, -1403	0.157
Bmp7	-540, -946, -1186, -1225, -1285	0.573
Col10a1	-162, −243, -253, -337, -426, -824,	2.217
Col11a1	-392, -712, -764, -820, -1420	0.24
Col2a1	-269, -475, -914, -928. -1122, -1176, -1235	0.159
Col9a1	-294, -427, -674, -1048	0.624
Ctnnb1	-88, -530, -940, -1340	2.065
Gdf5	-31, -80, -109, -234, -946, -970	0.157
Hif1a	-218, -629, -741, -1045,	6.24
Igf1	-268, -295, -310, -403, -512, -818	0.61
Il1b	-16, -69, -238, -252, -301, -664, -684, -755, -948, -1144, -1336	3.463
Il4	-54, -72, -157, -179, -237, -356, -432, 450, -634, -898, -1170, -1241	0.694
Il6	-173, -275, -289, -575, -640, -852,	3.239
Il10	-138, -144, -276, -404, -482, -1253, -1430	0.765
Il13	-130, -153, -200, -223, -920, -935, -982, -998, -1139	0.705
Il17a	-512, -685, -833, -1252	2.234
Mmp13	-140, −146, 219, -298, -363, -458, -916, -1127, -1163, -1180	3.642
Nog	-47, -257, -285, -396, -710, -858, -906, -1472	0.756
Tgfb1	-284, -1144, -1153, -1410	0.198
Timp1	-154, -263, -350, -910	0.731
Timp3	-230, -262, -276, -303, -517, -642, -655, -1041, -1277	0.737
Tnfa	-54, -77, -181, -404, -507, -840, -1379	1.956

*The locations are relative to the transcription start site which designated as +1.
†Fold change of mRNA expression in *Nfat1*^{-/-} cartilage, the expression level of wild-type (WT) cartilage was normalized to 1.0.

factor-1alpha, HIF-1alpha).[9,28] The specificity of the NFAT1 ChIP assay was confirmed by using three different negative controls including the normal mouse IgG, cross-linked *Nfat1*^{-/-} chromatins without the NFAT1 binding domain, and a pair of negative primers (NegPri) located in the *Acan* gene body with no NFAT1 binding sites (Figs 3f and 3g).

NFAT1 regulates promoter activities of its target genes in chondrocytes. We first validated the levels of NFAT1 binding to the promoter of *Col2a1*, *Bmp7* (representing anabolic genes), *Il1b*, and *Adamts5* (representing catabolic genes), and their specificity by conventional PCR. These genes were chosen because they had been proposed as major anabolic or catabolic genes in AC and showed high levels of NFAT1 binding in our ChIP assays (Fig 3a to 3d). The PCR data demonstrated effective pull-down of NFAT1-DNA fragments by the NFAT1 antibody in WT chondrocytes, but not in *Nfat1*^{-/-} chondrocytes (Fig 4a and 4b). Promoter luciferase reporter assays were then performed to evaluate the transcriptional activity of NFAT1 binding to the DNA fragments located in the promoter region of these genes in cultivated articular chondrocytes isolated from WT or *Nfat1*^{-/-} femoral heads (Fig. 4c). Empty control vectors had no luciferase activity, while the plasmids containing DNA fragments with NFAT1 binding sites in the promoter region of *Col2a1*, *Bmp7*, *Il1b*, and *Adamts5* showed significantly higher luciferase activity in WT chondrocytes than those in *Nfat1*^{-/-} chondrocytes that lack the functional NFAT1 protein. Transfection of luciferase reporter plasmids bearing mutated NFAT1 binding

DNA sequences into cultured WT chondrocytes resulted in very low luciferase activity close to those in *Nfat1*^{-/-} chondrocytes (Fig. 4d), confirming that NFAT1 regulates promoter activity of its target genes in chondrocytes by binding its specific DNA sequence.

The luciferase activities of *Bmp7* and *Adamts5* were significantly higher (p < 0.05) than the baseline from the empty control vector in cultured *Nfat1*^{-/-} chondrocytes (Fig. 4d). This could be explained by the presence of other NFAT family member(s) in *Nfat1*^{-/-} chondrocytes. *Nfat2, Nfat3*, and *Nfat4* mRNAs are also expressed at a low level in articular chondrocytes,[8] NFAT1 to NFAT4 can be activated via the same signalling (calcium-calcineurin) pathway and share common DNA binding sequences.[1,2,30] Thus, NFAT2-4 may be responsible for the low level of luciferase activities in *Nfat1*^{-/-} chondrocytes.

It is noteworthy that the expression level of *Il1b* and *Adamts5* mRNA determined by qPCR was increased in *Nfat1*^{-/-} AC,[7] whereas the promoter activity of *Il1b* and *Adamts5* genes determined by luciferase assay was decreased in *Nfat1*^{-/-} chondrocytes. IL1b and ADAMTS5 expression levels were increased in *Nfat1*^{-/-} AC because NFAT1 normally suppresses their expression in AC cooperating with cofactors; loss of NFAT1 may activate other transcription factor(s) to upregulate catabolic gene expression.[5] The promoter activity of *Il1b* and *Adamts5* was low in *Nfat1*^{-/-} chondrocytes because the mutated NFAT1 protein in *Nfat1*^{-/-} chondrocytes lacks the NFAT1-DNA binding domain and is unable to produce appropriate promoter activity.

Fig. 3a

Fig. 3b

Fig. 3c

Fig. 3d

Fig. 3e

Fig. 3f

Fig. 3g
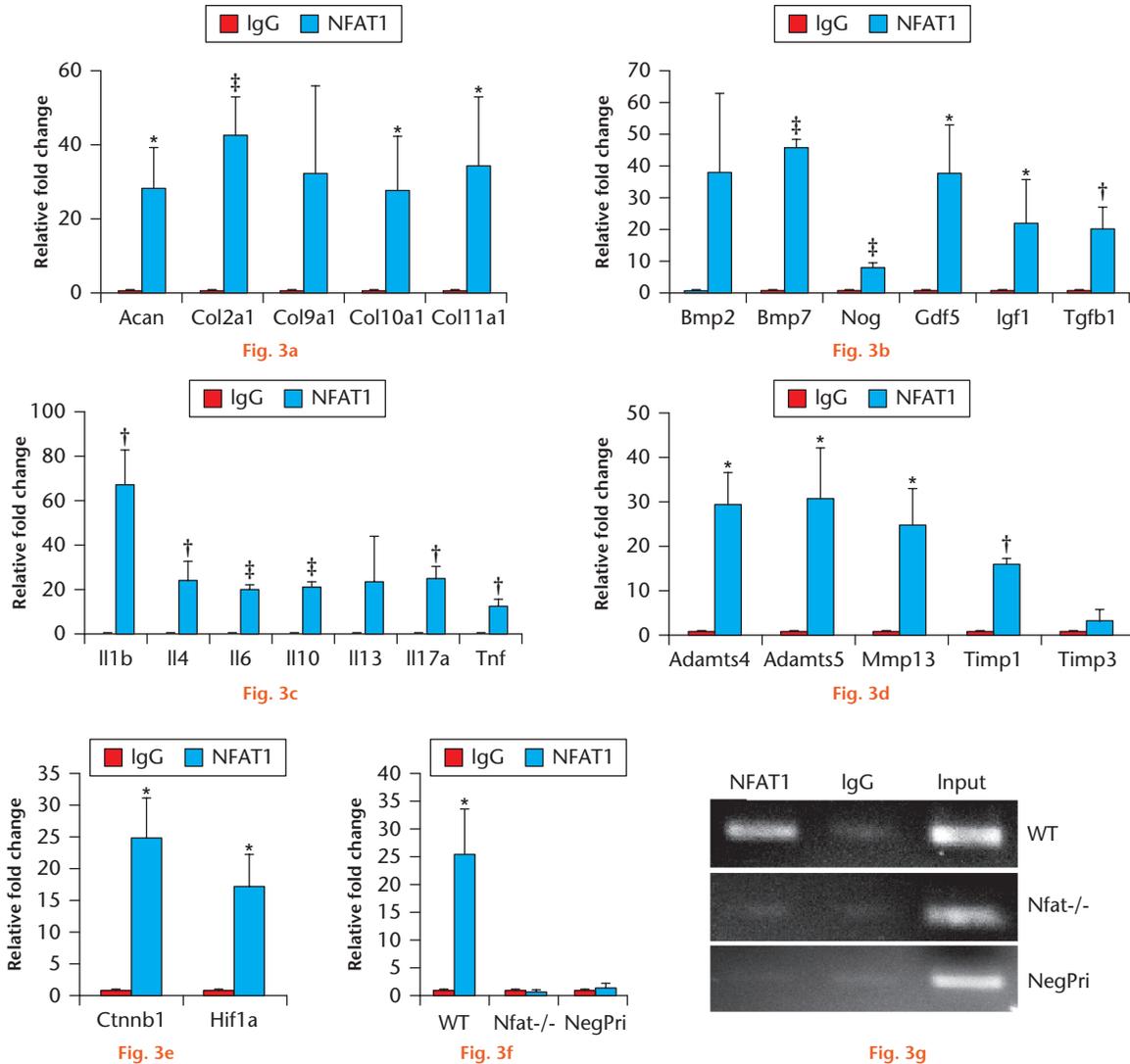
Chromatin immunoprecipitation (ChIP) assays followed by quantitative polymerase chain reaction (qPCR) quantification demonstrate the binding level of nuclear factor of activated T cells 1 (NFAT1) to the promoter region of a) cartilage matrix genes, b) growth factor genes, c) cytokine genes matrix-degrading proteinase and their inhibitor genes, d) and transcription factor genes, e) using chromatin prepared from the articular cartilage of three- to four-month-old wild-type (WT) mice. The specificity of ChIP assay is confirmed by normal mouse IgG for each gene. Crosslinked chromatin prepared from *Naft1⁻/⁻* articular cartilage lacking functional NFAT1 protein and a pair of negative primers (NegPri) located in the *Acan* gene body with no NFAT1 binding sequences are used as additional negative controls for (f) qPCR and (g) agarose gel electrophoresis. The relative binding level of IgG to input has been normalized to 1.0. n = 3. *p < 0.05, †p < 0 .01, ‡p < 0.001.

These results suggest that NFAT1 may maintain AC homeostasis by directly binding to and regulating the transcription of its target genes in articular chondrocytes. Therefore, NFAT1 deficiency triggers an imbalanced expression of anabolic and catabolic genes in AC in favour of matrix catabolism at the initiation stage of OA.

Discussion

OA is the most common form of joint disease. No disease-modifying pharmacologic therapy is currently available, largely because the pathogenic mechanisms of OA remain unclear. Previous studies have demonstrated that aberrant gene expression in joint tissue plays an important role in the development of OA. Those genes can be generally divided into two groups: anabolic genes responsible for the synthesis of cartilage matrix; and catabolic genes contributing to cartilage degradation. However, a large number of OA clinical trials targeting one of those molecules have been unsuccessful,[31,32] leading to the pursuit of upstream factors that regulate the expression of multiple molecules.[7,33]

The current study has revealed for the first time that NFAT1 not only directly binds to the promoter of specific anabolic and catabolic genes, but also regulates the promoter activities and expression of its target genes in articular chondrocytes, thereby maintaining balanced anabolic and catabolic activities of chondrocytes to protect AC against OA. NFAT1 deficiency causes an
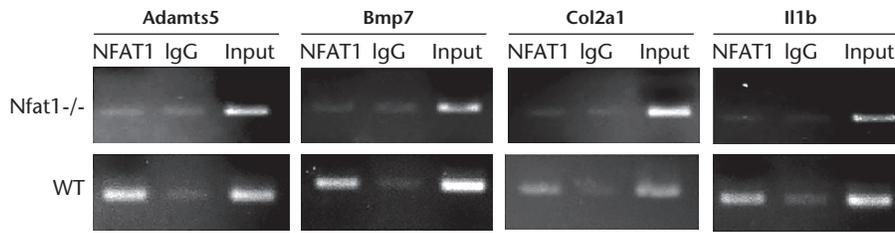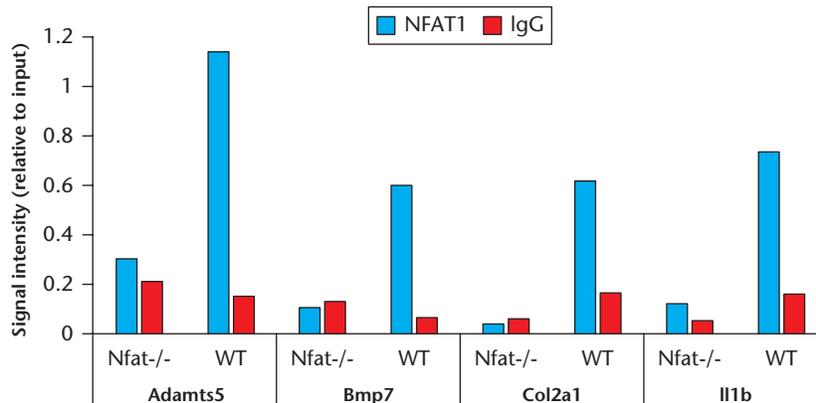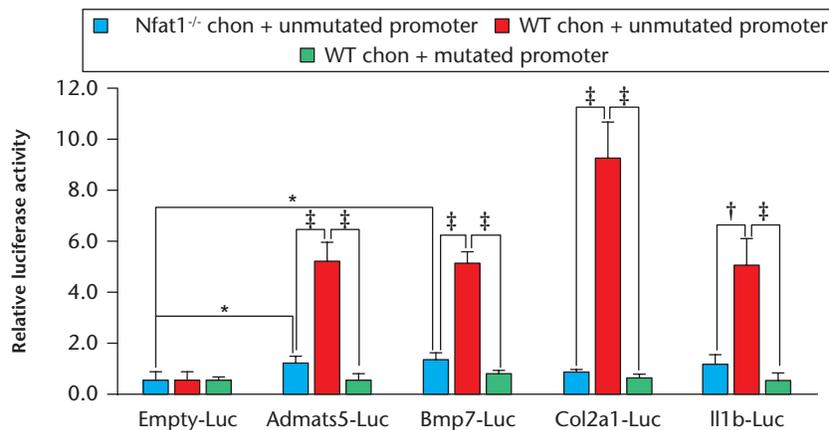
Fig. 4a

Fig. 4b

Fig. 4c

Fig. 4d

Promoter luciferase reporter assay. a) Representative agarose gel electrophoresis images of ChIP-PCR show effective binding of NFAT1 to the promoter region of *Col2a1, Bmp7, Il1b*, and *Adamts5* genes in wild-type (WT) chondrocytes, but not in *Nfat1*^{-/-} chondrocytes. b) The intensity of the ChIP-PCR bands of ChIPed DNA samples from the gel images of a) was quantified using ImageJ software. c) A schema of the firefly luciferase reporter constructs of the DNA fragments in the promoter region of *Col2a1, Bmp7, Il1b*, and *Adamts5* genes with the position relative to their transcription start site and the primer sequences used for PCR cloning into the multiple cloning site (MCS) of a pGL3 vector. d) Luciferase activities of WT or *Naft1*^{-/-} chondrocytes were transiently transfected with empty luciferase vectors (unmutated promoter) or vectors with mutated NFAT1 binding sequences in the promoter region (mutated promoter) of *Col2a1, Bmp7, Il1b*, and *Adamts5* genes. Nfat1-/- chon: Nfat1-/- articular chondrocytes; WT chon: wild-type articular chondrocytes. Renilla luciferase activities were used for normalization. n = 3. *p < 0.05, †p < 0.01, ‡p < 0.001.
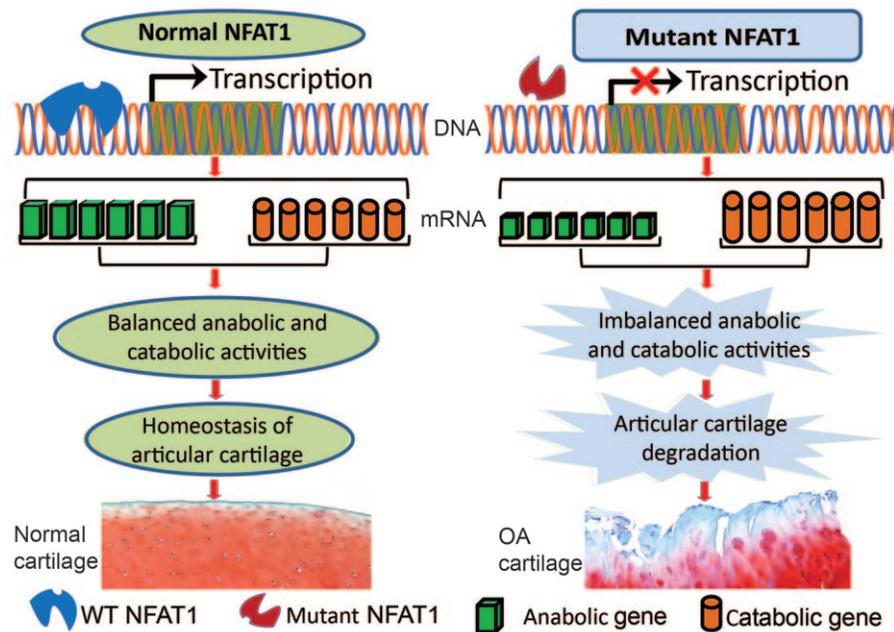
Fig. 5

A diagram showing the possible regulatory role of nuclear factor of activated T cells 1 (NFAT1) in the maintenance of articular cartilage (AC) homeostasis and the molecular mechanism of NFAT1 deficiency-induced cartilage degradation. The wild-type (WT) NFAT1 protein binds to the promoter region and regulates the expression of specific anabolic and catabolic genes in WT chondrocytes, and possibly also in synovial cells. In contrast, the mutant NFAT1 protein loses the ability to bind to the promoter or regulate the expression of its target genes, which leads to cartilage degradation and predisposes affected joints to osteoarthritis (OA).

imbalanced expression of anabolic and catabolic genes in articular chondrocytes in favour of matrix catabolism, leading to AC degradation and OA. Our previous studies demonstrated that forced expression of NFAT1 using lentiviral vectors partially or completely rescued the abnormal expression of catabolic and anabolic genes in primary *Nfat1*^{−/−} articular chondrocytes isolated from three-month-old mice.[7] Given the importance of the NFAT1 target genes to cartilage homeostasis and OA pathogenesis, anti-OA agents using NFAT1 as a target could be more effective than drug candidates that target a single catabolic or anabolic molecule.

It is not completely clear why NFAT1 deficiency affects the function of articular chondrocytes,[7,8] while activation of NFAT2 regulates the terminal differentiation of osteoclasts.[3] It has been proposed that the transcriptional activity of NFATs may be activated or deactivated depending on their binding partners, which include AP-1 (composed of Fos and Jun proteins), MEF2, GATA proteins, and histone deacetylases (HDACs). By coupling with different partners, NFAT members may play distinct roles in different types of cells.[30,34,35] It would be appropriate to identify specific binding partners for each NFAT member under different conditions in future studies.

One of the limitations of this study is the lack of ChIP-seq analysis to identify unknown downstream genes regulated by NFAT1. Future ChIP-seq analysis would be beneficial whenever ChIP-seq grade anti-NFAT1 antibodies

become available. In addition, potential NFAT1 target genes in the synovium are not examined in this study due to technical difficulties in obtaining pure synovial membrane from mouse joints. Since OA is a disease of the whole joint,[36] future investigations on the regulatory role of NFAT1 in other joint tissues, particularly the synovium, would further improve our understanding of the pathogenic mechanisms of OA.

Despite these limitations, this study succeeds in the goal of determining the ability of NFAT1 to bind to and regulate the transcription of specific target genes in articular chondrocytes during the initiation stage of OA. This has substantially advanced our understanding of the molecular mechanism of NFAT1 deficiency-induced OA. Based on the findings from this study and our previous reports,[7,8] the possible regulatory role for NFAT1 in the maintenance of AC homeostasis and the molecular mechanism of NFAT1 deficiency-induced OA are proposed in Figure 5.

In conclusion, we report for the first time that NFAT1 directly binds to 21 target genes and regulates their transcription in articular chondrocytes. NFAT1 deficiency triggers defective transcriptions of both anabolic and catabolic genes in articular chondrocytes favouring cartilage catabolism, leading to osteoarthritic cartilage degradation. Therefore, NFAT1 could be a more promising target for OA therapy than other anti-OA drug candidates that target a single anabolic or catabolic molecule.

Supplementary material

ë A table with a full list of gene names

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- M. Zhang: Designed the study, Conducted the experiments and collected the data, Analyzed and interpreted the data, Wrote the manuscript.
- Q. Lu: Conducted the experiments and collected the data.
- T. Budden: Conducted the experiments and collected the data.
- J. Wang: Designed the study, Analyzed and interpreted the data, Wrote the manuscript.