LEF1-mediated MMP13 gene expression is repressed by SIRT1 in human chondrocytes

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ABSTRACT: Reduced SIRT1 activity and levels during osteoarthritis (OA) promote gradual loss of cartilage. Loss of cartilage matrix is accompanied by an increase in matrix metalloproteinase (MMP) 13, partially because of enhanced LEF1 transcriptional activity. In this study, we assessed the role of SIRT1 in LEF1-mediated MMP13 gene expression in human OA chondrocytes. Results showed that MMP13 protein levels and enzymatic activity decreased significantly during SIRT1 overexpression or activation by resveratrol. Conversely, MMP13 gene expression was repressed in chondrocytes transfected with SIRT1 siRNA or treated with nicotinamide (NAM), a sirtuin inhibitor. Chondrocytes challenged with IL-1β, a cytokine involved in OA pathogenesis, enhanced LEF1 protein levels and gene expression, resulting in increased MMP13 gene expression; however, overexpression of SIRT1 during IL-1β challenge impeded LEF1 levels and MMP13 gene expression. Previous reports showed that LEF1 binds to the MMP13 promoter and transactivates its expression, but we observed that SIRT1 repressed LEF1 protein and mRNA expression, ultimately reducing LEF1 transcriptional activity, as judged by luciferase assay. Finally, mouse articular cartilage from Sirt1−/− presented increased LEF1 and MMP13 protein levels, similar to human OA cartilage. Thus, demonstrating for the first time that SIRT1 represses MMP13 in human OA chondrocytes, which appears to be mediated, at least in part, through repression of the transcription factor LEF1, a known modulator of MMP13 gene expression.—Elayyan, J., Lee, E.-J., Gabay, O., Smith, C. A., Qiq, O., Reich, E., Mobasheri, A., Henrotin, Y., Kimber, S. J., Dvir-Ginzberg, M. LEF1-mediated MMP13 gene expression is repressed by SIRT1 in human chondrocytes. FASEB J. 31, 3116–3125 (2017). www.fasebj.org

KEY WORDS: cartilage • osteoarthritis • inflammation • IL-1β • catabolism

Articular cartilage undergoes age-related degenerative changes leading to the joint disease osteoarthritis (OA). Gradual loss of hyaline joint cartilage during OA is evoked through chronic synovitis, which involves the accumulation of proinflammatory cytokines, as IL1β, TNF-α, and IL6 within the joint (1–5), subsequently leading to a steady increase in matrix metalloproteinases (MMPs) and a disintegrin and MMP with thrombospondin motifs (ADAMTS) proteins, which further promote cartilage destruction (6–12). Recent experimental attempts

ABBREVIATIONS: ACAN, aggrecan gene; ADAMTS, a disintegrin and metalloproteinase with thrombospondin-like motifs; BMP, bone morphogenic protein; ChIP, chromatin immunoprecipitation; COL2A1, collagen-2 α-1 gene; FBS, fetal bovine serum; FGF, fibroblast growth factor; GDF, growth differentiation factor; GSK3β, glycogen synthase kinase-3β; hESC, human embryonic stem cell; KO, knockout; LEF, lymphoid enhancer factor; MMP, matrix metalloproteinase; NAM, nicotinamide adenine mononucleotide; OA, osteoarthritis; Res, resveratrol; qPCR, quantitative PCR; siRNA, small interfering RNA; SIRT1, silent mating type information regulation 2 homolog 1 (sirtuin-1); SOX9, sex-determining-region-on-the-Y-chromosome-related Box-9; WB, Western blot; WT, wild type

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succeeded in halting cartilage destruction in mouse OA models using intra-articular injections of a natural Sirt1 activator, resveratrol (Res) (13). Correspondingly, additional in vivo studies (14) demonstrated that Sirt1 exerts an anti-inflammatory effect in cartilage during OA. Moreover, mechanistic studies have provided further support that SIRT1 promotes chondrocyte survival through various pathways (15–19)—in particular, deacetylation of p65/ RelA—which reduces iNOS gene expression after IL-1β challenge of chondrocytes (20). Despite the wealth of information regarding the beneficial effects exerted by SIRT1 in maintaining chondrocyte survival and attenuating inflammation, very little is known about the impact of SIRT1 on cartilage destruction during OA.

Nevertheless, several reports have shown that the Wnt/β-catenin signaling pathway is involved in IL-1β-mediated cartilage degeneration (21–26). These studies initially identified a 3′ regulatory promoter site of MMP13, to which lymphoid enhancer factor (LEF)-1 binds, thus inducing MMP13 gene expression (25). Moreover, Yun et al. (26) showed that IL-1β augments LEF1 levels and induces nuclear translocation of NF-κB in chondrocytes. Consequential studies by this group revealed that IL-1β induces NF-κB and activator protein 1 (AP-1) levels in chondrocytes and that both proteins interact with LEF1 to facilitate MMP13 gene expression via a DNA looping mechanism, rather than gene activation mediated by the canonical Wnt pathway.

Reduced SIRT1 activity has been found to correlate with increased expression of various cartilage-degrading enzymes, such as MMP13 and ADAMTS4 (27), but little is known of the mechanism by which this is achieved; moreover, there is no indication to date that LEF1 may be an intermediate factor facilitating this effect. These correlative observations (27), led us to hypothesize that SIRT1 may be an indirect negative regulator of MMP13 via its conceivable capacity to repress LEF1. Indeed, this report shows that SIRT1 reduced MMP13 gene expression and LEF1 protein levels in human primary chondrocytes. Furthermore, inflammation of the joint may lead to SIRT1 truncation (27), possibly reducing SIRT1 chromatin binding (28), sequentially lifting its inhibitory effects on LEF1 gene expression and promoting downstream MMP13 gene expression.

**MATERIALS AND METHODS**

**Reagents, cell culture, and transfections**

Experimental procedures on mice were performed in accordance with National Institutes of Health (NIH) Animal Research Advisory Committee (Bethesda, MD, USA) and based on the Association for Assessment and Accreditation of Laboratory Animal Care International (Frederick, MD, USA) guidelines. 129-Sirt1tm1Mcbymice were purchased from RIKEN BioResource Center (Ibaraki, Japan), carrying a Sirt1 heterozygote genotype (29). 129-Sirt1tm1Mcbymice were outbred with C57BL/6J mice to generate wild-type (WT) and Sirt1-knockout (KO) mice. Mice were raised in 12 h light/dark cycles and received food and water ad libitum. Male and female mice were euthanized at 1 or 12 wk for analysis of articular joints.

Human chondrocytes were isolated from patients undergoing total knee arthroplasty. Non-OA knee samples were provided by National Disease Research Interchange (Philadelphia, PA, USA). The average age was 62 yr (range, 52–71). Chondrocyte isolation and culture were performed as described elsewhere (30). Monolayer cultures were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin, and 50 μg/ml streptomycin (Biologic Industries, Beit-Haemek, Israel). The cells were treated with the proinflammatory cytokines IL1β (5 ng/ml) and TNF-α (50 ng/ml; both from Peprotec, Rehovot, Israel) in defined serum-free medium (BIO-MPF 1; Biologic Industries, Beit-Haemek, Israel). Conditioned medium was filtered with a 10-kDa cutoff Amicon filter (EMD-Millipore, Billerica, MA, USA) and processed for immunoblot analysis.

Retroviral infections for Sirt1 and pHan (control) were performed (31). All transient transfection experiments were initiated on 50% confluent monolayer cultures. Plasmids (4 μg) were transfected by Amaxa nucleofection for human chondrocytes (Amaxa, Basel, Switzerland), according to the manufacturer’s protocol. The human pCDNA_SIRT1 expression plasmid was a kind gift of Prof. Danny Reenberg (New York University School of Medicine, New York, NY, USA) The SIRT1 expression plasmid (Pbabe-puro.SIRT1) was purchased from Addgene (27023; Cambridge, MA, USA). Small interfering RNAs (siRNAs) for SIRT1 and LEF1 were purchased from Ambion (Thermo Fisher Scientific, Waltham, MA, USA) and used according to the manufacturer’s recommendations. Sirt1 activity was modulated by the inhibitor nicotinamide (NAM, 10 mM) or the activator Res (1 μM; Sigma-Aldrich, St Louis, MO, USA).

**Direct chondrogenesis differentiation protocol**

MAN7 human embryonic stem cells (hESCs) were cultured as previously described by Oldershaw et al. (32) and Cheng et al. (33) with slight modifications. hESCs were cultured in a feeder-free system on vitronectin-coated (Thermo Fisher Scientific) tissue culture plates with mTESR1 medium (StemCell Technology, Vancouver, BC, Canada) and subcultured using EDTA passage. For differentiation toward chondroprogenitors, a defined 3-stage protocol was used (32). In brief, hESCs were transferred to fibronectin (EMD-Millipore)-coated tissue culture wells (d 0) and initially differentiated toward primitive streak mesoderm by addition of Wnt3a, activin A, and fibroblast growth factor (FGF)-2 (d 1–3), followed by FGF2, bone morphogenic protein (BMP)-2 (substitution for BMP4; unpublished data), and follistatin, to promote mesoderm-like cells (d 4–7). Finally, cells were cultured in FGF2, growth differentiation factor (GDF)-5, and BMP2 to promote chondrogenesis (d 8–10) and levels of FGF2 and GDF5 (d 11–13). Samples were collected every day for gene expression analysis.

**RNA isolation and PCR analysis**

Total RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA, USA) after the manufacturer’s recommendations. Oligo dT was used as the primer in the reverse-transcription reaction. Real-time quantitative PCR (qPCR) reactions were performed with 10 ng of cDNA and Syber Green mix (BioRad Laboratories, Hercules, CA, USA). All reactions were performed with iCycler software (Bio-Rad, Hercules, CA, USA) and subcultured using EDTA passage. Real-time quantitative PCR (qPCR) reactions were performed with 10 ng of cDNA and Syber Green mix (Bio-Rad, Hercules, CA, USA). All reactions were performed with iCycler software (Bio-Rad, Hercules, CA, USA) and subcultured using EDTA passage. Total RNA was extracted from cells using the RNeasy kit (Qiagen, Valencia, CA, USA) after the manufacturer’s recommendations. Oligo dT was used as the primer in the reverse-transcription reaction. Real-time quantitative PCR (qPCR) reactions were performed with 10 ng of cDNA and Syber Green mix (Bio-Rad Laboratories, Hercules, CA, USA). All quantitative analyses were performed with iCycler software (Bio-Rad, Hercules, CA, USA). All RNA samples were treated with DNase I before the PCR reactions. Primers used: GAPDH-forward (F): 5′-TACTAGGTTTT-

TACGCGCC-3′, reverse (R): 5′-TCAAAACCCGGAGCCAGAG-
GCAC-3′; SIRT1-F: 5′-CAGGTTAGGAGCCGCTGTTA-3′, R: 5′-

CTAAACTGAGACCTCTGCA-3′; MMP-1-F: 5′-AATCTGAGG

TCAACTGAGACCTCTGCA-3′; MMP-3-F: 5′-TCCCTCCAGAAAGCT-

TGAACCTGAA-3′, R: 5′-AAACCTTAGGTGATCGGCT-3′; MMP-8-F: 5′-ATCCCTGCTCATGCTTCTGCACC-3′, R: 5′-GTTTGGTACATGTTCCATCCGGCA-3′; MMP-13-F:

5′-AGTTTGCAGACGGCTACCTGAGAT-3′, R: 5′-TTTGGCAGTACCTCTTAAGGCGGA-3′; ADAMTS4-F: 5′-ACAAAAGTGGCAAGGAGGCT-3′, R: 5′-AGGCTGTGGACCGGTAAAGGAA-3′; ADAMTS5-F: 5′-TTCAAGCTCACGCGCTGCAACTG-3′; LEFI-F: 5′-AATAAGCTCCGGTGTTG-3′, R: 5′-ATGGGTAGGGTTGCGCTGAATC-3′. Primers for determining chondrogenesis were: COL2A1-F: 5′-CGGTGGCGAGCTTCACTCCT-3′; COL2A1-R: 5′-GGCAATGGCAACTG-3′.

**Chromatin immunoprecipitation**

To perform chromatin immunoprecipitation (ChiP) assays, we treated human chondrocytes (P0–P2) with 5 ng/ml IL1β for 24 h, washed them with PBS, and cross-linked them with 1% formaldehyde for 10 min at room temperature. Cells were then lysed [lysis buffer; 10 mM EDTA, 50 mM Tris-HCl (pH 8.0), 1% SDS] and sonicated (Vibra Cell; Sonics and Materials, Newtown, CT, USA) at 50 cycles of 95% amplitude for 30 s, followed by a 45 s incubation on ice, based on Bar Oz et al. (34). Samples were then processed using the Low Cell ChiP kit (Diagenode, Liège, Belgium). After DNA isolation, qPCR reactions were performed within the 3′ region of genomic human MMP13 (16,566-16,625 bp) with primers flanking the conserved LEF1 binding site (CTTTGA) was chosen for amplification (F: 5′-AGCATCTGGAGATACCGTTATTG-3′; R: 5′-CCACGACCGCATACATATAG-3′). Human IGX1A Negative Control (Qiagen, Hilden, Germany) containing an open-reading-frame–free intergenic region, was used as a negative control for all ChiP samples.

**Protein and immunoblot analyses**

Cell lysis and the generation of protein extracts were performed as has been described (31). The protein extracts were then electrophoretically resolved by SDS-PAGE (10–20 μg protein/lane) and transferred onto PVDF membranes for immunoblot analysis. The blots were processed (19–21) and then probed with the indicated antibodies. The antibodies were used for β-actin (47778; Santa Cruz Biotechnology, TX, USA), α-tubulin (T5168; Sigma-Aldrich), SIRT1 (07-131; EMD-Millipore), Ac-H3K9/14 (A-4021-050; EMD-Millipore), 2MeH3K9 (07441; EMD-Millipore), MMP13 (sc-81547; Santa Cruz Biotechnology), LEFI (2230; Cell Signaling Technology), and β-catenin (ab16051; Abcam, Cambridge, United Kingdom). Immunoblots were developed using secondary antibodies to either alkaline phosphatase (BCIP/NBT as a color developer) or horse radish peroxidase (chemiluminescence) and exposed to preflashed X-ray film. All immunoblots were scanned in high resolution, and band intensity was determined with ImageJ software (NIH, Bethesda, MD, USA). Semiquantitative band intensities were normalized to the corresponding housekeeping protein appearing on the blot.

**MMP and SIRT1 enzyme activity**

MMP Activity Assay Kit (ab11246; Abcam) uses a fluorescence resonance energy transfer quenched peptide, as a generic MMP activity indicator. Upon MMP cleavage, the fluorescence is recovered and read by a fluorescence microplate reader at 490 nM (excitation) and 525 nm (emission) per microgram protein. SIRT1 enzymatic activity was assessed by use of A Fluor de Lys fluorescence assay kit (Enzo Life Sciences, Farmingdale, NY, USA) (31). Values are presented as substrate conversion per microgram protein.

**Reporter assay**

The TOPFlash or TOPFlash luciferase reporter plasmid (EMD-Millipore) was cotransfected in human chondrocytes with a control vector (pcDNA3) or the SIRT1/LEFI expression vectors, via Amaxa Nucleofector Technology Amaxa Biosystems, Cologne, Germany) in accordance with the manufacturer’s instructions. Two days after transfection, cells were lysed using the passive lysis buffer (Promega, Madison, WI, USA) and luciferase activity in the extracts was measured using the dual luciferase assay system (Promega). Cotransfection with the Renilla vector allowed normalization of the assays for differences in transfection efficiency.

**Immunohistochemistry**

Human and murine samples were subjected to 2 d of tissue fixation in 4% formalin and 14 d of decalcification with 10% EDTA. Samples were dehydrated with a graded series of ethanol washes, embedded in paraffin, and sectioned into 5-μm slices. Sections were digested with 1 mg/ml hyaluronidase in PBS at pH 6 (H3506; Sigma-Aldrich) for 1 h at 37°C and stained with a DAB substrate kit (DAB057) after overnight incubation with MMP13 and LEFI primary antibody. Zytocem Plus (Zytomed Systems, Berlin, Germany) horseradish peroxidase polymer conjugated anti-rabbit (ZUC032) was used as a secondary antibody. Negative controls were incubated with secondary antibody alone and counterstained with hematoxylin.

To determine OA histopathology in murine samples, sections were stained with 0.5% Safranin O and 0.1% Fast green after using Weigert’s iron hematoxylin (1.15973; Merck, Darmstadt, Germany). OA histopathology was carried out based on Glasson et al. (35).

**Statistical analysis**

Statistical significance was assumed for confidence levels greater than 95% (P < 0.05). Mann-Whitney analysis was performed to determine the differences between 2 equivalent treatments within a group (n > 4). Error bars indicate the standard deviation around the mean value, unless otherwise indicated.

For immunohistochemistry the percentage of cells per field stained positive was determined. Stained cells are reflective of greater than 10-fold intensity above the background (as determined by scanning densitometry). An average of 10 fields from 3 sections of 6 separate murine WT and Sirt1−/− mice, as well as OA and non-OA cartilage samples were assessed (n = 6). Each field was blindly read by 2 different individuals.

**RESULTS**

**Overexpression of SIRT1 in human chondrocytes leads to repression of MMP3, -8, and -13 and ADAMTS4 gene expression**

Inspection of primary human chondrocytes obtained from OA and non-OA donors showed increased MMP13 tissue staining and mRNA expression in OA-derived tissue (Fig. 1A), which also corresponded with a reduction in
SIRT1 activity in OA. To determine whether SIRT1 may negatively regulate MMP13 expression, murine Sirt1 was then stably overexpressed in human OA chondrocytes by retroviral expression vector. In line with previous reports (31), stable overexpression of murine Sirt1 in human chondrocytes was confirmed via immunoblot analysis and associated with decreases in H3K9/14 acetylation, as compared to unchanged methylation of dimethyl-H3K9 and increased Sirt1 activity (Fig. 1B). Next, we analyzed mRNA from chondrocyte lines for catabolic gene expression (Fig. 1C). Cells overexpressing Sirt1 displayed significant decreases in MMP13 gene expression (5-fold), along with MMP3 and -8, whereas MMP1 gene expression remained unaffected compared to vector control, Sirt1 also repressed ADAMTS4, whereas ADAMTS5 was unaffected. Ectopic overexpression of SIRT1 (~90% transfection efficiency; Fig. 2A), resulted in a reduction in cellular and secreted MMP13 protein, which was additionally reflected by mRNA levels and MMP13 enzymatic activity (Fig. 2C). Treating passage 0 chondrocytes with Res (1 μM), an activator of Sirt1, reduced cellular MMP13 protein level, mRNA expression and activity, as compared to untreated controls (Fig. 2B, D). Res treatment generated a 60% increase in Sirt1 activity (Fig. 2B). Overall, these data imply that SIRT1 may repress MMP13 gene expression, as well as other pro-catabolic genes in chondrocyte lines.

Down-regulating SIRT1 in human chondrocytes leads to induction of MMP13

To investigate this observation further, we performed the reverse experiment, in which SIRT1 siRNA was transfected into chondrocytes to reduce SIRT1 protein levels. SIRT1 protein and mRNA levels dropped significantly after SIRT1 siRNA transfection (Fig. 3A). As a result, MMP13 gene expression and activity increased
We next treated passage 0 chondrocytes with (10 mM), which is a direct inhibitor of SIRT1 (36, 37). After NAM treatment of chondrocytes, we observed a 70% reduction in SIRT1 enzymatic activity and a corresponding 75% increase in acetylation of histone H3K9/K14 (data not shown). Figure 3 shows clear increases in MMP13 mRNA, cellular protein levels, and enzymatic activity in the NAM-treated cells. This NAM-mediated increase in MMP13 mRNA occurred even in cells that overexpressed SIRT1, indicating that NAM can overcome the repressive effects of SIRT1 on MMP13 gene expression.

**Induction of MMP13 by IL-1β is partially repressed by SIRT1 overexpression**

As mentioned, data from other studies have established that LEF1 is necessary for MMP13 gene expression during IL-1β challenge (21–26). Thus far, we have demonstrated that SIRT1 repressed MMP13 gene expression; however, the mechanism by which this is achieved is still unclear. We next attempted to decipher whether SIRT1 reverses the effect of significantly. We next treated passage 0 chondrocytes with (10 mM), which is a direct inhibitor of SIRT1 (36, 37). After NAM treatment of chondrocytes, we observed a 70% reduction in SIRT1 enzymatic activity and a corresponding 75% increase in acetylation of histone H3K9/K14 (data not shown). Figure 3B shows clear increases in MMP13 mRNA, cellular protein levels, and enzymatic activity in the NAM-treated cells. This NAM-mediated...
LEF1-mediated MMP13 gene expression in an inflammatory milieu exerted by IL-1β (21–26). IL-1β up-regulates MMP13 gene expression 5-fold, consistent with previous reports (8) (Fig. 4A). Overexpressing SIRT1 with IL-1β induction significantly reduced MMP13 at the level of mRNA, protein, and enzymatic activity by ~5-fold as compared to IL-1β stimulation with pcDNA control (Fig. 4). These data indicate that IL-1β induces MMP13 through a mechanism at least partly influenced by the action of SIRT1, given that intact SIRT1 significantly lowered the maximum amount of MMP13 induced by IL-1β stimulation. In previous work, we found that stimulation of chondrocytes with IL-1β, TNFα, or both elicits SIRT1 truncation (27, 38), leading to its reduced chromatin binding of COL2A1 (28). The truncated SIRT1 variant may possess impaired chromatin binding and thus render diverse expression of its gene targets.

The transcription factor LEF1 is a transactivator of MMP13 and is down-regulated by SIRT1

To identify the underlying mechanism by which SIRT1 inhibits MMP13 gene expression, we focused on the LEF1 transcription factor, given that it is implicated in IL-1β-mediated cartilage degeneration in arthritis (21–26). The transcription factor LEF1 has been shown to positively regulate MMP13 expression in chondrocytes via an enhancer sequence at the 3' region of the gene (25). Overexpression of LEF1 in chondrocytes led to increases in both MMP13 protein and mRNA levels (Fig. 5A). When LEF1 siRNA was transfected into human chondrocytes, LEF1 and MMP13 protein (Fig. 5B) and mRNA levels were significantly reduced. These data, supported by previous findings, demonstrate a close correlation between LEF1 and MMP13 gene expression.

When LEF1 levels were assessed in SIRT1-expressing cells, it was apparent that both LEF1 protein (Fig. 5C) and mRNA levels (Fig. 5D) were reduced in the presence of SIRT1. When endogenous SIRT1 levels were reduced by a SIRT1 siRNA, both LEF1 protein and mRNA levels were elevated significantly (Fig. 5E), corresponding to increases in MMP13 levels (as in Fig. 3A). To assess transcription activity of LEF1, we used the TOPFlash construct containing a promoter consisting of LEF1 sites fused to a luciferase-encoding gene. Cotransfection of SIRT1 with TOPFlash significantly reduced luciferase levels, as compared to pcDNA controls (Fig. 5F). Knocking down LEF1 with TOPFlash in chondrocytes showed an ~2-fold decrease in luciferase activity (Fig. 5G), supporting that SIRT1 overexpression indeed reduced luciferase intensity of TOPFlash via down-regulating LEF1 expression. As the control, we cotransfected TOPFlash with LEF1-expressing vector and observed a 2-fold increase in luminescence compared to pcDNA control (Fig. 5H). Taken together, these data indicate that SIRT1 represses LEF1 gene expression, which is tightly linked to MMP13 gene expression in human chondrocytes.

To further assess whether the SIRT1 repressive effects on LEF1 are conserved throughout chondrogenesis, we used a previously described 3-step protocol in which human embryonic stem cells are efficiently directed toward chondrogenesis (32, 33). At d 13 of chondrogenesis, SOX9, COL2A1, and ACAN gene expression was significantly increased compared to d 0 (Fig. 5I). During d 10 and 13 of chondrogenesis, SIRT1 gene expression levels were reduced 2-fold (Fig. 5J), whereas LEF1 gene expression was augmented 15-fold, compared to d 4 and 8. These data provide evidence that the repressive effect of SIRT1 on LEF1 levels is developmentally conserved.

MMP13 and LEF1 gene expression in articular cartilage of Sirt1 KO mice and human OA

To examine the role of SIRT1 in regulating MMP13 in vivo we explored the expression of this protease in an animal model, Sirt1<sup>−/−</sup> mice (29). We assessed 1-wk-old Sirt1<sup>−/−</sup> and WT mice for MMP13 and LEF1 levels in the superficial zone from articular cartilage of distal interphalangeal joints in the anterior paws. As shown by immunohistochemistry in Fig. 6A, the staining intensity for MMP13 and LEF1 appeared elevated in the articular cartilage of the Sirt1<sup>−/−</sup>.
mice, compared with the WT mice, which is quantified in graphs. Scoring 4–6-mo-old mice for OA severity in the medial tibial plateau showed significant increases in OA severity in Sirt1<sup>2/2</sup> mice in the lateral compartment, and a (30%) reduction in cartilage thickness in both medial and lateral compartments of the tibial plateau in KO mice compared with age-matched WT mice (Fig. 6B, C, respectively). Of note, among the WT mice, both males and females did not display significant differences in OA severity, in tibial and femoral condyles.

Because the levels of SIRT1 are reduced in human OA cartilage and chondrocytes (28, 38), LEF1 levels are expected to be elevated in the affected tissue, with a corresponding elevation in MMP13. As predicted and shown in Fig. 6D, LEF1 levels were indeed elevated in the cartilage from patients with OA. Moreover, this increase appears to be significant, as shown in the right panels. Accordingly, LEF1 protein levels were elevated in OA cartilage tissues, whereas SIRT1 levels were reduced. Furthermore, OA-derived chondrocytes treated with IL1β or IL1β/TNFα showed increased MMP13 expression (Fig. 6E), which was accompanied by truncated SIRT1 and increased LEF1 protein levels, as compared to untreated cells (Fig. 6F). ChIP analysis for the human MMP13 3′-region bearing the conserved LEF1 binding site did not show enrichment for SIRT1; rather, LEF1 increased significantly upon IL1β/TNFα challenge. These data suggest that during OA, reduced SIRT1 activity correlates with increased LEF1 levels, ultimately leading to enhanced MMP13 gene expression in chondrocytes.
DISCUSSION

In this study, we attempted to decipher the mechanism by which SIRT1 regulates MMP13 gene expression in human OA chondrocytes. Our results support previous observations regarding the pivotal role LEF1 plays in MMP13 gene expression (21–26) and add a new facet to our knowledge on LEF1 regulation, which until now had been unknown.
We report for the first time, to our knowledge, that SIRT1 is a negative regulator of LEF1 gene expression and thus indirectly represses MMP13 gene expression. During OA, proinflammatory conditions elicit loss of SIRT1 enzymatic activity via SIRT1 cleavage (27), potentially lifting its repressive effect on LEF1 and causing enhanced LEF1 gene expression, as we show in this report.

On a developmental level, Wnt signaling is critical for endochondral ossification and skeletal development. The canonical Wnt pathway consists of Wnt ligands interacting with the FRIZZLE receptor, inhibiting glycogen synthase kinase (GSK)-3β, and phosphorylation of β-catenin, resulting in β-catenin stabilization, which then enables its transcriptional transactivation together with T-cell factor (TCF)/LEF complex in the nucleus. In adulthood and aging, β-catenin activation is associated with enhanced cartilage ossification and degeneration via augmented MMP levels (39–42). Furthermore, procatabolic cytokines, such as IL-1β, induce Wnt protein expression, which elicits β-catenin activation (39). In fact, the Wnt protein family member Wnt7b is up-regulated in both RA and OA synovium (42), which supports its contribution to cartilage destruction. Moreover, it has been shown that inhibition of GSK3β results in stable cellular β-catenin and induces OA-like features in vivo (41). On the other hand, Ma and colleagues (39) established that Wnt/β-catenin (i.e., Wnt3a), in a LEF1-independent manner, possessed an anticanonical role and inhibited the expression of MMP-1,-3, and -13 under IL-1β stimulation in primary human, murine, and bovine articular chondrocytes. Therefore, there are several canonical and noncanonical effects of Wnt signaling that modulate OA pathogenesis.

Another mode of regulating β-catenin stability was reported by Simic et al. (43) who showed that SIRT1 deacetylates β-catenin and thus renders it stable and capable of transactivating its nuclear targets toward osteogenic differentiation. This SIRT1-related pathway is less likely to occur in OA, because SIRT1 possesses reduced activity in OA cartilage, which is expected to lead to maintenance of β-catenin acetylation levels and its exclusion from the nucleus. SIRT1 may affect not only β-catenin but also its downstream transactivating partners, as we showed in this study.

A growing body of evidence points to SIRT1 as a potent repressor of MMP13 gene expression in cultures of primary human OA chondrocytes (27, 44, 45). The results presented here show that SIRT1 represses LEF1 gene expression, which activates MMP13 gene expression. Studies have shown that SIRT1 can demonstrate a broad anti-inflammatory function in a variety of tissues (44–49). For example, in chronic obstructive pulmonary disorder, SIRT1 exerts a protective effect, partially because of its capacity to down-regulate MMP9 (49), and modulating NFκB activity during the disease (48). In addition to the known function of SIRT1 in repressing MMP9 in macrophages and monocytes (49), we demonstrated that SIRT1 represses MMP13, as well as MMP3 and -8, in chondrocytes. Thus, suggesting that part of the SIRT1 anti-inflammatory function is to repress the expression of these matrix-degrading enzymes, as was demonstrated here both in vitro and in vivo.

Although the exact mechanism, by which SIRT1 targets the LEF1 gene is not completely known, our data show that SIRT1 has a strong repressive effect on LEF1 expression, not only in articular cartilage, but also during chondrogenesis, indicating that this is a highly conserved mechanism. In conclusion, our data provide further support that activation of SIRT1 plays a positive role in blocking or reducing the severity of OA, in part through its ability to repress the expression of MMPs. Future efforts should be aimed at deciphering new mechanisms involved in this process.

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AUTHOR CONTRIBUTIONS

A. Mobasheri, Y. Henrotin, S. J. Kimber, M. Dvir-Ginzberg designed the research; J. Elayyan, E.-J. Lee, O. Gabay, O. Qi, E. Reich, and C. A. Smith performed research; A. Mobasheri, Y. Henrotin, S. J. Kimber, M. Dvir-Ginzberg, J. Elayyan, E.-J. Lee, O. Gabay, O. Qi, E. Reich, C. A. Smith contributed new reagents and analytic tools and clinical samples; S. J. Kimber, M. Dvir-Ginzberg, J. Elayyan, E.-J. Lee, O. Gabay, O. Qi, E. Reich, C. A. Smith analyzed the data and performed statistical analysis; and A. Mobashari, Y. Henrotin, S. J. Kimber, M. Dvir-Ginzberg, and O. Gabay wrote the paper; and all authors approved the final manuscript.

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