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Dysregulated circadian rhythm pathway in human osteoarthritis: NR1D1 and BMAL1 suppression alters TGF-β signaling in chondrocytes

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S U M M A R Y

Objectives: Circadian rhythm (CR) was identified by RNA sequencing as the most dysregulated pathway in human osteoarthritis (OA) in articular cartilage. This study examined circadian rhythmicity in cultured chondrocytes and the role of the CR genes NR1D1 and BMAL1 in regulating chondrocyte functions.
Methods: RNA was extracted from normal and OA-affected human knee cartilage ($n = 14$ each). Expression levels of NR1D1 and BMAL1 mRNA and protein were assessed by quantitative PCR and immunohistochemistry. Human chondrocytes were synchronized and harvested at regular intervals to examine circadian rhythmicity in RNA and protein expression. Chondrocytes were treated with small interfering RNA (siRNA) for NR1D1 or BMAL1, followed by RNA sequencing and analysis of the effects on the transforming growth factor beta (TGF-β) pathway.
Results: NR1D1 and BMAL1 mRNA and protein levels were significantly reduced in OA compared to normal cartilage. In cultured human chondrocytes, a clear circadian rhythmicity was observed for NR1D1 and BMAL1. Increased BMAL1 expression was observed after knocking down NR1D1, and decreased NR1D1 levels were observed after knocking down BMAL1. Sequencing of RNA from chondrocytes treated with NR1D1 or BMAL1 siRNA identified 330 and 68 significantly different genes, respectively, and this predominantly affected the TGF-β signaling pathway.
Conclusions: The CR pathway is dysregulated in OA cartilage. Interference with circadian rhythmicity in cultured chondrocytes affects TGF-β signaling, which is a central pathway in cartilage homeostasis.
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Introduction

Osteoarthritis (OA) involves destruction of articular cartilage and remodeling of other joint tissues[1]. The main OA pathogenesis pathways in cartilage include destruction of the extracellular matrix, cell death, abnormal cell differentiation and production of inflammatory mediators[2]. A large number of signaling mechanisms are abnormally activated in OA and contribute to cartilage damage. These include inflammation-related pathways such as NFκB and MAP kinases, Wnt, hypoxia, PI3K and transforming growth factor beta (TGF-β) pathways[1,3,4].

Global transcriptome analysis by RNA sequencing provides an unbiased approach to obtain vast amounts of information on genes and pathways that are abnormally activated or inhibited in disease[5]. We completed an RNA sequencing study on normal and OA human knee cartilage that led to the discovery that the circadian rhythm (CR) pathway was inhibited and the most significantly dysregulated pathway in OA. Among the differentially expressed genes in this pathway, *NR1D1* and *BMAL1* showed the largest degree of suppression in OA cartilage.

CR is critical in coordinating cell functions throughout all tissues[6,7]. In mammals, CR is a fundamental regulatory factor for many aspects of behavior and physiology, including sleep/wake cycles, blood pressure, body temperature and metabolism[6]. Disruption of CR leads to increased incidence of many diseases, such as cancer, metabolic disease, and mental illness[6]. CR is regulated by the central oscillator in the hypothalamic suprachiasmatic nucleus (SCN), and local oscillators throughout the body coordinate daily

cycles by integrating signals from the SCN with other internal and external time cues[8]. The pacemaker consists of a core group of genes with transcriptional-translational feedback loops that involve multiple clock genes such as CLOCK, BMAL1, NPAS2, PER1, 2 and 3, CRY1 and 2, and NR1D1[9,10]. These clock genes and their protein products function in a feedback loop resulting in a nearly 24-hour cycle. The transcription factor BMAL1 is the core driver of the molecular clock. Positive regulators (BMAL1, CLOCK, NPAS2) drive the expression of negative feedback regulators (PER, CRY, NR1D1), which in turn inhibit the expression and activity of the positive regulators[11].

NR1D1 encodes a member of the nuclear receptor family, and is expressed in liver, adipose tissue, and skeletal muscle[12–14]. It is a transcriptional repressor that is activated by heme[15,16], and recruits nuclear receptor co-repressor (NCoR)-–Histone Deacetylase (HDAC) 3 complexes to Rev-Erb response elements in enhancers and promoters of target genes[17–19]. BMAL1 encodes a main positive transcriptional regulator of the circadian oscillation, which functions as a heterodimeric complex with CLOCK[20]. NR1D1 transcription is activated by BMAL1/CLOCK through its binding to E-box in NR1D1 promoter[21]. In turn NR1D1 acts as the major regulator of BMAL1 by repressing its transcription[19,22], thus forming a negative feedback loop to maintain circadian rhythmicity. Aside from its function in CR control, these genes are also involved in the control of metabolism, autophagy, and inflammatory responses[9,12,19,23–27]. Interestingly, even cells isolated from peripheral tissues generate a CR in culture using the same clock factor network[7,28]. These rhythmically expressed genes control the expression of many other genes (clock controlled genes), which in turn drive cascades of rhythmic gene expression. At least 4–10% of total cellular transcripts in any given organ are thought to oscillate in a circadian manner and this set of oscillating genes has a tissue specific pattern[29,30].

The objectives of this study were to study CR in cultured chondrocytes and determine the consequences of CR gene dysregulation in chondrocyte function. This study is the first to determine in a systematic approach (1) which CR genes are expressed in normal and OA cartilage, (2) how expression of these genes is regulated, (3) whether cartilage cells display endogenous CR and (4) whether clock genes are involved in regulating expression of genes that are associated with OA pathogenesis.

Materials and methods

Cartilage donors

Normal human knee cartilage tissues were procured by tissue banks from five female (age 26–57 years, mean 39 years) and 18 male (age 18–44 years, mean 30 years) donors (approved by Scripps Institutional Review Board) and processed within 24–72 h post mortem. Full thickness cartilage was harvested for RNA isolation from identical locations on the medial femoral condyles. OA-affected cartilage was harvested from the tissue removed during knee replacement surgery from 10 female (age 61–82 years, mean 69 years) and six male (age 66–84 years, mean 71 years) donors.

Tissue processing and RNA isolation

Cartilage was stored at −20°C in Allprotect Tissue Reagent (Qiagen, Valencia, CA) immediately after resection from the subchondral bone. For RNA isolation, cartilage was pulverized in a 6770 Freezer/Mill Cryogenic Grinder (SPEX SamplePrep, Metuchen, NJ), and homogenized in Qiazol Lysis Reagent (Qiagen) using 25 mg tissue per 700 μl Qiazol. RNA was isolated using the miRNeasy Mini

kit (Qiagen) with on-column DNAse digestion, followed by removal of proteoglycans using RNAmate (BioChain Institute, Newark, CA). RNA from cultured chondrocytes was extracted using Direct-zol RNA MiniPrep Kit (Zymo Research, Irvine, CA).

RNA sequencing and data analysis

RNA from eight normal (two female, six male) and 10 OA (five female, five male) cartilage donors was sequenced using 125–150 ng of total RNA as input. mRNA libraries were prepared using the Encore Complete RNA-Seq DR Multiplex System 1-8 and 9-16 (NuGen, San Carlos, CA) with 16 unique indexed adapters (L2V6DR-BC2-L2V6DR-BC16). Two lanes of an Illumina HiSeq 2000 instrument were used to generate a total of 8–30 million single-end 100 bp reads.

Raw data were checked for quality with the software FastQC (v0.10.1) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). We mapped the RNA-seq reads for each library to the UCSC human hg19 reference genome using TopHat2 (v2.0.9)[31]. Read abundances were estimated using Cufflinks (v2.1.1) as Fragments Per Kilobase of exon per Million fragments mapped (FPKM)[32]. Cuffdiff2 was used to calculate differentially expressed genes between normal and OA samples[33]. Genes with a q-value <0.05 were considered significantly differentially expressed and were included in the downstream pathway analysis. signaling pathway impact analysis (SPIA) was conducted using the Bioconductor packages SPIA and Graphite, using the differentially expressed gene list and their log2 fold changes as input[34,35]. Pathway databases included in the analysis include KEGG, Biocarta, NCI and Reactome. Pathways were considered significantly differentially expressed if the pGFWER were <0.05.

Quantitative polymerase chain reaction (qPCR)

RNA was extracted from normal and OA human cartilage samples as well as from cultured chondrocytes and gene expression levels were analyzed by qPCR. The following pre-designed TaqMan gene expression assays (Life Technologies) were used: NR1D1 (Hs00253876_m1), BMAL1 (Hs00154147_m1), TGFBR1 (Hs00610320_m1), TGFBR2 (Hs00234253_m1), TGFBR3 (Hs01114253_m1), TGFB1 (Hs00998133_m1), TGFB2 (Hs00234244_m1), TGFB3 (Hs01086000_m1), ID2 (Hs041787239_m1), TNC (Hs01115665_m1), and ELN (Hs00355783_m1).

Immunohistochemistry

Immunohistochemistry was performed to assess protein expression patterns in human and mouse cartilage using anti-NR1D1 antibody (Abcam ab174309, Cambridge, MA) and anti-BMAL1 antibody (Thermo Scientific PA1-523, Waltham, MA). Rabbit IgG (1 μg/ml) was used as a negative control in all experiments. For human cartilage, expression patterns were compared between normal and OA samples. In C57 BL/6 mice, we analyzed young normal and aged knees as a model of aging-related OA. We also analyzed knees from mice with surgically induced OA by destabilization of medial meniscus and medial collateral ligament resection[36]. The methods for tissue processing and immunohistochemistry were described earlier[36].

Western blotting

At indicated time points, cultured human chondrocytes were lysed in RIPA buffer supplemented with Halt protease inhibitor cocktail and phosphatase inhibitor cocktail (Thermo Scientific) and samples were analyzed by western blotting as previously

described[37]. The following antibodies were used: anti-GAPDH (Life Technologies AM4300), anti-Rev-Erbα (Cell Signaling 13418), anti-BMAL1 (Bethyl Laboratories A302-616A, Montgomery, TX).

Circadian rhythmicity in cultured chondrocytes

Cultured chondrocytes were maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% calf serum supplemented with penicillin and streptomycin. Medium was changed to 0.5% serum 16 h before synchronization with 100 nM dexamethasone. T0 was defined as 24 h after dexamethasone application to the medium. Dexamethasone-containing media was removed and replaced with serum-free media at the time points indicated in figure legends. Cells were collected every four hours for the time course analysis of gene and protein expression. For subsequent experiments with synchronization, cells were collected at T0, T8, T16, T24, T36, and T48 according to the highest and lowest expression time points of *NR1D1* and *BMAL1*.

Small interfering RNA (siRNA) knockdown of NR1D1 and BMAL1 in chondrocytes

siRNAs for *NR1D1* (s18386), *BMAL1* (s1616) and negative control (AM4635) were purchased from Life Technologies. Human normal chondrocytes were transfected with siRNA using Lipofectamine RNAiMAX transfection reagent (Life Technologies) at a concentration of 12.5 pmol/ml. Three siRNAs targeting different regions were tested in advance to knock down *NR1D1* (s18386, 5940, s18387), and the single most effective siRNA (s18387) was used in subsequent experiments. Since we were able to achieve sufficient knock down by the first siRNA tested, we did not further explore other siRNAs for *BMAL1*. Effects of NR1D1 and BMAL1 knock down on CR genes were analyzed by qPCR, and relative gene expressions were compared to a control group treated with negative control siRNA. We also performed a global analysis of gene expression using next generation sequencing of RNA from chondrocytes that had been transfected with NR1D1 and BMAL1 siRNA (harvested at T24 and T12, respectively to account for their peak expression) with corresponding samples treated with control siRNA ($n = 2$ for each time point). NEBNext Ultra Directional RNA Library Prep Kit for Illumina (New England Biolabs, Ipswich, MA) was used for library preparation. RNA sequencing and data analysis was performed as described above for the RNA samples from normal and OA human cartilage using miRNeasy Mini kit (Qiagen). Quality control for the raw data was performed as described above. The raw RNA-seq reads were aligned to the UCSC human hg19 genome using the STAR aligner (v2.3.0)[38]. Read counts were quantified using the Python package HTSeq-Count (v0.6.1) with UCSC RefSeq hg19 annotation (Release 57). Differential expression analysis of the knockdown vs control was conducted on raw count data normalized by the Bioconductor Limma package using the Bioconductor package DESeq2[39]. Genes were considered significantly differentially expressed if they had an adjusted *P* value of <0.05. SPIA was performed on the differentially expressed genes as described above.

Effect of TGF-β1 on CR gene expression

To test the effect of knocking down NR1D1 and BMAL1 on the chondrocyte response to TGF-β, recombinant human TGF-β1 (PeproTech, Rocky Hill, NJ) was added to the culture medium of chondrocytes from four independent donors with normal cartilage at a concentration of 10 ng/ml 48 h after siRNA transfection. Chondrocytes were collected 6 h later for RNA extraction and qPCR as described above. The time point was selected according to a preliminary experiment comparing 6 h and 24 h after TGF-β1 stimulation, resulting in maximum effect at the early time point.

Statistical analysis

The qPCR values and positively stained cell counts in immunohistochemistry were normally distributed and equally variable. The mRNA expression values for NR1D1 and BMAL1 between normal and OA human cartilage, and between cultured chondrocytes with or without TGF-β1 treatment were analyzed by Student's *t*-test. The comparison between groups in rates of NR1D1 and BMAL1 positive cells in immunohistochemistry slides were performed by multiple comparison using ANOVA with Bonferroni adjustment. Ratios of positively stained cells in each group (18−24 months old, 30−36 months old, and surgically induced OA) were compared against 6 months old samples, therefore three hypotheses with a desired $\alpha = 0.05$ were tested. For experiments with cultured chondrocytes treated with siRNA, statistically significant differences in qPCR values were determined with multiple comparison using Dunnett's test. Samples treated with negative control siRNA were used as control condition. When TGF-β1 was added to the culture medium, samples with both TGF-β1 and negative control siRNA treatment, were considered as the control condition. The results are reported as mean ± S.E.M. *P* values less than 0.05 were considered significant.

Results

Expression patterns of clock genes and proteins in normal and OA human cartilage

RNA sequencing data from eight normal and 10 OA human cartilage samples were analyzed for dysregulated pathways and this showed that the CR was the most significantly dysregulated pathway in OA cartilage. Among genes listed in the CR pathway in KEGG data base, the mRNA levels of *NR1D1*, *BHLHE40*, *BMAL1(ARNTL)*, *PER1*, *PRKAG2*, *PER2*, *RORA*, *NPAS2*, and *CRY2* were significantly lower in OA while *RBX1* was significantly increased in OA cartilage (Table I). Among these genes, *NR1D1*, *BHLHE40* and *BMAL1* showed the largest differences between normal and OA, with a log2 fold change >2.4. Since there is a known close interaction between *NR1D1* and *BMAL1* as core clock genes, we chose to further focus on the role of these two genes in cartilage homeostasis.

Quantitative PCR was performed to validate RNA sequencing results on human normal and OA cartilage ($n = 14$ each). The expression levels of *NR1D1* and *BMAL1* were confirmed to be significantly lower in OA cartilage [Fig. 1(A)]. Protein expression of NR1D1 and BMAL1 in cartilage was assessed by immunohistochemistry in both human and mice cartilage. NR1D1 protein was

Table I
Differentially expressed CR pathway genes in normal and OA human cartilage. Among genes listed in KEGG database for CR pathway, NR1D1, BHLHE40, and BMAL1 showed the largest differences between normal and OA cartilage

Gene	Normal	OA	log2FC	q-Value
NR1D1	384.80	56.79	−2.76	0.0030
BHLHE40	405.97	65.06	−2.64	0.0030
BMAL1	8.03	1.51	−2.41	0.0088
PER1	131.68	28.29	−2.22	0.0053
PRKAG2	23.04	6.63	−1.80	0.0030
PER2	17.59	5.68	−1.63	0.0088
RORA	22.94	10.19	−1.17	0.0161
NPAS2	8.82	4.08	−1.11	0.0280
CRY2	16.57	7.89	−1.07	0.0332
RBX1	46.48	108.46	1.22	0.0104
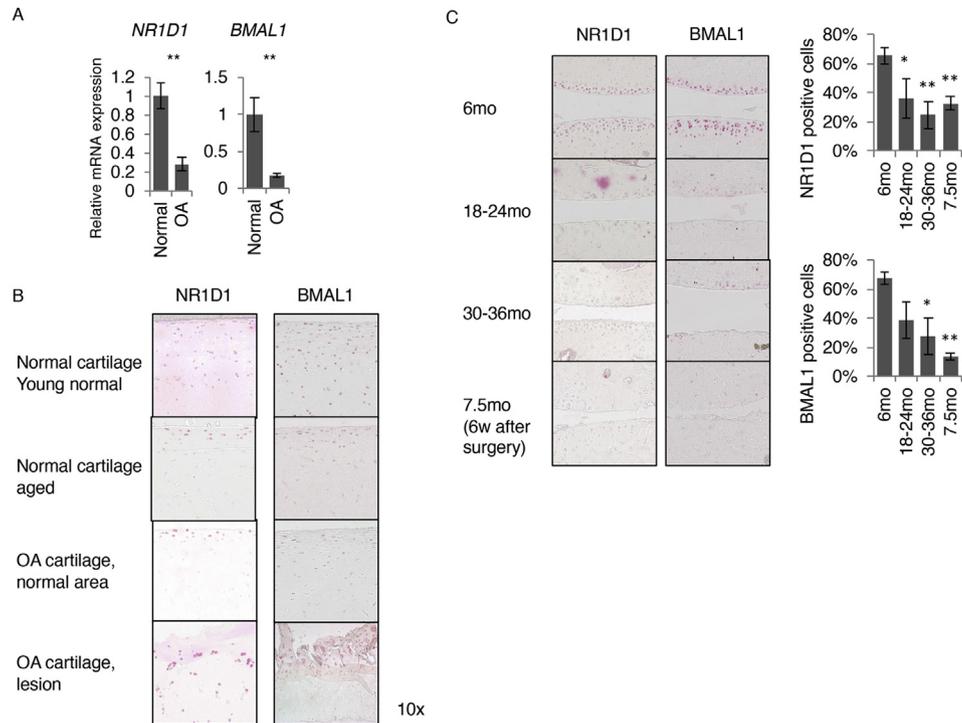
Fig. 1. Expression of NR1D1 and BMAL1 mRNA and protein in cartilage. (A) qPCR was performed on 14 normal and 14 OA human articular cartilage samples (*$P < 0.05$, **$P < 0.01$). (B) NR1D1 and BMAL1 Protein expression was analyzed by immunohistochemistry. Representative images are shown for young normal, aged normal and OA normal appearing and OA lesion area. Images are representative of 5–8 samples for each tissue type. NR1D1 and BMAL1 positive cells were observed predominantly in the superficial and upper-mid zone of normal cartilage. Protein expression was reduced in OA cartilage even in the normal areas, although strong expression was observed in the cluster cells. (C) Immunohisto-chemistry of mouse cartilage. Six mouse joints for each sample type were analyzed. Bar graphs indicate percentages of positive cells. NR1D1 and BMAL1 positive cells were distributed mainly in the superficial to upper-mid zone. Expression levels were significantly reduced with aging and with induction of surgical OA.

expressed throughout the human cartilage tissue, most strongly in the superficial and mid zones [Fig. 1(B)]. In OA cartilage, NR1D1 positive cells were diminished even in areas of preserved full thickness cartilage, with few positive cells remaining in the su-perficial zone. In lesions with fibrillations, strong staining was observed in chondrocyte clusters. In young normal mice, NR1D1 protein distribution was similar as in normal human tissue, with positive cells mainly in the superficial and upper mid zone. Sta-tistically significant differences were detected between 6 months old normal knees and all other groups in NR1D1 expression, while BMAL1 expression was significantly different in 6 months old normal knees and 30–36 months old aged knees, as well as knees with surgically induced OA. Both NR1D1 and BMAL1 pre-sented reduced expression with aging and in surgically induced OA, compared to young normal knees [Fig. 1(C)].

Rhythmicity of gene expression and regulation of clock genes in cultured human chondrocytes

A spontaneous robust circadian rhythmicity was observed in cultured human chondrocytes where *NR1D1* had peak expression at T24 and T48 after synchronization, whereas *BMAL1* fluctuated in a slightly earlier opposite phase with highest expression at T16 and T40 [Fig. 2(A)]. The rhythmicity was confirmed for both NR1D1 and BMAL1 protein expression by western blotting [Fig. 2(B)].

Knock down of *NR1D1* and *BMAL1* was used to examine the interaction of clock genes in cultured chondrocytes. Effective siRNA-mediated knock down in protein levels was confirmed by western blotting for NR1D1 and BMAL1 [Fig. 3(A)]. When trans-fected with specific siRNA 48 h before synchronization, the expression levels of *NR1D1* and *BMAL1* were repressed

throughout the time course up to T48. *NR1D1* still displayed rhythmic fluctuation with reduced peak expression at T24 and T48. *BMAL1* siRNA completely disrupted the rhythmic expression pattern of *BMAL1* [Fig. 3(B)]. *BMAL1* knock down reduced the level of NR1D1 mRNA and protein, and *NR1D1* knock down increased *BMAL1* transcription [Fig. 3(B)]. These changes were mainly observed at T24 and 48, when the expression level of *BMAL1* was low. In relation to other genes in the CR pathway, *NR1D1* knock down increased *BHLHE40* from T16 to T48, while having subtle effect on *PER2*, *RORA*, *RORC*, and *RBX1*. In contrast, *BMAL1* knock down increased *BHLHE40* at T8 and decreased its expression at T24, while the expression level of *PER2* was reduced at T0, T24 and T36. *RORA*, *RORC*, and *RBX1* were not significantly affected by *BMAL1* knock down (Supp.1). These results establish that a cell autonomous circadian rhythmicity exists in cultured chon-drocytes and demonstrate the interdependence of CR genes in their temporal expression patterns.

Genome-wide impact of CR gene knock down in cultured human chondrocytes

To determine the role of CR genes in regulating global gene expression patterns, we used next generation RNA sequencing on normal human articular chondrocytes that were transfected with siRNA against NR1D1 and BMAL1. This unbiased approach revealed 330 and 68 differentially expressed genes, respectively, as compared to control siRNA (Table II). Importantly, a large number of these differentially expressed genes ($n = 50$) were altered by both siRNAs treatments (Supp. Table S2). Most (48 of these 50) genes were up regulated and only two genes were down regulated compared to treatment with control siRNAs. The
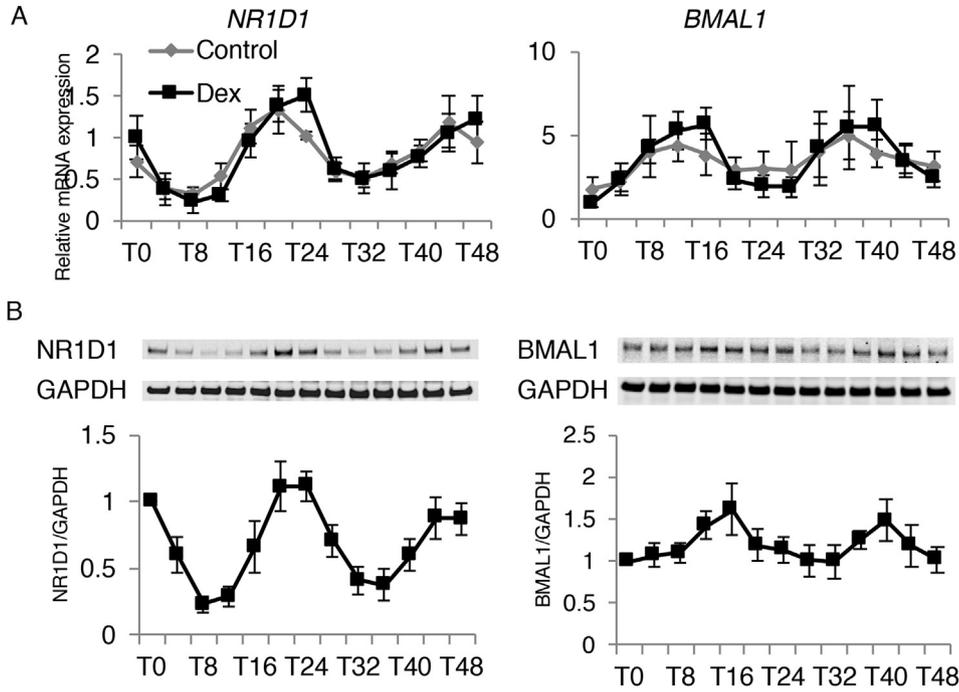
Fig. 2. Rhythmicity of NR1D1 and BMAL1 expression in cultured chondrocytes. (A), (B) Chondrocytes isolated from five individual donors were analyzed with and without dexamethasone (DEX) synchronization. qPCR and western blotting were performed on samples collected at 4 h intervals. T0 indicates 24 h after DEX was added to the culture medium. (A) NR1D1 mRNA expression peaked at T24 and T48, with lowest expression observed at T8 and T32. BMAL1 expression followed a reversed phase. (B) NR1D1 protein fluctuated in an identical pattern with the mRNA expression. BMAL1 protein expression had less fluctuation, with approximately 4 h delayed phase from mRNA expression.
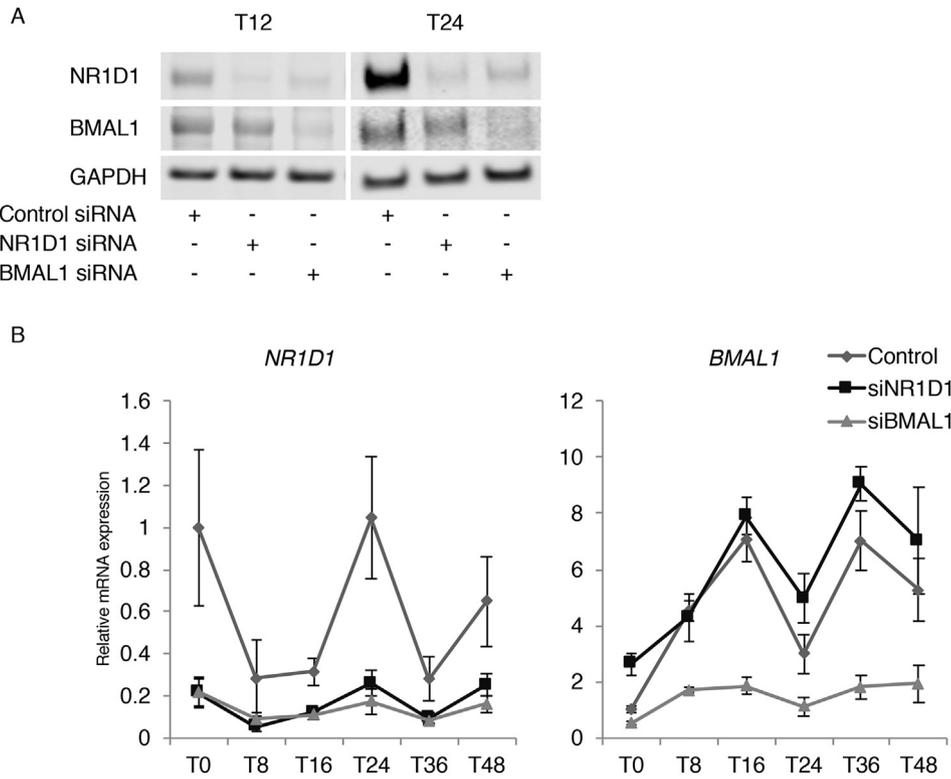
Fig. 3. Effect of NR1D1 and BMAL1 knock down on expression of NR1D1 and BMAL1. (A) Western blotting was performed on cultured chondrocytes from seven individual donors after siRNA transfection, collected at T12 and T24 after DEX synchronization. Effective knock down of both NR1D1 and BMAL1 was confirmed at both time points. Knocking down BMAL1 decreased the expression level of NR1D1, while knocking down NR1D1 had less effect on BMAL1 protein level. (B) qPCR was performed on samples after siRNA transfection, and RNA collected at time points as indicated. *NR1D1* expression was reduced at all time points by knocking down NR1D1 or BMAL1, while still showing rhythmicity. *BMAL1* expression was reduced and dysregulated by knocking down BMAL1, whereas knocking down NR1D1 resulted in increased expression levels of BMAL1 after T24.

Table II
Pathways affected by NR1D1 or BMAL1 knock down. RNA sequencing was performed on samples after siRNA transfection for NR1D1 and BMAL1. Among the differentially expressed pathways between NR1D1 or BMAL1 knock down and control, the TGF-β signaling pathway was significantly altered by both siRNA treatments

NR1D1 knock down (330 differentially expressed genes)	
Pathway name	pG
Protein processing in endoplasmic reticulum	1.89E−06
Viral carcinogenesis	1.03E−05
Oocyte meiosis	0.0016
TGF-beta signaling pathway	0.0017
BMAL1 knock down (68 differentially expressed genes)	
Pathway name	pG
TGF-beta signaling pathway	0.0088
p53 signaling pathway	0.0093
Focal adhesion	0.0103
Retrograde endocannabinoid signaling	0.0103
Oocyte meiosis	0.0131

global probability value from the SPIA was significant for four pathways in NR1D1 knock down, and for five pathways in BMAL1 knock down (Table II). Among the differentially expressed pathways, the TGF-β signaling pathway was significantly altered by both siRNA treatments. Altered expression levels of TGF-β signaling pathway genes were validated by qPCR analysis of RNA from additional chondrocyte cultures that were treated with the siRNAs [Fig. 4(A)]. While expression levels of TGFBR1, TGFB1, TGFB2 and TGFB3 were increased, levels of TGFBR2 and TGFBR3 were reduced.

Interactions between CR genes and TGF signaling in cultured human chondrocytes

TGF-β1 treatment significantly reduced NR1D1 mRNA expression, while increasing BMAL1 mRNA expression level [Fig. 4(B)]. Knocking down NR1D1 or BMAL1 before TGF-β1 treatment affected expression levels of TGF-β pathway genes (Supp. Table S3, Supp. 4) in the same manner as the previously mentioned experiment without TGF-β stimulation. The expression levels of some TGF-β induced genes, such as *ELN* and *TNC* were increased by knocking down NR1D1 or BMAL1 [Fig. 4(C)].

Discussion

Since the report of circadian rhythmicity in the mitosis of epiphyseal cartilage by Simmons[40], several authors have described daily variation in endochondral ossification, extracellular matrix synthesis, chondrocyte proliferation and cartilage growth[41]. Attempts to seek diurnal variations in OA markers have also been made[42], but the correlation between CR and cartilage homeostasis in mature articular cartilage has remained largely unclear. In a recent study, Gossan *et al.* reported that 619 genes (3.9% of the expressed genes) displayed circadian pattern of expression in cartilage. This included genes involved in cartilage homeostasis and survival, as well as genes with potential importance in the pathogenesis of OA. Several clock genes were disrupted in the early stages of cartilage degeneration in a mouse model of OA[30], thus suggesting the role of CR in maintaining cartilage homeostasis. Furthermore, Kc *et al.* reported that environmental disruption of CRs by altered
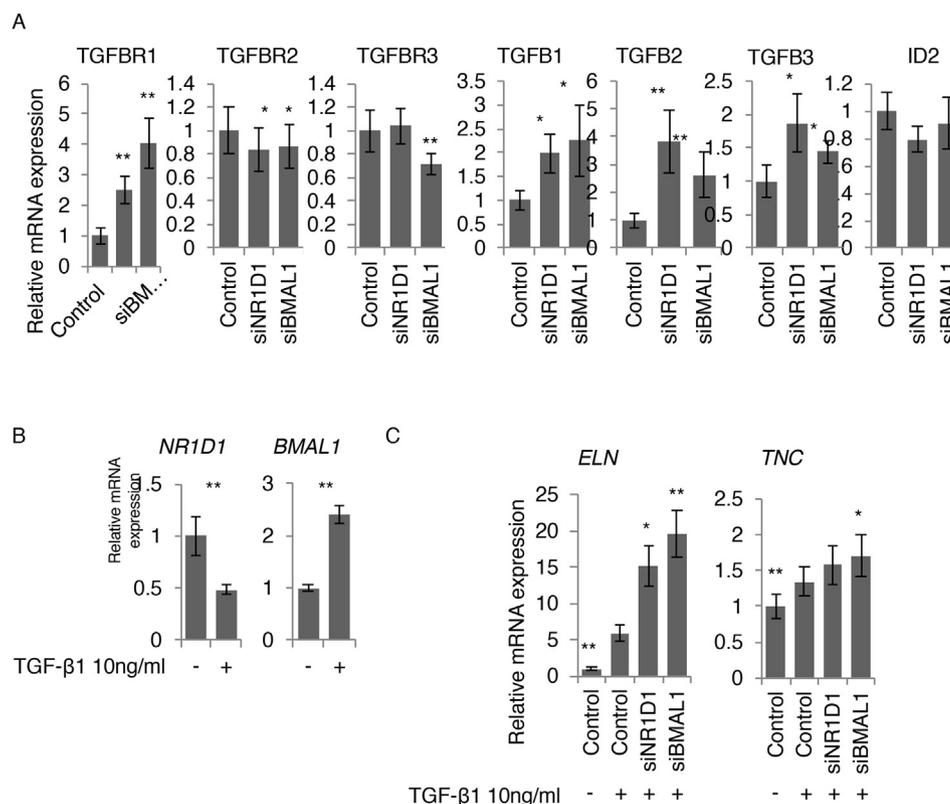
Fig. 4. Correlation of NR1D1 and BMAL1 knock down and TGF-β signaling. (A) qPCR for TGF-β signaling pathway genes was performed on cultured chondrocytes isolated from three individual donors after siRNA transfection for NR1D1 and BMAL1. (B) qPCR for NR1D1 and BMAL1 were performed on cultured chondrocytes isolated from four individual donors after TGF-β1 stimulation. TGF-β1 treatment significantly reduced NR1D1 expression and significantly increased BMAL1 expression. (C) qPCR was performed for ELN and TNC after siRNA transfection with and without TGF-β1 stimulation ($*P < 0.05$, $**P < 0.01$).

light:dark cycle promoted osteoarthritic changes in mouse knee joint[43].

Our results indicate a time-dependent variability of mRNA expression in cultured chondrocytes with a clear circadian rhythmicity up to 48 h with nearly opposite phase in NR1D1 and BMAL1. This observation is in agreement with previous reports using different cell types, as well as mice cartilage[8,22,30,44–47]. We identified lower expression levels of multiple CR genes by genome wide RNA sequencing in OA, and bioinformatics analysis revealed that CR is the most dysregulated pathway in human OA cartilage. Down regulation of NR1D1 and BMAL1 mRNA expression levels in OA cartilage was confirmed by qPCR, and expression of corresponding proteins was reduced with age and with the induction of surgical OA in a mouse model, before severe cartilage degradation occurred. This seems to be inconsistent from the report of Chaturvedi et al., proposing that NR1D1 (Rev-ERbAα) expression was highest among all the nuclear receptors expressed in OA cartilage, thus assuming over expression of this gene to be involved in OA pathogenesis[48]. Our RNA sequencing data also revealed that NR1D1 expression was highest among the 48 nuclear receptors in both normal and OA cartilage (Supp. 5). However, the expression level in OA cartilage was significantly lower than the expression level in normal cartilage (Fig. 1), which the previous report was not able to identify, as it did not include analysis of normal cartilage. Recently, Dudek et al. performed a series of experiments on BMAL1, reporting the rhythmicity of this gene in chondrocytes and showing the effect of knock down and knock out of this gene on cartilage degeneration[49]. Our results are in agreement with their report, further adding data on NR1D1.

Results from previous reports show altered expression levels of CR genes by aging, induction of mechanical stress, TNF-α, and development of OA[30,50,51]. Our results indicate that the CR pathway is overall down regulated in OA. Although each CR gene is potentially affected by external stimuli such as inflammatory mediators, it is surprising that both the positive and negative limbs of the feedback loop are down regulated. Interestingly, nearly 70% of gene expression changes induced by BMAL1 knock down were common to those influenced by NR1D1 knock down, despite its opposing function. The most likely interpretation seems to be that the major impact caused by down regulation of the main positive stimulator BMAL1, due to whatever external cause (e.g., aging, mechanical stress, cytokines) outside the circadian feedback loop, subsequently induces the down regulation of the whole CR pathway. Indeed, down regulation of RORC and RORA, the only positive regulators of BMAL1[52], may contribute to forming a feedback loop leading to further down regulation of BMAL1. Knock down of the negative regulator including NR1D1 would theoretically counteract BMAL1 knock down by stimulating BMAL1 transcription, but the effect seems to be masked by a larger effect of BMAL1 down regulating stimuli. However, our results suggest that most of the final output of BMAL1 knock down is mediated via NR1D1 and other downstream genes, rather than a direct effect of BMAL1.

TGF-β is a potent multi-functional regulator of cell growth and differentiation abundantly expressed in cartilage. The TGF-β pathway has been identified as a key signaling pathway in OA, but evidence for both protective and catabolic roles of TGF-β has been reported[3,53]. TGF-β activation in subchondral bone is suggested to contribute to the development of OA[54], but conversely, loss of TGF-β signaling in cartilage induces chondrocyte hypertrophy and ultimately results in cartilage degeneration[55]. TGF-β is implicated in all stages of chondrogenesis, including mesenchymal condensation, proliferation of chondroblasts and the deposition of cartilage-specific ECM molecules[4].

Our RNA sequencing results on NR1D1 and BMAL1 siRNA treated chondrocytes identified TGF-β signaling pathway as a common differentially expressed pathway. In particular, ELN and TNC, previously reported to be induced by TGF-β[56,57], were among the most overexpressed genes after NR1D1 and BMAL knock down. Our in vitro experiments show that (1) TGF-β signaling genes are affected by NR1D1 and BMAL1 alteration, (2) TGF-β itself alters NR1D1 and BMAL1 expression, (3) alteration in NR1D1 and BMAL1 expression would affect TGF-β downstream gene expressions, such as ELN and TNC. We investigated whether there are potential effects on SMAD signaling but did not observe any difference in the levels of SMAD2/3, Smad1,5,8 or in TGF-β induced SMAD phosphorylation after NR1D1 or BMAL1 knock down.

This link between CR genes and TGF-β signaling is in agreement with previous reports[26,58], and suggest that alterations in circadian rhythmicity may result in abnormal ECM protein synthesis, potentially contributing to OA pathophysiology. However, at this point we were not able to further investigate the direct mechanism of interaction between TGF-β and clock genes, and further research is necessary.

Biomarkers of OA show diurnal variation[42,59–61]. While some of this variation may be attributed to physical activity or food consumption, the present findings that cartilage has an intrinsic CR suggests that it also may be a determinant of OA biomarker fluctuation.

This study has several limitations. First, we were unable to control the time of human sample collection. Since NR1D1 and BMAL1 expression level present large variation across the day, it is possible that the comparison between normal and OA would have been influenced by the time of death and/or sample collection. However, the difference of expression level between normal and OA was significantly large, thus suggesting that the baseline expression level is reduced in OA and this reduction has a bigger impact on cartilage homeostasis than the daily rhythmic change. Secondly, aging is a considerable factor that may have influenced our gene expression level differences between normal and OA human cartilage samples. Gossan et al. presented that the circadian clock becomes less robust during aging and the oscillation amplitude is reduced in aged mice[30]. However, our observation of reduced NR1D1 and BMAL1 protein in mice surgical OA model indicates that the reduction of NR1D1 and BMAL1 occurs by OA progression, independent from aging. Finally, we do not know whether CR gene expression patterns in articular cartilage change after death. However, our samples were collected at an early time point within 72 h after death or resection during knee replacement surgery, and we consider only minimum changes would occur.

In conclusion, we show that expression of NR1D1 and BMAL1 is reduced in OA cartilage. NR1D1 and BMAL1 present circadian rhythmicity in cultured chondrocytes with an opposite phase. Reduced expression of NR1D1 and BMAL1 in chondrocytes affects TGF-β signaling.

Author contributions
ML, RA, TS, AS conceived of the study, and participated in its design and coordination.

RA, MS, KF, AS performed gene and bioinformatics expression analyses.

YA, YM, OA, TT, YT carried out histology, immunohistochemistry and cell culture experiments and performed quantitative analysis.

All authors read and approved the final manuscript.

Dr. Lotz had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

Ethics approval
This study was conducted with the approval of the Human Subjects Committee and the Institutional Animal Care and Use Committee at The Scripps Research Institute.

Conflict of interest

Role of funding

Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.joca.2016.11.007.

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