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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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SUMMARY

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.

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

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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