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ORIGINAL ARTICLE

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Identification of clock as a mechanosensitive gene by large-scale DNA microarray analysis: downregulation in osteoarthritic cartilage

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Abstract The chondrocytes of cartilage have so far been reported to include three mechanoresponsive genes: cartilage matrix protein (CMP, matrilin-1), type X collagen, and Indian hedgehog (Ihh). In fact, all of the genes identified in these chondrocytes are associated solely with mechanical stress. In this study we examined the how mechanical stress influenced the patterns of gene expression of chondrocytes in three-dimensional (3D) sponges in order to better understand the mechanisms that control the chondrocyte phenotype during the development of osteoarthritis. We cultured mouse chondrocytes in 3D sponge as a model of mechanical stress and isolated total RNA for a large-scale DNA microarray analysis covering 12000 genes. We analyzed the pattern of gene expression in relation to gene localization in cellular components such as the cytoplasm, cytoskeleton, and nucleus. Immunoblotting of osteoarthritis cartilage were performed using polyclonal anti-clock antibody. The biological rhythm of mRNA of clock and clock-related genes was analyzed by real-time polymerase chain reaction (PCR). Clock gene was confirmed by real-time PCR to validate the microarray data. Other clock-related genes such as Per1 and Per2 were also expressed in chondrocytes, exhibiting biological rhythm after serum shock. The large-scale DNA microarray indicated that clock and other genes functionally associated with mechanical stress play an essential role in regulating the biological rhythm of chondrocyte metabolism in osteoarthritis cartilage.

Key words Cartilage · Clock · Mechanical stress · Osteoarthritis

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Introduction

Mechanical stress is an important factor to consider in analyses of skeletal development and osteoarthritis. The genes that relate to mechanical stress overall remain to be identified, however. Conventional analyses of the regulation and functions of genes have been driven by highly focused studies targeting individual genes and proteins in a methodical, step-by-step fashion. In DNA microarray, DNA probes representing cDNA clones are arrayed onto glass slides and hybridized with fluorescence-labeled cDNA targets. The microarray technology provides a format for the simultaneous measurement of the expression levels of thousands of genes in a single hybridization assay. Our study was designed to determine changes of the gene expression profile in mouse chondrocytes undergoing mechanical-stretch. We compared the gene expression profiles of chondrocytes after mechanical stretch using cDNA microarrays containing 12000 sequence-verified mouse genes. The results identified an association in with mechanical stress in one gene, that is, clock. Clock, a gene expressed in the brain, heart, lung and peripheral tissue, is confirmed to be regulated by circadian biological rhythm and has a known influence on traits as diverse as body size and temperature on molecular clock.[1,2] Circadian rhythmicity plays important roles not only in basic cellular functions such as membrane excitation and energy metabolism, but also in behavioral events such as sleep–waking cycles. In mammals, all these circadian rhythms are generated at the cellular level by a circadian core oscillator composed of an autoregulatory transcription–translation-based feedback loop involving clock, per1, and per2 genes. Previous studies have not clarified whether the clock genes expressed in chondrocytes are functionally related to mechanical stress. Biological rhythm and associated gene expressions are crucial to the maintenance of a stable body metabolism. In this paper we analyze the role of the clock gene in the regulation of biological rhythm during the development of osteoarthritis.

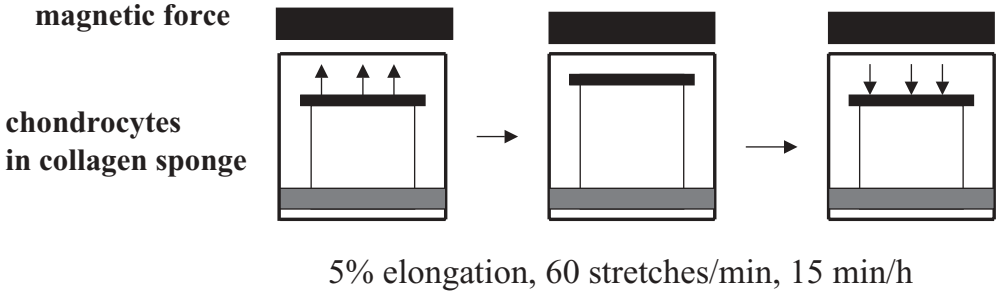

Fig. 1. Mechanical stress induced by magnetic force in chondrocytes in a 3D sponge

Materials and methods

Isolation and culture of newborn mouse chondrocytes

Chondrocytes were isolated from 8 newborn mice within 1 week of birth. After collecting the cells from rib cages digested in collagenase D (3 mg/ml) for 6 h, we seeded them onto plates (1×10^6 cells/plate) and cultured them overnight in DMEM containing 10% FCS and 1% penicillin-streptomycin to prepare a three-dimensional (3D) chondrocyte culture system for mechanical stimulation studies.

Mechanical stimulation of chondrocytes in the 3D chondrocyte culture system

Chondrocytes were cultured in 3D collagen sponges by the same method used in our earlier mechanical stress experiments.[3] The mechanical stress was applied by physiologically stretching the chondrocytes. Briefly, 100 µl of cell suspension was applied to $2 \times 2\times$ 0.25-cm Gelform sponges (Upjohn, Kalamazoo, MI, USA) presoaked with Hanks' balanced salt solution (HBSS). After overnight incubation, the sponges were stretched with an intermittent pattern (5% elongation, 60 stretches/min, 15 min/h) for 4 days with a Bio-stretch device (ICCT Inc., Markham, ON, Canada) for microarray in 10% FCS DMEM (Fig. 1). This procedure was found to generate matrix deformation to a degree comparable with that observed in vivo.[3] The chondrocytes were centrifuged and suspended in plating medium at 1×10^7/ml.

Real-time quantitative polymerase chain reaction (PCR)

Total RNA of chondrocytes cultured in stretched and nonstretched sponges was extracted with an RNeasy mini kit (Qiagen, Hilden, Germany). The mRNAs of clock were quantified by real-time quantitative reverse transcriptase (RT)–PCR with a Perkin–Elmer ABI Prism 7700 sequence detection system to validate the data from the large-scale DNA microarray using the same sample as the microarray. The following primers were used: clock, 5′-TGTCAGA TTAGTAACACCTC-3′ and 5′-TATTATTGGTGGTGC CCTGT-3′; Per1, 5′-ATGAGTGGTCCCCTAGAAGG-3′ and 5′-GCCATTGCTGTTTGCATCAG-3′; Per2, 5′-TGTGTGCGGCTTAGATT-3′ and 5′-TGCTGCTCTTG CACCTTGAC-3′; GAPDH, 5′-CTGGCGCTGATACG TCGGTG-3′ and 5′-GTTCACACCCATGACGAACA-3′. Osteopontin primers are 5′-TGGACGACGATGATGAC GAT-3′ and 5′-TTGGAGTGAAAGTGTCTGCT-3′.

DNA microarray

Upon completing an RT reaction of 5 µg total RNA using aminoallyl dUTP, the cDNA were hydrolyzed and cleaned, then monofunctional dye NHS-esters Cy5 and Cy3 were coupled with cDNA for stretched chondrocytes and cDNA nonstretched chondrocytes (as control), respectively. Twelve thousand BMAP genes were used for the microarray. After removing quenched cye-dyes with a Qiaquick PCR purification kit, the cDNA were hybridized using 20× standard saline citrate, polyA (10 mg/ml), and 10% sodium dodecyl sulfate (SDS) in a microarray chamber overnight. Data collected by scanning with a GenePix 400A scanner were analyzed by IPLab software. Two gene tips were placed on each glass slide, and each experiment was performed in duplicate (i.e., two glass slides and four gene tips).

Biological rhythm by clock, Per1, and Per2 in chondrocytes

After treatment with 50% serum shock, we measured the mRNA expression levels of clock, Per1, and Per2 mRNA using real-time PCR.[4] Mouse chondrocytes (1×10^6) were seeded, washed with phosphate-buffered saline [PBS(–)] three times, incubated with 50% bovine serum for 30 min, washed again, and incubated with 10% Dulbecco's modified Eagle medium for 2, 4, 6, 12, 24, or 48 h. Total RNA was collected from the cultured chondrocytes using an RNeasy kit to analyze the mRNA levels of clock, Per1, Per2, and osteopontin as negative control by real-time PCR.

Immunoblotting

Articular cartilage was collected from eight osteoarthritis patients during total knee arthroplasties with informed consent. The patients were aged from 52 to 71 years old (mean 63.5 years). Four of the patients were assessed as grade II and the other four were assed as grade IV by Kellgren–Lawrence X-ray grading of osteoarthritis.[5] Immunoblotting with anti-clock polyclonal antibody was performed to

compare the levels of protein expressed by the clock gene in cell lysates collected from chondrocytes of the grade II and grade IV osteoarthritis samples. Proteins from the chondrocytes were applied in the same amounts by using anti-CD44 pAb (Bender MedSystems, Vienna, Austria) for control. The samples used for the non-reducing condition were mixed with standard 2× SDS gel loading buffer, while those used for the reducing condition were mixed with a loading buffer containing 5% beta-mercaptoethanol and 0.05 M dithiothreitol. The samples were boiled for 10 minutes before loading onto 10% SDS–polyacrylamide gel electrophoresis (PAGE) gels. After electrophoresis, the proteins were transferred onto Immobilon-PVDF membrane (Millipore, Bedford, MA, USA) in 25 mM Tris, 192 mM glycine, and 15% methanol. The membranes were blocked in 2% bovine serum albumin fraction V (Sigma, St. Louis, MO, USA) in PBS for 30 min and then probed with antibodies. The primary antibody used was an anti-clock polyclonal antibody (diluted 1:5000) (Affinity BioReagents, Golden, CO, USA). Horseradish peroxidase-conjugated goat antirabbit IgG (H + L) (Bio-Rad Laboratories, Melville, NY, USA) at a 1:3000 dilution was used as a secondary antibody. The immunoreactive proteins were visualized by applying ECL Western blotting detection reagents (Amersham, Arlington Heights, IL, USA) and exposing the membrane to Kodak X-Omat AR film. The molecular weights of the immunoreactive proteins were determined against two different sets of protein marker ladders.

Results

Large-scale DNA microarray data

Results from the microarray data analysis for upregulated genes on day 4 indicated the expression of Pak3 binding protein in membrane, ubiquitin-specific protease 25 in the cytoplasm, and transcription factor CA150 in the nuclei (Table 1). The analysis for downregulated genes at the same time point revealed the expression of collagen alpha 2(VI) chain precursor and glutamate receptor, NMDA1 in the membrane, and transcription factor 4 and clock genes in the nuclei (Table 2). Among these, the rate of clock gene downregulation was found to be 0.267 times that after the mechanical stress.

Table 1. Upregulated genes in chondrocytes after 4 days of mechanical stress (grouped by chondrocyte localization)

Location	Description	Ratio
Cytoplasmic	COA transferase	2.118
Cytoplasmic	SEC61a, transport protein	2.018
Cytoplasmic	Ubiquitin specific protease 25	2.002
Membrane	β2 microglobulin	2.154
Membrane	Pak3 binding protein	2.044
Nuclear	TBP-associated factor, RNA polymerase II	2.544
Nuclear	T-cell death associated gene	2.023

Validation of large-scale DNA microarray by real-time-PCR

Next, we conducted real-time PCR studies to validate the accuracy of the microarray data by comparing the mRNA of the clock gene in the nonstretched chondrocytes with that in the genes subjected to mechanical stress in the mRNA samples (0.298 times in the stretched chondrocytes). According to the data from the large-scale DNA microarray, the rate of downregulation of the clock gene in the stretched chondrocytes was 0.267 times that in the nonstretched chondrocytes (Fig. 2). We thus confirmed the accuracy of the microarray analysis for quantifying mRNA changes.

Biological rhythm by clock, Per1, and Per2 in chondrocytes

After the treatment with serum shock, we measured the mRNA expressions of clock, Per1, and Per2 using real-time PCR. We found, as a result, that clock expressed the biological rhythmic curve (Fig. 3a) to almost the same degree as Per1 (Fig. 3b) and Per2 (Fig. 3c). Among these three biological clock genes, Per2 in chondrocytes expressed the biological rhythm with exceptional accuracy over a 24-h period. Our findings thus confirm the function of these genes in regulating metabolism of chondrocytes by setting a biological rhythm. Findings on the mRNA expression of osteopontin in our control study did not suggest the development of biological rhythm in response to treatment of serum shock (Fig. 3d).

Immunoblotting revealed a suppression of the clock gene in severe osteoarthritic cartilage

We selected clock, a protein related to biological rhythm, to investigate whether mechanoresponsive genes are involved in osteoarthritis. The levels of clock protein indicated the expression in the four samples of cartilage with grade II osteoarthritic and downward expression in the four samples

Table 2. Downregulated genes in chondrocytes after 4 days of mechanical stress (grouped by chondrocyte localization)

Location	Description	Ratio
Matrix	Collagen α 2(VI) chain precursor	0.251
Membrane	Glutamate receptor, NMDA1	0.265
Membrane	Cation channel	0.271
Mitochondria	Adenylate kinase 2	0.196
Nuclear	Centromere autoantigen B	0.239
Nuclear	Rhombotin-1 (LIM)	0.257
Nuclear	Transcription factor 4	0.259
Nuclear	Orphan nuclear receptor	0.258
Nuclear	Retinoic acid repressible protein	0.263
Nuclear	Clock	0.267
Nuclear	Transcription factor NF-AT	0.278
Secreted	Semaphorin 3B	0.266

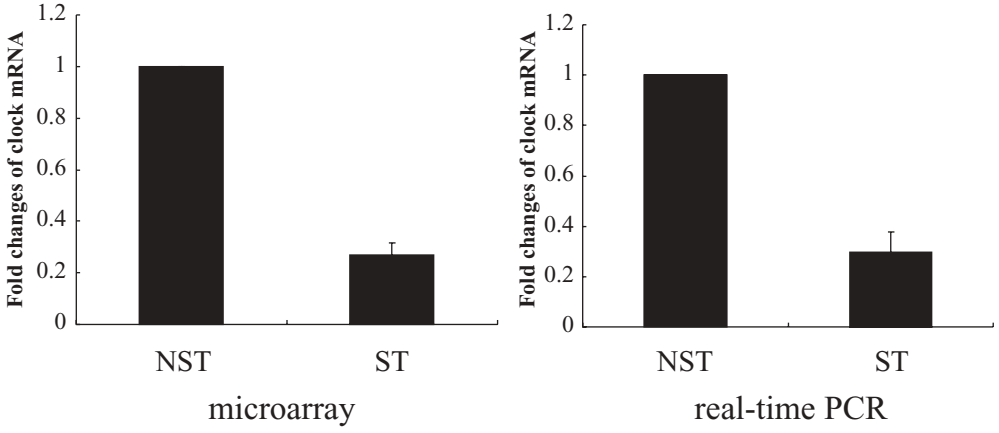

Fig. 2. Validation of large-scale DNA microarray data with clock compared to real-time polymerase chain reaction (*PCR*). *NST*, non-stretched culture; *ST*, stretched culture

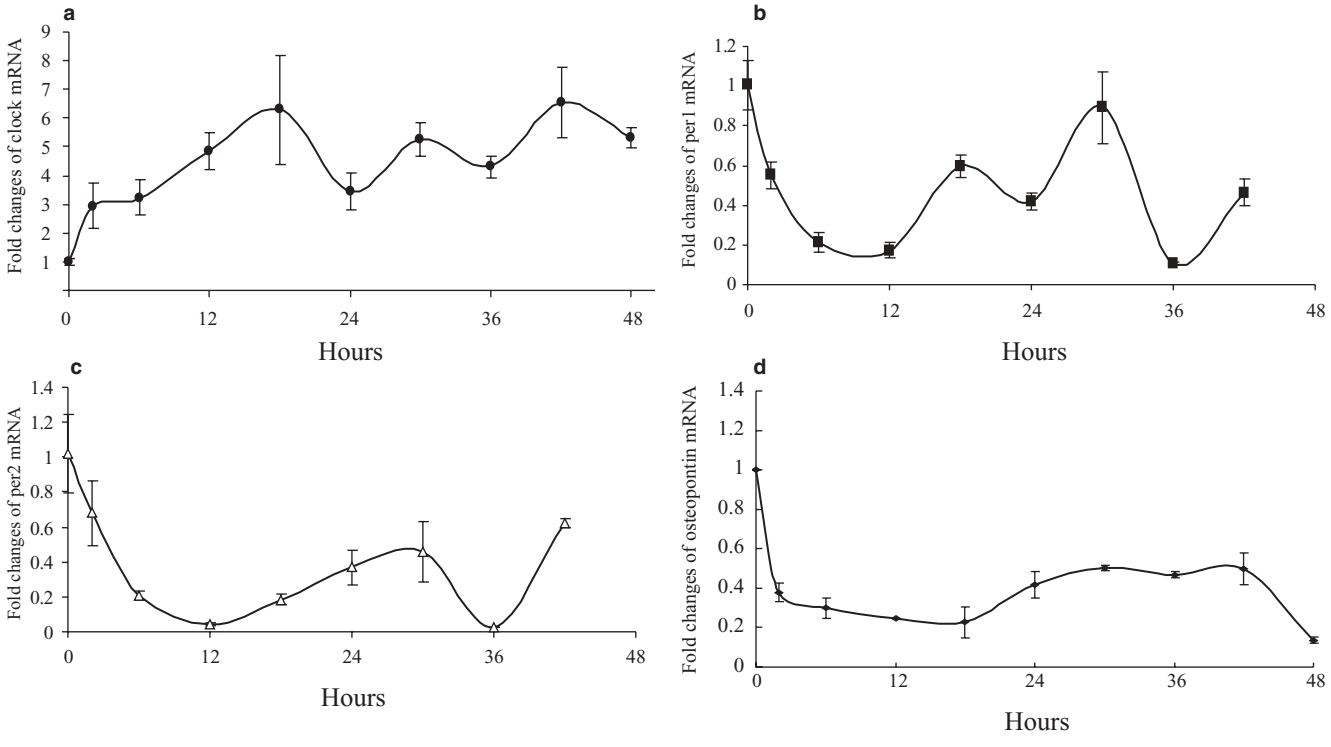

Fig. 3. **a** Biological rhythm of expression of clock mRNA by real-time reverse transcription–polymerase chain reaction (RT–PCR). **b** Biological rhythm of expression of per1 mRNA by real-time RT–PCR. **c** Biological rhythm of expression of per2 mRNA by real-time RT–PCR. **d** Negative control of biological rhythm with osteopontin mRNA by real-time RT–PCR

of cartilage with grade IV osteoarthritis. Thus, clock protein was confirmed to be more suppressed in the cartilage with severe osteoarthritis than in that with mild osteoarthritis cartilage (Fig. 4). Measurements of the densities of the blotting bands indicated a 75% downregulation in the protein level. These data suggest that the level of gene expression is similar to the level of protein expression.

Discussion

In this study we confirmed the role of the clock genes in functional responses to mechanical stress. Clock genes have an established involvement in the physiological changes accompanying jet lag. Perlecan, another biological-clock gene, may play a role in chondrocyte growth and differentiation. In animal studies, perlecan knockout mice have been confirmed to develop chondrodysplasia with dyssegmental ossification. Mice lacking molecular-clock components such as the per genes in osteoblasts display high bone mass, suggesting that bone remodeling may also be subject to circadian regulation.[6] Our group found that clock, a gene regulated by circadian rhythm, was downregulated in the chondrocytes of cartilage with severe osteoarthritis. We thus have reason to believe that the clock gene also plays an important role in maintaining the normal metabolism of chondrocytes.

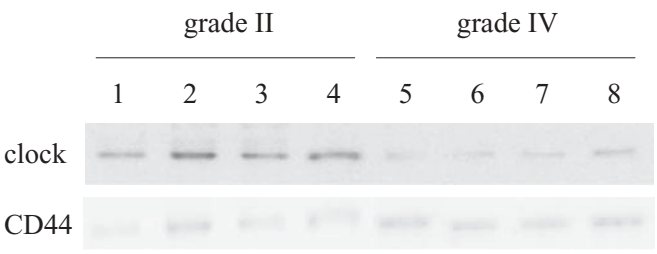

Fig. 4. Immunoblotting with polyclonal anti-clock antibody to cell lysate of osteoarthritis chondrocyte of Kellgren–Lawrence grade II and grade IV

The body is subjected to two types of mechanical stress, physiological and pathological. Having earlier observed a decrease in cell growth at 4 days after the induction of mechanical stress, our group simulated mechanical stress of the pathological type in this study using a bio-stretch machine. We observed downregulation of the clock gene after inducing this mechanical stress and then confirmed the finding by real-time PCR. Taken in sum, these findings suggest that the clock gene may play an important role in regulating physiological function in chondrocytes. In another study performed by mechanically stimulating human articular chondrocytes in vitro, the cells expressed enhanced levels of aggrecan mRNA and decreased levels of matrix metalloprotein (MMP)-3 mRNA.[7] The transduction process involves integrins, stretch-activated ion channels, and interleukin (IL)-4.[7] This chondroprotective response is absent in chondrocytes in osteoarthritis (OA) cartilage. Mechanical abnormalities leading to aberrant chondrocyte activity in diseased articular cartilage may be important in the progression of OA.[7] Other forms of mechanical stress such as hydrostatic pressure (HP) may also influence the cartilage metabolism in normal and pathological conditions, especially in weight-bearing areas of the skeletal system.[8] The incorporation of [^{35}S]sulfate into glycosaminoglycans (GAGs) was 1.3-fold higher and 1.4-fold higher than baseline control in samples exposed to constant HP and cyclic HP, respectively.[9] D'Lima et al. investigated apoptosis after mechanical stress on cartilage to elucidate the mechanisms behind the apoptosis of human chondrocytes observed in response to mechanical injury.[10] Another paper reported that inter-nucleosomal DNA fragmentation, an established marker of apoptosis, was detectable in 34% of chondrocytes at 96 h after mechanical loading at 14 MPa, versus only 4% of the chondrocytes of nonloaded explants.[11] Mechanical stress is known to induce osteoarthritis in articular cartilage. According to our data, clock was down regulated by mechanical stress and subsequently conferred an altered biological rhythm for the maintenance of chondrocyte metabolism. Thus, we found that the suppression of clock expression may contribute to the development of osteoarthritis by altering the cartilage metabolism.

Chondrocytes from deeper zones show higher rates of proliferation and collagen and proteoglycan synthesis than chondrocytes isolated from superficial and middle zones.[12,13] Furthermore, the proteoglycans synthesized by the chondrocytes from the deeper zones have higher keratin sulfate content, while those from the superficial zone are more sensitive to the catabolic effects of IL-1.[14] Progression through each of these phases is accompanied by profound changes in gene expression patterns.[15] Thus, the maintenance of the chondrocyte-specific phenotype plays a crucially important role in preserving the normal structure and biomechanical properties of articular cartilage, both under normal conditions and during repair of injured and diseased tissue. We found that clock was expressed in chondrocytes of the deeper zone by immunohistochemistry (data not shown). This site-specific expression of clock suggests that clock may be functionally involved in the maintenance of cartilage integrity via effects conferred on the chondrocyte metabolism. Among the current hypotheses to explain the changes in osteoarthritic chondrocytes, several investigators have proposed that phenotypic alterations of the cells occur in response to changing signals or matrix composition.[16,17] In histological findings, chondrocyte clusters were detected in the deeper zone of osteoarthritis cartilage. Clusters of chondrocytes in osteoarthritis cartilage have been shown to express type I and III collagens, proteins which can only be found at very low levels, if at all, in the normal articular chondrocytes.[18,19] The study of gene expression and histological change of OA cartilage may have implications for the understanding of the pathogenesis of osteoarthritis.

In conclusion, we have observed a profound change in the patterns of gene expression in chondrocytes cultured in 3D sponges and subjected to mechanical stress. Chondrocyte matrix, cytoplasmic genes, and nuclear genes were all expressed. The clock gene exhibited distinct properties among the genes investigated. In addition to being downregulated by mechanical stress in vitro, the clock gene was found to be downregulated in cartilage with severe osteoarthritis cartilage. Clock, an established regulator of biological rhythm, may therefore play a role in maintaining chondrocyte metabolism in tissues subjected to mechanical stress.

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