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The chondrocyte-intrinsic circadian clock is disrupted in human osteoarthritis

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**Abstract**

Peripheral clocks are essential for driving cell differentiation. In osteoarthritis, loss of the normal differentiated chondrocyte (cartilage cell) phenotype is causative of disease. We investigated whether clock gene expression differed in osteoarthritic compared to “healthy” chondrocytes and used RNAi to determine if the differences observed could affect chondrocyte phenotype. Following serum shock, PER2 expression was significantly higher, whereas BMAL1 expression was significantly lower, in osteoarthritic chondrocytes. Knockdown of BMAL1 in “healthy” chondrocytes was associated with higher cell proliferation and MMP13 expression, features characteristic of the osteoarthritic chondrocyte phenotype. Chondrocyte-intrinsic clock disruption may be a critical early step in osteoarthritis development.
Introduction

Osteoarthritis (OA) is the leading cause of disability in adults worldwide. Despite its prevalence there are currently no disease-modifying therapies to prevent or treat OA. Disease occurs when the resident cartilage cells (chondrocytes) lose their normal differentiated cell phenotype. In OA, chondrocytes re-enter the cell cycle and synthesise excessive amounts of cartilage-degrading matrix metalloproteinases (MMPs). The resultant cartilage loss results in pain and loss of joint mobility (Reviewed in (Goldring 2012)). Understanding the key molecular events governing the change in chondrocyte behaviour seen in OA is critical for understanding the disease process.

During development, peripheral circadian clocks have a fundamental role in controlling lineage-appropriate cell differentiation (Brown 2014). In cartilage, the chondrocyte-intrinsic clock is critical for driving the cell behavioural changes necessary for skeletogenesis (Takarada et al. 2012). Given the apparent crucial role of peripheral clocks in ensuring tissue-specific cell differentiation, we speculated that changes to peripheral clocks may lead to loss of the normal differentiated chondrocyte phenotype. We hypothesise that the chondrocyte-intrinsic clock is disrupted in human OA and that altered clock activity is permissive of the change to the catabolic chondrocyte phenotype central to OA development.

Materials and Methods

Tissue collection and cell culture

Tibial plateau were collected from seven OA patients undergoing total knee arthroplasty at the Nuffield Orthopaedic Centre, Oxford, United Kingdom or at Palmerston North Hospital, Palmerston North, New Zealand. Ethical approval was
Measurement of expression of circadian clock and OA-associated extracellular matrix components

Chondrocytes isolated from undamaged and damaged cartilage from each patient (n=7 patients) were plated in basal media at 50,000 cells/ml in standard, uncoated 96-well tissue culture plates for 18h to allow cells to adhere to the tissue culture plates. Cells were then serum starved by culturing in DMEM without FBS for 24h. Following serum starvation, cDNA was prepared from a subset of chondrocytes from each patient (t=0h) using a cells-to-cDNA kit as per the manufacturer’s instructions (Ambion, Austin, TX). Remaining chondrocytes were media changed to basal medium. At four-hourly intervals over a 24h period, subsets of the cultured chondrocytes (n=7 patients) were harvested for cDNA synthesis. mRNA levels of BMAL1, CLOCK, PER1, PER2, CRY1, CRY2, AGCN (aggrecan), Col2a1 (Type 2 collagen) and MMP13 (matrix
metalloproteinase 13) were measured by real time RT-qPCR (SYBR green) and commercially-available primers (Qiagen, UK).

**Real time quantitative PCR**

Real-time RT-qPCR reactions were performed using a ViiA7 (Life Technologies, Paisley, UK) and a LightCycler (Hoffman-LaRoche, Basel, Switzerland). Samples were run in duplicate with a coefficient of variation between duplicates of <1.0 cycle. Analysis was carried out using the delta-delta cT method (Livak&Schmittgen 2001).

**RNAi-mediated gene silencing**

Chondrocytes isolated from undamaged cartilage (n= 3 patients, age range 53-75yrs) were serum-starved for 24h then cultured for 18h in serum-free DMEM containing 0.5% lipofectamine RNAimax (Life Technologies, Paisley UK) and 0.03pmol/μl siRNA (GE Healthcare, Fairfield, Connecticut, USA). Cells were cultured for a further 24h in basal medium for gene expression studies or 48h for cell proliferation assays.

**Determination of chondrocyte phenotype**

Compared to healthy articular chondrocytes, OA chondrocytes have a higher rate of cell proliferation and demonstrate markedly higher synthesis of matrix metalloproteinase 13 (MMP13), the enzyme largely responsible for the cartilage destruction in OA (Goldring 2012). To monitor chondrocyte phenotype, cell proliferation was measured using the Click-it® EdU AlexaFluor®-555 Imaging Kit following the manufacturer's instructions (Life Technologies, Paisley, UK). Expression of MMP13 was measured using real time RT-qPCR using commercially available primers (Qiagen, UK).
Statistical Analysis

Expression of clock genes and chondrocyte-specific markers in chondrocytes from undamaged and damaged cartilage over a 24h period were analysed using the general linear model with post-hoc Tukey-Kramer testing to determine the effect of time, disease and the interaction of time and disease using SAS 9.1® software, SAS Institute, Carey, N.C., USA. As a result, time-series data is expressed as least squared mean (LSMEAN) ± standard error. Time-series data for BMAL1 were log-transformed to avoid heteroscedasticity. Data for all other genes in the time series experiments conformed to the requirements of the general linear model without the need for transformation.

Data from the RNAi experiments were analysed by t-test using Prism 5.0, GraphPad Software, San Diego, California and are expressed as mean ± SD. A $p$-value of ≤0.05 was considered significant for all analyses.

Results

Expression of components of the circadian clock differs between human chondrocytes isolated from undamaged and damaged regions of cartilage

A circadian pattern of expression of all core clock genes was observed in chondrocytes isolated from damaged and undamaged regions of cartilage. No significant differences in expression of CRY1 (Fig 1a), CRY2 (Fig 1b), CLOCK (Fig 1c) or PER1 (Fig 1d), were observed between cells isolated from undamaged versus damaged tissue. In contrast, expression of PER2 was 3-fold higher at t=0h ($p=0.001$) and 1.7-fold higher at t=4h ($p=0.03$, Fig 1e) in chondrocytes isolated from damaged
compared to undamaged tissue. Peak expression of *BMAL1* (at t=8h) was 2.6-fold lower in chondrocytes isolated from damaged compared to undamaged tissue ($p=0.05$, Fig 1f).

*Expression of cartilage extracellular matrix components changes over a 24h period and differs between chondrocytes isolated from undamaged and damaged regions of cartilage.*

A key feature distinguishing OA chondrocytes from normal articular chondrocytes is increased production of extracellular matrix components and matrix-degrading enzymes. We found expression of *AGCN* (Fig 2a), *COL2A1* (Fig 2b) and *MMP13* (Fig 2c) varied over a 24h period in chondrocytes isolated from both damaged and undamaged cartilage. Expression of *AGCN* was 1.9-fold higher immediately following serum starvation (0h, $p=0.001$) and 2.1-fold higher 4h following serum starvation ($p=0.002$) in chondrocytes isolated from damaged cartilage compared to those isolated from undamaged cartilage (Fig 2a). Similarly expression of *COL2A1* was 3.4-fold higher at 0h ($p=0.006$) and 5.5-fold higher at 4h ($p=0.0002$) following serum starvation in chondrocytes isolated from damaged compared to undamaged cartilage (Fig 2b). In contrast, there was no significant difference in level of expression of *MMP13* immediately following serum starvation (0h) in chondrocytes isolated from the two tissue regions however expression was 2.3-fold higher in chondrocytes from damaged cartilage 8h following serum starvation ($p<0.0001$).

*Knockdown of BMAL1 in primary human chondrocytes results in increased cell proliferation and increased MMP13 gene expression.*
Given that *BMAL1* knockout mice are prone to arthropathy (Kondratov et al. 2006), we hypothesised that a reduction in *BMAL1* expression in chondrocytes may cause at least some of the changes in chondrocyte behaviour seen in OA. Using RNAi we knocked down *BMAL1* (si*BMAL1*) in chondrocytes isolated from macroscopically normal (undamaged) cartilage (Figure 3a). Compared to healthy adult chondrocytes, OA chondrocytes display an increased level of cell proliferation and increased expression of the cartilage-degrading enzyme matrix metalloproteinase 13 (MMP13) (Goldring 2012). We found level of cell proliferation was 20% higher (*p*=0.01) (Figure 3b) and expression of *MMP13* 6-fold higher in si*BMAL1*-treated chondrocytes compared to controls transfected with non-targeting siRNA (*p*=0.02) (Figure 3c).

**Discussion**

To our knowledge this is the first study to demonstrate that the chondrocyte-intrinsic circadian clock is altered in human OA and that BMAL1 appears to have a role in maintaining the normal differentiated chondrocyte phenotype.

Knee OA commonly develops in a localised region of tibial plateau cartilage before spreading to encompass much if not all of the cartilage in the joint. This regional pattern of disease development means that both macroscopically normal cartilage (containing chondrocytes displaying the "healthy" phenotype) and visibly damaged cartilage (containing chondrocytes displaying the "osteoarthritic" phenotype) is present within the tissue obtained from each patient (Snelling et al. 2014). In the present study, expression of the core clock genes was compared between chondrocytes isolated from the two cartilage regions in tissue obtained from seven patients. Following clock re-setting by serum-shock we found that peak expression of *BMAL1* was 2.6-fold lower
whereas peak expression of \textit{PER2} was 1.7-fold higher in chondrocytes isolated from damaged compared to undamaged cartilage. Whether these differences reflect a change in peak amplitude or a change in the phasing of expression of these two genes is yet to be determined. However this study demonstrates that differences in the expression of peripheral clock components can occur between cells within different regions of the same tissue. This finding suggests the existence of a highly localised means of controlling clock gene expression in peripheral tissues allowing significant differences in clock gene expression between cells located in relatively close proximity to one another within a tissue.

That \textit{BMAL1} expression was found to be altered in chondrocytes isolated from damaged (OA) cartilage in the present study is particularly salient as \textit{BMAL1} knockout mice develop multiple degenerative conditions including arthropathy (Kondratov et al. 2006). Our finding that knockdown of \textit{BMAL1} in chondrocytes isolated from undamaged cartilage (which display the “healthy” phenotype \textit{in vitro}) resulted in higher levels of cell proliferation and higher \textit{MMP13} expression (phenotypic changes characteristic of OA) suggests that reduced \textit{BMAL1} expression may be a causative factor contributing to the loss of normal chondrocyte phenotype in human arthropathy.

There is substantial data to indicate that the circadian clock controls cell proliferation (Khapre et al. 2010, Masri et al. 2013). However the effect of \textit{BMAL1} on proliferation appears to be context-dependent. Both knockdown (Khapre et al. 2014, Bouchard-Cannon et al. 2013), as well as over-expression (Lin et al. 2013) of \textit{BMAL1} have been associated with increased cell proliferation in various cell types. These apparent conflicting results are likely a reflection of the complexity of cell cycle regulation. Both
the mTOR (mammalian/mechanistic target of rapamycin) (Khapre et al. 2014) and Wnt (Lin et al. 2013) signalling pathways are known to interact with BMAL1. The level of activity of these, and other cell cycle regulating pathways, at the time of BMAL1 knockdown or over-expression is likely to be important for determining the overall effect of altered BMAL1 levels on cell proliferation.

Interestingly, we found that expression of two key cartilage matrix components, type 2 collagen and aggrecan, showed time-of-day fluctuations indicating cartilage matrix synthesis is under circadian control. This is conducive with previous reports of a circadian pattern of expression for several genes encoding cartilage-degrading enzymes in primary murine chondrocytes as well as a human chondrocyte cell line including MMP14, ADAMTS4 (A Disintegrin and Metalloproteinase with Thrombospondin Motifs 4) and ADAMTS9 (Gossan et al. 2013). In the present study we found that expression of MMP13, the major cartilage-degrading enzyme implicated in OA (Goldring 2012), varied over the course of a 24h period suggesting it is also under circadian control. Recently BMAL1 knockdown has been shown to lead to increased MMP13 expression in murine cardiomyocytes (Ingle et al. 2015). Our finding that BMAL1 knockdown also leads to increased expression of MMP13 in human chondrocytes suggests that disrupted circadian clock-mediated control of extracellular matrix degradation may be an important contributor to OA pathogenesis. Gossan et. al. (2013) also observed that expression of both Stat3 and TGFβR3 oscillated over a 24h period in primary murine chondrocytes (Gossan et al. 2013). The products of both of these genes are involved in transcriptional regulation of several cartilage degrading enzymes including MMP13 and MMP14 providing a possible mechanism for circadian control of cartilage turnover (Gossan et al. 2013).
Disruption to the chondrocyte-intrinsic circadian clock may represent a novel mechanism enabling the switch to the abnormal chondrocyte phenotype which is a critical event in OA development. Peripheral circadian clocks are known to have an essential role in ensuring tissue-appropriate cell differentiation (Brown 2014). Findings from the present study indicate that the cell-intrinsic circadian clock is also important for maintenance of phenotype in differentiated cells; a discovery likely to have implications for understanding the pathogenesis of a variety of degenerative diseases.

**Declaration of Interest**

All authors declare no conflict of interest. This study was funded by the Palmerston North Medical Research Foundation, Arthritis Research UK (20087) and the NIHR Oxford Musculoskeletal Biomedical Research Unit.

**References**


Figure Legends

**Figure 1** Expression of the core components of the molecular circadian clock differs in chondrocytes isolated from damaged compared to undamaged mRNA levels of A CRY1 B CRY2 C CLOCK D PER1 E PER2 and F BMAL1 in chondrocytes isolated from undamaged and damaged cartilage following re-feeding after serum starvation. Data are expressed as LSMEANS ± SE. Significant differences between chondrocytes from undamaged and damaged tissue are indicated by the symbol *.

**Figure 2** Expression of cartilage extracellular matrix components differs between chondrocytes isolated from damaged compared to undamaged tissue and varies over a 24h period mRNA levels of A aggrecan B type 2 collagen (COL2A1) and C matrix metalloproteinase 13 (MMP13) following serum starvation in chondrocytes isolated from damaged compared to undamaged cartilage. Data are expressed as LSMEANS ± SE. Significant differences between chondrocytes from undamaged and damaged tissue are indicated by the symbol *.

**Figure 3** Knockdown of BMAL1 in human chondrocytes results in phenotypic changes similar to those observed in osteoarthritis.

A Expression of BMAL1 in chondrocytes isolated from undamaged cartilage following transfection with BMAL1 siRNA (siBMAL1) or non-targeting control siRNA (siCntrl). B Level of cell proliferation (determined by the percentage of 5-ethynyl-2-deoxyuridine (EdU)-positive cells) and C mRNA levels of MMP13 in siBMAL1-and siCntrl-transfected chondrocytes. Data are expressed as mean ± SD. Significant differences are indicated by the symbol *.