

1 **The chondrocyte-intrinsic circadian clock is disrupted in human osteoarthritis**

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1 **Abstract (100 words max for short communication)**

2 Peripheral clocks are essential for driving cell differentiation. In osteoarthritis, loss of
3 the normal differentiated cartilage cell (chondrocyte) phenotype is causative of
4 disease. We investigated whether clock gene expression was altered in osteoarthritic
5 chondrocytes and used RNAi to determine if changes in the chondrocyte-intrinsic
6 clock altered chondrocyte phenotype. Following serum shock, *PER2* expression was
7 higher whereas *BMAL1* expression was lower in osteoarthritic chondrocytes.
8 Knockdown of *BMAL1* in “healthy” chondrocytes was associated with higher cell
9 proliferation and *MMP13* expression, features characteristic of the OA chondrocyte
10 phenotype. Chondrocyte-intrinsic clock disruption may be a critical early step in the
11 development of OA.

12

1 **Introduction (5000 words total article for short communication)**

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

11
12 During development, peripheral circadian clocks have a fundamental role in
13 controlling lineage-appropriate cell differentiation (Brown 2014). In cartilage, the
14 chondrocyte-intrinsic clock is critical for driving the cell behavioural changes
15 necessary for skeletogenesis (Takarada et al. 2012). Given the apparent crucial role
16 of peripheral clocks in ensuring tissue-specific cell differentiation, we speculated that
17 changes to peripheral clocks may lead to loss of the “normal” differentiated
18 chondrocyte phenotype. We hypothesise that the chondrocyte-intrinsic clock is
19 disrupted in human OA and that altered clock activity is permissive of the change to
20 the catabolic chondrocyte phenotype central to OA development.

21

22 **Materials and Methods**

23 *Tissue collection and cell culture*

24 Tibial plateau were collected from seven OA patients undergoing total knee
25 arthroplasty at the Nuffield Orthopaedic Centre, Oxford, United Kingdom or at

1 Palmerston North Hospital, Palmerston North, New Zealand. Ethical approval was
2 granted by the Oxfordshire Research Ethics Committee (09/H0606/11) and Massey
3 University Human Ethics Committee Southern A (13/83). Patients were male and
4 female aged 53ys-82yrs. Chondrocytes were isolated from both macroscopically
5 normal (undamaged) and visibly damaged (osteoarthritic) cartilage from each patient
6 by collagenase digestion using standard protocols. Thus seven paired samples of
7 “osteoarthritic” and “normal” chondrocytes were obtained. Chondrocytes were used
8 at P0 and P1 for experiments. Unless otherwise stated, cells were cultured in basal
9 medium (DMEM with 10% FBS) at 37°C in 5% CO₂, 95% air.

10

11 *Measurement of circadian clock gene expression*

12 Chondrocytes isolated from undamaged and damaged cartilage from each patient
13 (n=7 patients) were plated in 96-well plates. Following 24h serum starvation, cDNA
14 was prepared from a subset of chondrocytes from each patient (t=0h) using a cells-
15 to-cDNA kit as per the manufacturer's instructions (Ambion, Austin, TX). Remaining
16 chondrocytes were media changed to basal medium. At four-hourly intervals over a
17 24h period, subsets of the cultured chondrocytes (n=7 patients) were harvested for
18 cDNA synthesis. mRNA levels of *BMAL1*, *CLOCK*, *PER1*, *PER2*, *CRY1* and *CRY2*
19 were measured by real time RT-qPCR (SYBR green) and commercially-available
20 primers (Qiagen, UK).

21

22 *Real time quantitative PCR*

23 Real-time RT-qPCR reactions were performed using a ViiA7 (Life Technologies,
24 Paisley, UK) and a LightCycler (Hoffman-LaRoche, Basel, Switzerland). Samples

1 were run in duplicate with a coefficient of variation between duplicates of <1.0 cycle.
2 Analysis was carried out using the delta-delta cT method (Livak&Schmittgen 2001).
3

4 *RNAi-mediated gene silencing*

5 Chondrocytes isolated from undamaged cartilage (n= 3 patients) were serum-starved
6 for 24h then cultured for 18h in serum-free DMEM containing 0.5% lipofectamine
7 RNAimax (Life Technologies, Paisley UK) and 0.03pmol/μl siRNA (GE Healthcare,
8 Fairfield, Connecticut, USA). Cells were cultured for a further 24h in basal medium
9 for gene expression studies or 48h for cell proliferation assays.
10

11 *Determination of chondrocyte phenotype*

12 Cell proliferation was measured using the Click-it® EdU AlexaFluor®-555 Imaging Kit
13 following the manufacturer's instructions (Life Technologies, Paisley, UK).
14 Expression of *MMP13* was measured using real time RT-qPCR using commercially
15 available primers (Qiagen, UK).
16

17 *Statistical Analysis*

18 RNA levels of clock genes were analysed by repeated measures analysis with post-
19 hoc Tukey-Kramer testing using SAS 9.1®, SAS Institute, Carey, N.C., USA. Data
20 for *BMAL1* were log-transformed to ensure compliance with the general linear model.
21 All other data were analysed by t-test using Prism 5.0, GraphPad Software, San
22 Diego, California. A *p*-value of ≤0.05 was considered significant.
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1 Results

2 *Expression of components of the circadian clock differs between human* 3 *chondrocytes isolated from undamaged and damaged regions of cartilage*

4 A circadian pattern of expression of all core clock genes was observed in
5 chondrocytes isolated from damaged and undamaged regions of cartilage. No
6 significant differences in expression of *CRY1* (Fig 1a), *CRY2* (Fig 1b), *CLOCK* (Fig
7 1c) or *PER1* (Fig 1d), were observed between cells isolated from undamaged versus
8 damaged tissue. In contrast, expression of *PER2* was 3-fold higher at t=0h ($p=0.001$)
9 and 1.7-fold higher at t=4h ($p=0.03$, Fig 1e) in chondrocytes isolated from damaged
10 compared to undamaged tissue. Peak expression of *BMAL1* (at t=8h) was 2.6-fold
11 lower in chondrocytes isolated from damaged compared to undamaged tissue
12 ($p=0.05$, Fig 1f).

13 14 *Knockdown of BMAL1 in primary human chondrocytes results in increased cell* 15 *proliferation and increased MMP13 gene expression.*

16 Given that *BMAL1* knockout mice are prone to arthropathy (Kondratov et al. 2006),
17 we hypothesised that a reduction in *BMAL1* expression in chondrocytes may cause
18 at least some of the changes in chondrocyte behaviour seen in OA. Using RNAi we
19 knocked down *BMAL1* (si*BMAL1*) in chondrocytes isolated from macroscopically
20 normal (undamaged) cartilage (Figure 2a). Compared to healthy adult chondrocytes,
21 OA chondrocytes display an increased level of cell proliferation and increased
22 expression of the cartilage-degrading enzyme matrix metalloproteinase 13 (MMP13).
23 We found level of cell proliferation was 20% higher ($p=0.01$) (Figure 2b) and
24 expression of *MMP13* 6-fold higher in si*BMAL1*-treated chondrocytes compared to
25 controls transfected with non-targeting siRNA ($p=0.02$) (Figure 2c).

1 Discussion

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

5
6 Knee osteoarthritis commonly develops in a localised region of tibial plateau
7 cartilage before spreading to encompass much if not all of the cartilage in the joint.

8 This regional pattern of disease development means that both macroscopically
9 normal cartilage (containing chondrocytes displaying the “healthy” phenotype) and
10 visibly damaged cartilage (containing chondrocytes displaying the “osteoarthritic”
11 phenotype) is present within the tissue obtained from each patient (Snelling et al.
12 2014). In the present study, expression of the core clock genes was compared
13 between chondrocytes isolated from the two cartilage regions in tissue obtained from
14 seven patients. Four hours following clock re-setting by serum-shock we found that
15 expression of *BMAL1* was 2.6-fold lower whereas expression of *PER2* was 1.7-fold
16 higher in chondrocytes isolated from damaged compared to undamaged cartilage.
17 Whether these differences reflect a change in peak amplitude or a change in the
18 phasing of expression of these two genes is yet to be determined. However this
19 study demonstrates that differences in the expression of peripheral clock
20 components can occur between cells within different regions of the same tissue. This
21 finding suggests the existence of a highly localised means of controlling clock gene
22 expression in peripheral tissues allowing significant differences in clock gene
23 expression between cells located in relatively close proximity to one another within a
24 tissue.

25

Commented [SS1]: I think this is really interesting!

1 That *BMAL1* expression was found to be altered in chondrocytes isolated from
2 damaged (OA) cartilage in the present study is particularly salient as *BMAL1*
3 knockout mice develop multiple degenerative conditions including arthropathy
4 (Kondratov et al. 2006). Our finding that knockdown of *BMAL1* in chondrocytes
5 isolated from undamaged cartilage (which display the phenotype of healthy articular
6 chondrocytes) resulted in higher levels of cell proliferation and higher *MMP13*
7 expression (phenotypic changes typical of OA) suggests that reduced *BMAL1*
8 expression may contribute to the loss of normal chondrocyte development of human
9 arthropathy.

10
11 There is substantial data to indicate that the circadian clock controls cell proliferation
12 (Khapre et al. 2010, Masri et al. 2013). However the effect of *BMAL1* on proliferation
13 appears to be context-dependent. Both inhibition (Khapre et al. 2011) as well as
14 promotion (Zeng et al. 2014) of cell proliferation have been observed following
15 *BMAL1* knockdown. Findings from the present study may aid in understanding the
16 complexity of *BMAL1*-mediated cell cycle control given that our knockdown
17 experiments were performed in human chondrocytes isolated from undamaged
18 cartilage, a population of cells which are normally quiescent.

19
20 A circadian pattern of expression has previously been observed for several genes
21 encoding cartilage-degrading enzymes in a murine chondrocyte cell line (Gossan et
22 al. 2013). More recently *BMAL1* knockdown has been shown to lead to increased
23 *MMP13* expression in murine cardiomyocytes (Ingle et al. 2015). Given that *MMP13*
24 has a major role in the cartilage degradation in OA, our finding that *BMAL1*
25 knockdown also leads to increased expression of *MMP13* in human chondrocytes

1 suggests that disrupted circadian clock-mediated control of extracellular matrix
2 degradation may be an important contributor in OA pathogenesis.

3
4 Disruption to the chondrocyte-intrinsic circadian clock may represent a novel
5 mechanism enabling the switch to the abnormal chondrocyte phenotype which is a
6 critical event in OA development. Peripheral circadian clocks are known to have an
7 essential role in ensuring tissue-appropriate cell differentiation. Findings from the
8 present study indicate that the cell-intrinsic circadian clock is also important for
9 maintenance of phenotype in differentiated cells.

10

11 **Declaration of Interest**

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

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17 **Figure Legends (2)**

18 ***Figure 1 Expression of the core components of the molecular circadian clock***
19 ***differs in chondrocytes isolated from damaged compared to undamaged***
20 ***cartilage.***

21 Primary human chondrocytes were isolated from macroscopically normal
22 (undamaged) and osteoarthritic (damaged) regions of cartilage obtained from
23 osteoarthritic patients undergoing knee arthroplasty (n=7). Following serum
24 starvation, cells were collected at 4 hourly intervals over a 24h period and

1 expression of core circadian clock genes analysed by real time RT-qPCR. mRNA
2 levels of **A** *CRY1* **B** *CRY2* **C** *CLOCK* and **D** *PER1* were markedly similar between
3 chondrocytes isolated from damaged and undamaged cartilage. However mRNA
4 levels of **E** *PER2* at 0h and 4h were significantly higher ($p=0.001$ and $p=0.03$
5 respectively) and mRNA levels of **F** *BMAL1* were significantly lower at 8h ($p=0.05$) in
6 chondrocytes isolated from damaged compared to undamaged cartilage. Data are
7 expressed as LSMEANS \pm SE.

8

9 **Figure 2 Knockdown of *BMAL1* in human chondrocytes results in phenotypic**
10 **changes similar to those observed in osteoarthritis.**

11 Primary human chondrocytes isolated from undamaged cartilage (n=3 patients) were
12 transfected with siRNA targeting *BMAL1* (si*BMAL1*) or non-targeting control siRNA.
13 **A** Expression of *BMAL1* as determined using real time RT-qPCR was significantly
14 lower in si*BMAL1*-transfected cells compared to controls ($p=0.007$). **B** Level of cell
15 proliferation (determined by the percentage of 5-ethynyl-2-deoxyuridine (EdU)-
16 positive cells) was significantly higher 48h post-transfection in cells transfected with
17 si*BMAL1* compared to controls ($p=0.01$). **C** mRNA levels of *MMP13* were
18 significantly higher in si*BMAL1* cells compared to controls ($p=0.02$) 24h post-
19 transfection. Data are expressed as mean \pm SD.

20