REVIEW

Timing metabolism in cartilage and bone: links between circadian clocks and tissue homeostasis

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Abstract

The circadian system in mammals is responsible for the temporal coordination of multiple physiological and behavioural processes that are necessary for homeostasis. In the skeleton, it has long been known that metabolic functions of chondrocytes, osteoblasts and osteoclasts exhibit intrinsic circadian rhythms. In addition, results from animal models reveal a close connection between the disruption of circadian rhythms and skeletal disorders such as rheumatoid arthritis, osteoarthritis and osteoporosis. In this review, we summarise the latest insights into the genetic and biochemical mechanisms linking cartilage and bone physiology to the circadian clock system. We also discuss how this knowledge can be utilised to improve human health.

Introduction

Circadian (from the Latin *circa diem*, meaning 'about a day') rhythms in behaviour and physiology are a hallmark of life on earth. The 24-h environmental cycles generated by the planet’s rotation around its axis have been wired into the molecular machinery of cells from all domains of life (Bell-Pedersen et al. 2005). This endogenous timekeeping mechanism, known as circadian clock, allows organisms to anticipate the periodic fluctuations brought on by the 24-h solar cycle, aligning biological functions with external changes (Pittendrigh 1993).

Skeletal homeostasis is an intrinsically dynamic process whose complexity not only derives from the number of molecules involved and their multifaceted interactions, but also from their precise spatial and temporal control. Remarkably, physiological functions such as longitudinal bone growth, bone remodelling, chondrocyte metabolism and cartilage matrix turnover exhibit 24-h rhythms, being controlled by the peripheral circadian clocks present in most of the cell types in cartilage and bone (Dudek & Meng 2014, Yang & Meng 2016). Alterations in the circadian rhythm of skeletal biology are associated with the development of various disorders, including osteoarthritis (OA), rheumatoid arthritis (RA) and osteoporosis (OP) (Kawai & Rosen 2010, Gibbs & Ray 2013, Berenbaum & Meng 2016).

In this review, we summarise the current understanding of the links between the circadian clock and skeletal metabolism. Specifically, we discuss how the circadian clock is implicated in the regulation of several facets of cellular metabolism that are essential for bone and cartilage homeostasis. We also elaborate on the utilisation of animal models to dissect the roles of circadian pathways in skeletal metabolism and pathophysiology of diseases. Finally, we provide an overview of the latest advances in chronotherapy and other translational aspects of circadian biology and discuss how these can be...
used to improve existing therapies and develop new ones for musculoskeletal conditions.

**Circadian rhythms in mammals: a coordinated network of hierarchical oscillators**

The mammalian circadian system comprises a multitude of oscillators organised in a hierarchy (Fig. 1) (Honma 2018). The central clock, located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus, is directly entrained by photic information transmitted to the SCN via the retinohypothalamic tract (Gooley et al. 2001, Hattar et al. 2002, Panda et al. 2002a). Non-photic stimuli such as behavioural arousal, melatonin and serotonergic activation are also capable of resetting the central clock; these cues are usually conveyed to the SCN via the geniculohypothalamic tract and the dorsal and median raphe nucleus (Challet & Pévet 2003, Dibner et al. 2010). The SCN clock regulates daily oscillations in mammalian behaviour and physiology, including locomotor activity, sleep–wake cycles, blood pressure, body temperature, pineal melatonin secretion and adrenal corticosterone release (Moore & Eichler 1972, Moore & Klein 1974, Eastman et al. 1984, Kramer et al. 2001, Scheer et al. 2005, Buijs et al. 2014). Additionally, the central clock acts as a pacemaker that relays crucial timing information to autonomous peripheral oscillators through neural and humoral outputs, such as parasympathetic and sympathetic innervation and glucocorticoid hormones (Balsalobre et al. 2000, Albrecht 2012, Buijs et al. 2014).

Circadian rhythms in the expression of genes and proteins have been reported in cells and tissues from across all organ systems. Genome-wide transcriptome studies have demonstrated that between 3 and 16% of the transcripts detected in peripheral tissues are rhythmically expressed (Panda et al. 2002b, Storch et al. 2002, Zvonic et al. 2006, Gossan et al. 2013, Dudek et al. 2016, 2017, Yang et al. 2017). In fact, a systems-level analysis of the murine transcriptome indicated that more than half of the genes encoding proteins are rhythmic in at least one tissue (Zhang et al. 2014). Notably, there is little overlap between the genes under circadian control in each tissue, demonstrating that the temporal orchestration of cellular metabolism is tissue specific (Mohawk et al. 2012, Buhr & Takahashi 2013, Zhang et al. 2014). This is reflected in the broad range of molecular pathways that are coordinated by the circadian clock, ranging from photoreception in the retina to xenobiotic detoxification in the liver and kidneys (Gachon et al. 2006, Storch et al. 2007). These tissue-specific gene expression patterns are presumably related to the gradual emergence of circadian rhythms during development through mechanisms that are deeply interlocked with cellular differentiation processes (Reppert & Schwartz 1986, Davis & Gorski 1988, Jud & Albrecht 2006, Yagita et al. 2010, Umemura et al. 2017).

Peripheral clocks rely on a unique combination of timing cues to fine-tune cellular functions. Although the central clock does not drive peripheral oscillators,
information originating in the SCN is essential for the synchronisation of phases between cells of the same tissue and establishing a stable phase interval between different tissues (Yoo et al. 2004, Guo et al. 2006). Nonetheless, the maintenance of systemic synchrony in mammals seems to be far more complex than initially suggested (Kowalska & Brown 2007, Husse et al. 2015). This is evidenced by parabiosis studies between intact and SCN-lesioned mice demonstrating that nonneural cues are sufficient to sustain circadian rhythms in the liver and kidneys, but not in the heart, spleen and muscle (Guo et al. 2005). In addition, the loss of synchrony in peripheral tissues caused by deletion of BMAL1 in the SCN was distinctively rescued by light/dark cycles and restricted feeding depending on the peripheral oscillator (Izumo et al. 2014). Nonetheless, more studies are necessary to fully comprehend how peripheral clocks interact with each other and with the SCN and how this regulation is achieved.

**The molecular circadian clock**

On a molecular level (Fig. 2), the circadian clock consists of intricate self-regulatory transcription-translation...
feedback loops whose interactions govern the rhythmic expression of clock-controlled genes (Reppert & Weaver 2002, Mohawk et al. 2012). The positive arm of the main feedback loop is formed by the transcriptional factor CLOCK (circadian locomotor output cycles kaput) and its heterodimeric partner BMAL1 (brain and muscle Arnt-like protein-1) (King et al. 1997, Gekakis et al. 1998, Lowrey & Takahashi 2011). The CLOCK-BMAL1 complex binds to E-box elements in the promoter region of target genes, including Period (Per1, Per2 and Per3) and Cryptochrome (Cry1 and Cry2), which are members of the negative arm of the feedback loop (Gekakis et al. 1998, Kume et al. 1999, Shearman et al. 2000). In the cytosol, PER and CRY interact with each other to form a complex and are phosphorylated by serine/threonine casein kinase 16 (CK1δ) and CK1ε (Lee et al. 2001, Gallego & Virshup 2007). This PER-CRY repressor complex is then translocated into the cell nucleus where it represses its own transcription by interacting with CLOCK-BMAL1 (Lee et al. 2001, Lowrey & Takahashi 2011). As repression progresses, specific E3 ubiquitin ligase complexes ubiquitinate PER and CRY proteins, marking them for degradation by the proteasome (Gallego & Virshup 2007, Preußner & Heyd 2016). Once the turnover of the repressor complex reaches a certain threshold, negative feedback is relieved and transcription of Per and Cry starts anew, initiating a new circadian cycle.

In addition to Per and Cry, the CLOCK-BMAL1 complex also activates the transcription of NR1D1 (nuclear receptor subfamily 1, group D, member 1) and NR1D2 (nuclear receptor subfamily 1, group D, member 2) that encode the nuclear receptors REV-ERBsα and REV-ERBsβ, respectively. Rhythmic expression of REV-ERBsα leads to the repression of Bmal1 thereby inducing a rhythm in these genes that is in antiphase with Per expression (Preitner et al. 2002). Retinoic acid-related orphan receptor (ROR) proteins (RORα and RORγ) compete with REV-ERBs for their shared DNA-binding elements (Preitner et al. 2002, Sato et al. 2004, Zhang et al. 2015), which results in the transcriptional activation of Bmal1 by RORα and its repression by REV-ERBsα (Solt et al. 2011). In this way, the REV-ERB–ROR feedback loop interconnects the positive and negative arms of the main feedback loop.

A third transcriptional loop driven by CLOCK-BMAL1 involves the proline and acidic amino acid-rich basic leucine zipper (PAR-bZIP) factors D-box-binding protein (DBP), thrytroph embryonic factor (TEF) and hepatic leukaemia factor (HLF). These proteins interact with the repressor NFIL3 (nuclear factor interleukin-3 regulated), which is driven by the REV-ERB/ROR loop, at sites containing D boxes (Mitsui et al. 2001, Gachon et al. 2004). Together, these integrated feedback loops generate intricate transcription–translation circuits with various phases of expression that vary according to the combination of cis elements in the promoters and enhancers of target genes (Ueda et al. 2005).

Articular cartilage

Articular cartilage (AC) is present in the articular surfaces of diarthrodial joints. AC is a specialised connective tissue that consists of a dense extracellular matrix (ECM) comprising protein fibres (collagen and elastic fibres) and ground substance (proteoglycans, glycosaminoglycans and glycoproteins), surrounding a sparse population of chondrocytes, the only cell type in this tissue (Mescher 2016). The regenerative potential of AC is inherently limited, meaning that once damaged this tissue is very difficult to repair (Iwamoto et al. 2013). Articular chondrocytes live in a challenging physicochemical environment: no innervation, no blood or lymphatic vessels, high osmotic pressure, acidic pH and low oxygen levels (Ng et al. 2017). As these cells rarely divide, cartilage homeostasis depends on the fine balance between chondrocyte anabolism and catabolism and their temporal regulation (Archer & Francis-West 2003). Disruption of chondrocyte metabolism has been linked to musculoskeletal disorders such OA and RA, which are characterised by a shift towards chondrocyte catabolism and progressive ECM degeneration (Goldring & Marcu 2009).

Evidence of circadian rhythms in articular cartilage

Circadian variations in cartilage were first described by studying the mitotic activity of chondrocytes in the epiphyseal growth plate of mice and rats, with the highest mitotic index occurring in the early morning (Simmons 1962, 1964a, Kember & Walker 1971, Stevenson et al. 1990). Several studies have also reported that the incorporation of [35S]sulphate, [3H] proline, [3H]galactose, [14C]proline/[14C]glycine or [3H] thymidine increased during the light phase in rat and mouse growth plates (Simmons 1964a,b, Walker & Kember 1972, Raugstad et al. 1979, Russell et al. 1983, Igarashi et al. 2013). In patients with knee OA and RA, the levels of cartilage oligomeric matrix protein (COMP) in serum presented a circadian variation (Andersson et al. 2006). Day/night variations could also be observed...
for other biomolecules related to cartilage metabolism in OA patients, namely aggrecan, type II collagen, hyaluronic acid, keratan sulphate and transforming growth factor-β (TGF-β) (Kong et al. 2006). Nonetheless, the lack of a normal control group in these studies limits the interpretation of these findings.

In the last decade or so, bioluminescence imagining techniques have greatly improved our knowledge of the molecular clocks driving circadian rhythms in various tissues. These techniques make use of reporter mice harbouring the firefly luciferase gene fused in-frame with the 3’ end of the Per2 gene (PER2::Luc), which permits real-time monitoring of circadian rhythms (Yoo et al. 2004). In 2013, Okubo and colleagues proved that the articular and epiphyseal cartilage of femoral bone from PER2::Luc mice have strong circadian oscillations in ex vivo culture for several months (Okubo et al. 2013). Similar approaches have revealed circadian rhythms in explant cultures of cartilage isolated from the xiphoid process and the femoral head of PER2::Luc mice (Gossan et al. 2013, Dudek et al. 2016). Circadian rhythms can also be observed in human primary and immortalised cell lines of chondrocytes transduced with lentiviral clock gene reporters (Gossan et al. 2013, Dudek et al. 2016).

Global transcriptome analyses have confirmed the rhythmic expression of core clock genes in cartilage isolated from the xiphoid process and femoral head of mice and from the ribs and femoral head of rats (Gossan et al. 2013, Honda et al. 2013, Dudek et al. 2016). By collecting samples from mice and rats constantly kept in darkness it has been possible to identify global circadian patterns of gene expression (Gossan et al. 2013, Honda et al. 2013). In both organisms, approximately 4% of the expressed genes showed statistically significant circadian rhythms (Gossan et al. 2013, Honda et al. 2013). Interestingly, many of these rhythmic genes are involved in cellular processes that are crucial for the maintenance of cartilage homeostasis, such as ECM structural components (e.g. aggrecan and collagen) and remodelling enzymes (e.g. Adats1, Adats4 and Adams59), calcium-dependent metalloproteinases (e.g. Mmp14), inhibitors of metalloproteinases (e.g. Timp3 and Timp4) and growth factors (e.g. Tgfa, Tgfbr3 and Egf) (Gossan et al. 2013, Honda et al. 2013, Yang & Meng 2016). Most importantly, these studies point towards a circadian regulation of cartilage metabolism with a time-of-day-dependent segregation of anabolic and catabolic pathways (Gossan et al. 2013, Honda et al. 2013).

Links between dysregulation of circadian rhythms and articular cartilage homeostasis

In recent years, evidence has emerged that disruption of circadian rhythms by environmental or genetic factors may contribute to the development of skeletal disorders. In aged mice, the amplitude of circadian oscillations is reduced approximately 40% in comparison to young mice; and it is known that age is one of the main risk factors for the development of OA (Hugle et al. 2012, Gossan et al. 2013). This reduction in the amplitude of circadian oscillations could impact on the regulation of downstream genes involved in cartilage metabolism. It has also been reported that long-term environmental disruption of light/dark cycles that mimics many years of rotating shift work or chronic jet lag promotes the development of an OA-like phenotype in murine knee cartilage (Kc et al. 2015).

In mice, targeted deletion of Bmal1 in chondrocytes (Col2a1-Bmal1−/−) abolished the time-dependent expression of many rhythmic genes and led to early-onset cartilage degeneration (Table 1) (Dudek et al. 2016). Most importantly, gene expression studies in Col2a1-Bmal1−/− mice demonstrated that Bmal1 is essential to maintain the balance between anabolic and catabolic factors in chondrocytes (Dudek et al. 2016). In the absence of this core clock component there is a dysregulation of the TGF-β and NFATC2 signalling pathways (Dudek et al. 2016). TGF-β plays a crucial role not only in cartilage development during embryogenesis but also in the maintenance of its functional and structural integrity throughout adult life (Finson et al. 2012, Wang et al. 2014). In aged murine chondrocytes this cytokine was associated with a diminished repair capacity (Scharstuhl et al. 2002, Blaney Davidson et al. 2005, Fortier & Miller 2006). Furthermore, dysregulation of the TGF-β signalling pathway has been correlated with an increased expression of MMP13 in human osteoarthritic cartilage (Blaney Davidson et al. 2009). The NFAT (nuclear factor of activated T cells) family of transcription factors also participates in several biological processes essential for cartilage health (Ranger et al. 2000, Greenblatt et al. 2013). NFATc2 has been shown to regulate the differentiation of adult mesenchymal stem cells into cartilage in mice (Ranger et al. 2000). In Nfatc2−/− mice, ectopic endochondral ossification is initiated at three months of age, and at a later stage extensive cartilage degradation may occur (Ranger et al. 2000, Wang et al. 2009). Loss of NFATc2 causes a metabolic imbalance in chondrocytes that is characterised by the overexpression of inflammatory (e.g. Il1b and Il6) and catabolic pathways.
Table 1  Summary of the phenotypes observed in articular cartilage from mouse models deficient in core circadian clock components.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Phenotype</th>
<th>Molecular changes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmal1−/−</td>
<td>Smaller body size</td>
<td>Decreased expression of Alp, Ihh and Col10a1 in rib growth plate</td>
<td>(Takarada et al. 2012)</td>
</tr>
<tr>
<td>Col2a1-Bmal1−/−</td>
<td>Age-related progressive knee cartilage degeneration</td>
<td>Decreased expression of Acan, Col2a1, Ctgf, Id3, Nfatc2/Nfatc2, Nr1d1, Per2, p-SMAD1/5, p-SMAD2, Serpine1 and Sox9/SOX9 in hip cartilage</td>
<td>(Dudek et al. 2016)</td>
</tr>
<tr>
<td>ClockΔ19</td>
<td>Progressive proteoglycan loss in knee cartilage</td>
<td>Increased expression of Il1b, Il6/IL6, Mcp1 and nuclear P65 in knee cartilage</td>
<td>(Yuan et al. 2019)</td>
</tr>
</tbody>
</table>

(e.g. Mmp13, Adamt5 and Timp1) and a decrease in anabolic signalling (e.g. Sox9, Acan and Col2a1) (Wang et al. 2009). Remarkably, expression of NFATc1 in lesioned cartilage from OA patients is downregulated in comparison with paired macroscopically normal samples (Greenblatt et al. 2013). Cartilage-specific ablation of NFATc1 in Nfatc2−/− mice induced the spontaneous development of an OA-like phenotype that recapitulates all the main features of human OA, that is, prevalence of matrix catabolism, osteophyte formation and subchondral bone changes (Greenblatt et al. 2013).

Mice carrying a mutation in Clock (ClockΔ19) presented accelerated cartilage degeneration in comparison to the wild type (Table 1) (Yuan et al. 2019). An increase in the expression of genes encoding proinflammatory cytokines such as IL-1β and IL-6 was also observed (Yuan et al. 2019). Interestingly, dysfunctional CLOCK decreased acetylation of NF-kB at Lys310 and increased its phosphorylation at Ser276 thereby promoting its translocation into the nucleus (Yuan et al. 2019). Future studies should investigate whether reduced NF-kB acetylation in a wild-type mouse recapitulates the same phenotype.

Analysis of gene expression patterns in human cartilage revealed that circadian rhythm pathways were among the most disrupted in OA patients (Akagi et al. 2017, Soul et al. 2018). Several studies have also demonstrated that expression of BMAL1/BMAL1 is significantly reduced in human osteoarthritic cartilage (Dudek et al. 2016, Snellling et al. 2016, Yang et al. 2016a, Akagi et al. 2017). Knockdown of BMAL1 in human chondrocytes altered TGF-β signalling and was associated with increased expression of catabolic factors (e.g. MMP1, MMP3, MMP13 and ADAMTS5) (Snellling et al. 2016, Yang et al. 2016a, Akagi et al. 2017, Khurana et al. 2019).

Disruption of rhythmic genes other than the core clock components has also been associated with abnormal cartilage metabolism. Autophagy, a cellular mechanism that allows the rapid elimination of abnormal proteins and/or organelles, is involved in the maintenance of AC cartilage by modulating chondrocyte function and survival (Bohensky et al. 2007, 2009). In OA, a decrease in chondrocyte autophagy was accompanied by an increase in cell death and matrix degradation (Caramés et al. 2012). Thus, apoptosis inhibition has been proposed as a therapeutic intervention for OA (Kim & Blanco 2007). Interestingly, expression of Xiap (X-linked inhibitor of apoptosis) was shown to be rhythmic in wild-type mouse xiphoid cartilage (Gossan et al. 2013). XIAP inhibits at least two members of the caspase family of cell death proteases, caspase-3 and caspase-7, and has shown anti-apoptotic activity in chondrocytes (Scott et al. 2005, Böhlm et al. 2010). Therefore, loss of Xiap rhythmicity during ageing might lead to an increase in apoptotic processes, contributing to OA pathogenesis.

Genes encoding matrix-degrading enzymes such as Adamt4 and Mmp14 were also found to be rhythmic in murine cartilage (Gossan et al. 2013, Honda et al. 2013). ADAMTS4 is one of the major proteases responsible for the degradation of cartilage proteoglycans, namely aggrecan (Verma & Dalal 2011). In human cartilage, this aggrecanase can be induced by proinflammatory cytokines and is upregulated in OA (Roach et al. 2005, Naito et al. 2007, Song et al. 2007). Another important protease is MMP14, a membrane-anchored enzyme that modulates the cellular availability of TGF-β (Velasco-Loyden et al. 2004) and participates in the activation of other metalloproteinases, namely MMP13 and MMP9, potentially amplifying its role in arthritis (Knäuper et al. 2002, Chellaiah & Ma 2013). Ablation of Mmp14 led to the development of severe arthritis in ageing mice that resembles both murine collagen-induced arthritis and human RA (Holmbeck et al. 1999).

Serum levels of parathyroid hormone (PTH) exhibit a circadian variation, with a peak occurring in the early
Circadian regulation of skeletal metabolism

In mouse, bioluminescence imaging has shown that PTH is capable of resetting PER2::Luc circadian oscillations in a time- and dose-dependent manner in the femoral head growth plate (Okubo et al. 2015). Both PTH and PTHrP (parathyroid hormone-related peptide) can exert physiological effects through PTH1R (PTH 1 receptor), a receptor that is expressed in the femoral head growth plate of mice (Okubo et al. 2015). The rate of chondrocyte differentiation is modulated by a negative feedback loop involving Indian hedgehog (IHH) and PTHrP. IHH, which is secreted by newly formed hypertrophic chondrocytes, stimulated the expression of PTHrP, which in turn retarded the formation of these cells (Vortkamp et al. 1996). Remarkably, expression of Ihh in the growth plate of mice exhibited circadian variation; and Bmal1−/− mice had decreased expression of Ihh in the growth plate (Table 1) (Takarada et al. 2012). In light of this evidence, it has been suggested that PTHrP also conveys time information to articular chondrocytes (Okubo et al. 2015), although definitive evidence is still lacking.

Bone

Bone is a specialised connective tissue that provides mechanical integrity for locomotion, protection to vital organs (e.g. brain, heart and lungs) and encloses the medullary cavities containing bone marrow wherein haematopoiesis occurs (Morgan et al. 2013, Ho et al. 2015). It also functions as a calcium and phosphate reservoir, being involved in the metabolic pathways associated with mineral homeostasis (Copp & Shim 1963). Bone is predominantly composed of inorganic materials of which calcium hydroxyapatite is the most abundant; the organic component, which is embedded in the calcified bone matrix, is enriched in type I collagen, but also includes proteoglycans, glycoproteins and calcium-binding proteins (Morgan et al. 2013).

To preserve its function and structure, bone undergoes two processes, modelling and remodelling, which depend on the coordinated action of the three major cell types present in the adult skeleton: osteoblasts, osteocytes and osteoclasts (Allen & Burr 2014). In bone modelling, the uncoupled action of osteoblasts and osteoclasts on separate surfaces ensures appropriate bone morphology and mass; it occurs primarily during childhood and at low rates throughout adult life in response to changes in mechanical loading (Allen & Burr 2014, Bartl & Bartl 2017). In contrast, bone remodelling is characterised by the coupled action of osteoclasts and osteoblasts on the same surface; it takes place in the mature skeleton as an adaption to mechanical loading and calcium and phosphate metabolism (Allen & Burr 2014, Bartl & Bartl 2017). Bone homeostasis can only be maintained when there is a strict temporal control of osteoclast-mediated bone resorption and osteoblast-mediated bone formation.

Evidence of circadian rhythms in bone

Initial studies into the circadian regulation of bone metabolism revealed that bone formation is diurnally regulated in rat metaphyseal bone; the most intense period of bone matrix mineralisation occurred during the night-time and was in antiphase with matrix synthesis (Simmons & Nichols 1966, Russell et al. 1984, Igarashi et al. 2013). Diurnal variations in the levels of serum markers of bone metabolism have also been reported, including N- and C-telopeptide, osteocalcin, pyridinoline, FGFR3, tartrate-resistant acid phosphatase, alkaline phosphatase, calcium, phosphorus, calcitonin and PTH (Gundberg et al. 1985, Greenspan et al. 1997, Srivastava et al. 2001, Shao et al. 2003, Kawai et al. 2014, Swanson et al. 2017).

More recently, peripheral clocks in bone have been investigated at the molecular level. Transcriptome analysis of cavarial bone from mice kept in a 12-h light/12-h darkness cycle revealed that 26% of the genes represented on the microarray exhibited a circadian expression profile (Zvonic et al. 2007). Many of these rhythmic genes were related to bone metabolism, including genes involved in BMP-, Wnt- and FGF-mediated signalling, as well as genes encoding matrix structural components (e.g. fibrillins and collagens) and remodelling enzymes (e.g. Adamts1, Adamts3 and Adamts5) (Zvonic et al. 2007, Yang & Meng 2016). To the best of our knowledge, genome-wide circadian transcriptome/proteome studies have not been performed in long bones or cells derived from it. Nonetheless, robust circadian oscillations of PER2::Luc have been identified in ex vivo cultures of both long (proximal femoral ends and radiuses) and flat bones (calvariae and scapulae) (Okubo et al. 2013). Despite these progresses, evidence for sustained molecular circadian rhythms in isolate bone cells (osteoblasts, osteoclasts and osteocytes) remains lacking.

Links between dysregulation of circadian rhythms and bone homeostasis

The molecular clock is an important regulator of bone metabolism and circadian rhythm disruption has
been increasingly recognised as a contributing factor to pathophysiological changes in bone. For instance, population-based studies indicated that rotating shift work, which is characterised by a chronic and repeated misalignment between internal clock time and external time cues, was associated with low bone mineral density in trabecular and cortical bones (Quevedo & Zuniga 2010), and a greater risk of hip and wrist fractures (Feskanich et al. 2009).

Leptin is a hormone predominantly secreted by adipocytes that participates in the regulation of bone remodelling, acting both on osteoblasts and osteoclasts to maintain bone mass constant (Ducy et al. 2000, Takeda et al. 2002, Elefteriou et al. 2005). Interestingly, mice lacking Per1 and either Per2 (Per1−/−Per2−/−) or the Per2 Per-Arnt-Sim (PAS) domain (Per1−/−Per2m/m) presented a significant increase in bone mass that affected vertebrae and long bones, suggesting that bone remodelling could be under circadian control (Table 2) (Fu et al. 2005). Cry1−/−Cry2−/− mice also exhibited a similar phenotype (Fu et al. 2005). Biochemical and histomorphometric analyses established that this high bone mass phenotype was linked to an increase in the number of osteoblasts, increased mineral apposition and bone formation rate (Fu et al. 2005). Evidence was also presented that leptin-mediated modulation of osteoblast function comprises two antagonistic pathways: on one hand, signalling through β2-adrenergic receptors upregulated Per1 and Per2, which in turn suppressed the expression of G1 cyclins and osteoblast proliferation; on the other hand, leptin acted through the AP-1 family of transcription factors to stimulate osteoblast proliferation and bone formation.

Table 2 Summary of the phenotypes observed in bone from mouse models deficient in core circadian clock components.

<table>
<thead>
<tr>
<th>Mouse strain</th>
<th>Phenotype</th>
<th>Molecular changes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bmal1−/−</td>
<td>Ectopic ossification of tendons and ligaments associated with bone insertion sites</td>
<td>Not shown</td>
<td>(Bunger et al. 2005)</td>
</tr>
<tr>
<td></td>
<td>Increased rates of bone formation and mineral apposition Increased number of osteoblasts</td>
<td>Not shown</td>
<td>(Fu et al. 2005)</td>
</tr>
<tr>
<td>Low bone mass</td>
<td>Not shown</td>
<td>(Kondratov et al. 2006, Samsa et al. 2016, Takarada et al. 2017)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Increased expression of Mmp9, CatK, Trap, Rank, Calcr and Rankl in femur</td>
<td>(Takarada et al. 2017)</td>
<td></td>
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<tr>
<td></td>
<td>Decreased number of active osteoblasts and osteocytes Reduced ability of bone marrow mesenchymal stem cells to differentiate into osteoblasts</td>
<td>Not shown</td>
<td>(Samsa et al. 2016)</td>
</tr>
<tr>
<td>Bmal1acs−/−</td>
<td>High bone mass Reduced bone resorption Decreased expression of Acp5 and Nfatc1</td>
<td>(Xu et al. 2016)</td>
<td></td>
</tr>
<tr>
<td>Bmal1acs−/+</td>
<td>Low bone mass Increased bone resorption</td>
<td>Not shown</td>
<td>(Takarada et al. 2017)</td>
</tr>
<tr>
<td>Per1−/−Per2−/−</td>
<td>High bone mass Increased number of osteoblasts, mineral apposition and bone formation rate</td>
<td>Not shown</td>
<td>(Fu et al. 2005)</td>
</tr>
<tr>
<td>Per1−/−Per2m/m</td>
<td>Decreased expression of Bmal1, Clock, Cry1, Per1, Per2 and Oxs Increased expression of Cnd1, Cnd3, Ccne, c-Fos, JunB and Fra2</td>
<td></td>
<td></td>
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<tr>
<td>Cry1−/−Cry2−/−</td>
<td>Not shown</td>
<td></td>
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</tr>
<tr>
<td>Per2m/m</td>
<td>High bone mass Increased osteoblast number and bone formation rate</td>
<td>Not shown</td>
<td>(Maronde et al. 2010)</td>
</tr>
<tr>
<td>Cry2−/−</td>
<td>High bone mass Normal osteoblast number, but decreased osteoclast activity</td>
<td>Not shown</td>
<td></td>
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Circadian regulation of skeletal metabolism

C F Gonçalves and Q-J Meng

(Maronde et al. 2010). In adult mice, ablation of Bmal1 resulted in a low bone mass phenotype that is characterised by significant reductions in bone mineral density and volume (Table 2) (Kondratov et al. 2006, Samsa et al. 2016, Takarada et al. 2017). These mice had a significantly lower number of active osteoblasts and osteocytes than wild-type littermates, which could be explained by a reduced ability of bone marrow mesenchymal stem cells to differentiate into osteoblasts (Samsa et al. 2016). Moreover, it has been reported that BMAL1 is involved in osteoclastogenesis by regulating the calcitriol-induced expression of Rankl in osteoblasts (Takarada et al. 2017). These findings contrast another study in Bmal1−/− adolescent mice that showed an increase in the number of osteoblasts, accompanied by increased rates of bone formation and mineral apposition (Fu et al. 2005). A recent study showed that tamoxifen-induced post-natal ablation of Bmal1 in adult mice did not recapitulate the skeletal phenotype presented by Bmal1−/− (Yang et al. 2016b). Altogether, these data suggest BMAL1 could have distinct roles in the regulation of bone homeostasis throughout development and post-natal stages.

The role of BMAL1 in bone remodelling processes is not limited to osteoblasts. In mice, targeted deletion of Bmal1 in osteoclasts led to the development of a high bone mass phenotype (Table 2) (Xu et al. 2016). The observed reduction in bone resorption was related to the direct upregulation of Nfatc1, a master regulator of osteoclast differentiation, by the CLOCK-BMAL1 complex (Kim & Kim 2014, Xu et al. 2016). Furthermore, it was demonstrated that expression of BMAL1 is reduced in bone marrow cells isolated from a type 2 diabetes rat model (Li et al. 2018). Complementary in vitro studies suggested that this core clock protein fine tunes the equilibrium between osteogenesis and osteoclastogenesis through modulation of the NF-κB signalling pathway (Li et al. 2018).

Hypophosphatemia during skeletal development impairs the mitochondria-mediated apoptosis of hypertrophic chondrocytes, leading to abnormal growth plate maturation and bone mineralisation (Sabbagh et al. 2005, Miedlich et al. 2010). Expression of clock genes in fracture calluses from hypophosphataemic mice was higher than that in control littermates, suggesting that phosphate metabolism is involved in the regulation of the circadian clock in bone (Noguchi et al. 2018). Remarkably, network analysis identified Ezh2 (enhancer of zeste homolog 2), a key epigenetic regulator of skeletal longitudinal growth, as one of the main molecules associated with hypophosphatemia (Dudakovic et al. 2015, Lui et al. 2016, Noguchi et al. 2018). Previous work revealed that EZH2 binds to the promoter region of Per1 and Per2; and targeted silencing of this histone methyltransferase disrupted circadian rhythms (Etxebarria et al. 2006). In adolescent mice, ablation of Ezh2 in bone marrow nestin-expressing cells resulted in premature cellular senescence and a depleted pool of mesenchymal progenitor cells; this was associated with impaired osteogenesis and a higher risk of developing osteoporosis later in life (Li et al. 2017).

Besides controlling mineral homeostasis, the skeleton is also an active modulator of energy metabolism (Kawai & Rosen 2010). CLOCK-BMAL1 heterodimers directly control the expression of Noc (nocturnin), a RNA deadenylase that participates in the post-transcriptional regulation of metabolic genes (Li et al. 2008, Stubblefield et al. 2012). In vitro studies demonstrated that Noc modulates the fate of bone marrow stromal cells by promoting adipogenesis and suppressing osteoblastogenesis (Kawai et al. 2010a). Noc−/− mice presented a high bone mass phenotype and a reduced number of adipocytes in the bone marrow, indicating a shift in stromal cell differentiation that favours osteoblastogenesis (Kawai et al. 2010a, Guntur et al. 2011). NOC has been shown to bind to PPARγ (peroxisome proliferator-activated receptor γ), promoting its nuclear translocation and activity (Kawai et al. 2010a). PPARγ activation favoured the differentiation of bone stromal cells into adipocytes and suppressed osteogenic signalling pathways such as BMP, TGF-β and WNT, as well as osteoblast-specific transcription factors (e.g. Runx2, Sox9, and Sp7) (Shockley et al. 2009). In vivo silencing of Pparg caused a reduction in the number of bone marrow adipocytes that was accompanied by increased trabecular bone formation (James et al. 2014). Furthermore, overexpression of the clock gene Rev-erba (a direct target of PPARγ) in bone mesenchymal stem cells inhibited cell proliferation and osteogenesis (Fontaine et al. 2003, He et al. 2015).

IGF-1 (insulin-like growth factor type 1) is an anabolic factor that plays an important role in the differentiation of mesenchymal stem cells into osteoblasts (Xian et al. 2012, Crane et al. 2013). In mouse femur, circadian expression of IGF1 was in antiphase with the clock-regulated transcription of Noc; and NOC was shown to interacting with the longer form of the 3′ untranslated region of IGF1 to reduce the expression of this growth factor (Kawai et al. 2010b). Circadian rhythms in IGF-1 signalling were...
perturbed in Cry-deficient mice; and it was proposed that CRY-mediated regulation of STAT5B (signal transducer and activator of transcription 5) phosphorylation could mediate the downregulation of Igf1 (Chaudhari et al. 2017). Moreover, IGF-1 can induce the phosphorylation of PTH1R in vitro, enhancing osteoblast to osteocyte transition and providing another link between the circadian clock, IGF-1 and PTH (Jubiz et al. 1972, Fuleihan et al. 1997, Qiu et al. 2018). More recently, it has been demonstrated that IGF-1-mediated signalling is sufficient to modulate the phase and amplitude of circadian rhythms in vivo and in vitro through increased PER synthesis (Crosby et al. 2019); future studies should seek to investigate whether these mechanisms are also relevant for skeletal homeostasis.

Systemic signalling through glucocorticoids is also a fundamental time cue for the circadian regulation of bone homeostasis. The addition of dexamethasone, a glucocorticoid hormone analogue, to osteoblasts and osteoclasts stimulated the rhythmic mRNA expression of circadian genes, as revealed by time course qPCR (Komoto et al. 2012, Fujihara et al. 2014). The circadian profile of osteoclast-specific genes such as Ctsk and Nfatc1 disappeared in bone tissue from adrenalectomised mice; and these oscillations were restored by intraperitoneal injection of dexamethasone (Fujihara et al. 2014). In addition, overexpression of the transcriptional regulator Gilz (glucocorticoid-induced leucine zipper) enhanced osteogenic differentiation of bone marrow stromal cells in vitro by blocking the transcription of Pparg (Shi et al. 2003, Zhang et al. 2008, Pan et al. 2014).

**Clinical relevance of circadian biology for skeletal disorders**

The numerous connections between the circadian clock and skeletal homeostasis bring about the possibility of targeting circadian rhythms to improve existing therapies and develop new routes. One approach is to adopt the concept of ‘chronotherapy’, in which the drug regimen is tailored to a patient’s circadian rhythm in order to maximise treatment effectiveness and reduce adverse effects (Selfridge et al. 2016). Over 40 years ago, it was reported that OA and RA symptoms can be improved by timing the intake of indomethacin and flurbiprofen; and treatment efficiency was correlated with the individual circadian variation of pain (Huskisson et al. 1970, Kowanko et al. 1981, Levi et al. 1985). More recently, it was demonstrated that timing teriparatide dosing affected the circadian rhythms of bone resorption markers in postmenopausal osteoporotic women (Luchavova et al. 2011). One of the most compelling examples of how circadian biology could be important when defining chronotherapeutic strategies comes from the use of glucocorticoids to treat RA. A landmark study revealed that coordinating glucocorticoid administration with the nocturnal rise in circulating levels of IL-6 substantially reduced joint pain and stiffness in comparison to standard morning administration (Arvidson et al. 1997). Subsequent clinical trials with modified-release prednisone which enables convenient bedtime administration demonstrated that this timed drug delivery noticeably increased treatment response rates and improved physical function (Buttgereit et al. 2008, 2010, 2013). These results emphasise the importance of implementing chronotherapy when designing clinical trials and evaluating treatment outcomes.

Another strategy to treat circadian disruptions is to use small molecules that directly target the clock or closely related pathways (Wallach & Kramer 2015). As the circadian clock is involved in the regulation of a multitude of signalling pathways, this approach might prove advantageous in comparison to polytherapy wherein two or more drugs are used to target distinct pathways. In fact, several compounds targeting clock proteins such as CRY1/2 and ROR have already been described, with beneficial effects in animal models for metabolic diseases (Hirota et al. 2012, He et al. 2016, Dierickx et al. 2019). For example, nobiletin is a naturally occurring polymethoxylated flavone present in citrus peels that enhances the amplitude of circadian rhythms in mouse (He et al. 2016). Remarkably, treatment of diet-induced obese mouse with nobiletin counteracted metabolic syndrome and increased energy expenditure and locomotor activity (He et al. 2016). RORs were identified as direct protein targets for nobiletin, acting as an agonist and enhancing Bmal1 transcription (He et al. 2016). Additionally, synthetic REV-ERB agonists have been shown to alter circadian expression patterns of metabolic genes in the liver, skeletal muscle and adipose tissue; and these changes resulted in increased energy expenditure (Solt et al. 2012). In the case of skeletal disorders, the therapeutic potential of small-molecule modulators remains largely unexplored; yet, these compounds offer promising approaches to modulate circadian timing and restore cellular homeostasis.

Lastly, behavioural interventions such as the maintenance of robust daily schedules in feeding and sleep/wake cycles have been increasingly recognised as potential approaches to strengthen circadian rhythms (Schroeder & Colwell 2013, Longo & Panda 2016). However, peripheral
circadian clocks seem to have evolved distinctive entrainment mechanisms by which the endogenous circadian clock is reset or synchronised with exogenous time cues, indicating the need to identify tissue-specific time cues (Guo et al. 2005, Izumo et al. 2014). Molecular components of the circadian clock have previously been identified as mechanosensitive in mammals and fruit flies (Kanbe et al. 2006, Simoni et al. 2014). In skeletal disorders, a balanced physical exercise schedule has been shown to be effective in improving musculoskeletal functions and reducing symptom severity (Roddy et al. 2005, Cooney et al. 2011, Moreira et al. 2014). From a circadian viewpoint, it is worth considering not only the duration and frequency of exercise, but also its timing; scheduled exercise that is optimised according to an individual’s chronotype, a human attribute that defines whether an individual prefers to be active and alert during the morning versus evening, is likely to be more effective than the random and irregular practice of physical exercise (Riley & Esser 2017, Duglan & Lamia 2019).

Concluding remarks

In modern 24/7 societies, the prevalence of circadian rhythm disruption is rising as more and more people have adopted nocturnal lifestyles. Nonetheless, circadian rhythm disruption is yet to be recognised as a major public health issue; and the circadian modulation of physiology has been largely overlooked in clinical research and practice.

Over the last decade or so, several lines of evidence have shed light onto the complex interactions between the circadian clock and the skeletal system (Fig. 3). Genomewide expression studies have established molecular clocks as pivotal modulators of the temporal segregation of metabolism in bone and cartilage cells. Studies in transgenic mouse models have linked the disruption of the molecular clock machinery to imbalances in processes such as cartilage matrix synthesis/degradation and bone formation/resorption, which often predispose the tissue to pathophysiological changes. In addition, epidemiological studies have also associated circadian rhythm disruption with a greater risk of developing skeletal disorders such as arthritis and osteoporosis.

Despite these recent advances, our understanding of the multiple levels of circadian regulation of skeletal metabolism and physiology remains largely unexplored. Future work will require conditional tissue-specific and cell-type-specific inactivation or overexpression of different molecular clock components to distinguish between gene-specific (e.g. during development) and post-natal clock-related functions. In addition, epidemiological studies should account for individual chronotype variability by including stratification approaches. Although the translational applications of circadian biology are only beginning to be explored, the clinical implementation of chronotherapy to treat RA resulted in significant benefits.
for patients. By taking into consideration the multifaceted role of the circadian clock in skeletal homeostasis, new therapies could be developed that will greatly enhance therapeutic index and bring us one step closer to precision medicine.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of this review.

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Circadian regulation of skeletal metabolism


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Circadian regulation of skeletal metabolism


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Circadian regulation of skeletal metabolism


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