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Review

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Co-regulation of circadian clock genes and microRNAs in bone metabolism

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Abstract: Mammalian bone is constantly metabolized from the embryonic stage, and the maintenance of bone health depends on the dynamic balance between bone resorption and bone formation, mediated by osteoclasts and osteoblasts. It is widely recognized that circadian clock genes can regulate bone metabolism. In recent years, the regulation of bone metabolism by non-coding RNAs has become a hotspot of research. MicroRNAs can participate in bone catabolism and anabolism by targeting key factors related to bone metabolism, including circadian clock genes. However, research in this field has been conducted only in recent years and the mechanisms involved are not yet well established. Recent studies have focused on how to target circadian clock genes to treat some diseases, such as autoimmune diseases, but few have focused on the co-regulation of circadian clock genes and microRNAs in bone metabolic diseases. Therefore, in this paper we review the progress of research on the co-regulation of bone metabolism by circadian clock genes and microRNAs, aiming to provide new ideas for the prevention and treatment of bone metabolic diseases such as osteoporosis.

Key words: Circadian rhythm; Circadian clock gene; MicroRNAs; Bone metabolism

1 Introduction

Bone is an important component of the locomotor system. Bone health depends on a dynamic balance between bone resorption and bone formation determined by the regular metabolic activities of various bone cells. Bone metabolism is influenced and regulated by both mechanical and biological factors (Tong et al., 2019). Some of these factors, such as the production and secretion of various hormones and cytokines, show regular chronobiological properties, which can vary with circadian rhythms. The bone cells themselves also undergo rhythmic activity with the circadian alternation (Gonçalves and Meng, 2019). These effects can be explained by the existence of a time-measuring device, the circadian clock. It exists in all organisms and regulates internal physiology to adapt to changes in the external environment. Circadian clock genes have now been used as therapeutic targets for many diseases and this has led to the creation and development of chronotherapy (Ruan et al., 2021). The body needs to conform to its internal circadian clock to stay healthy, and abnormal circadian rhythms will negatively affect the functioning of its systems. Shift workers and mice exposed to reversed circadian conditions show osteoporotic features such as bone microstructure degeneration, reduced bone mass, decreased bone strength, and increased bone brittleness (Schilperoort et al., 2020). An abnormal circadian rhythm affects bone health, which again suggests that bone metabolism can be regulated by the circadian clock, and that altered expression of circadian clock genes is the underlying cause. However, the mechanism whereby circadian clock genes regulate bone metabolism is still unknown.

MicroRNAs (miRNAs) are endogenous small RNAs involved in post-transcriptional repression of messenger RNA (mRNA) in eukaryotic cells. Their

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regulation of gene expression is considered a third epigenetic mechanism in addition to histone modification and DNA methylation (Bartel, 2004; Michou, 2018). MiRNAs are widely involved in the regulation of various physiological and pathological processes in organisms, including bone metabolism (Taipaleenmäki, 2018). Numerous studies have shown that miRNAs can effectively regulate bone resorption and formation by targeting key factors in bone metabolic signaling pathways (Ma et al., 2019; Zheng et al., 2019; Zhang et al., 2021). As a result, targeted therapies involving miRNAs have gradually been applied to the field of bone metabolic diseases. Both circadian clock genes and miRNAs can participate in the regulation of bone metabolism, but the mechanisms whereby they regulate bone metabolism remain to be clarified. Therefore, in this review we analyze the progress made in research on circadian clock genes and miRNAs and their co-regulation in bone metabolism, aiming to provide a comprehensive new approach for the prevention and treatment of bone metabolic diseases.

2 Circadian clock genes and their biological functions

The circadian clock is an intrinsic rhythm that regulates various physiological and behavioral activities of mammals in accordance with the 24-h daily diurnal alternation. The circadian clock that regulates circadian rhythms can be divided into a central clock and a peripheral clock. It is located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus and in some other brain regions and peripheral organs (Schibler et al., 2003). The production of two types of circadian clock depends on the transcription–translation feedback loops of specific genes, which we refer to as circadian clock genes. The main circadian clock genes identified in mammals and *Drosophila* so far include: (1) core circadian clock genes such as brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1 (*BMAL1*), circadian locomotor output cycles kaput (*CLOCK*), neuronal PAS domain protein 2 (*NPAS2*), period (*PER*), and cryptochrome (*CRY*); (2) peripheral circadian clock genes such as nuclear receptor subfamily 1 group D member 1 (*NR1D1*)/*REV-ERBα* and *NR1D2*/*REV-ERBβ*, retinoid-related orphan receptor (*ROR*, including *RORα*, *RORβ*, and *RORγ*), timeless (*TIM*), differentiated embryo chondrocyte (*DEC*), Duffy-binding protein (*DBP*), and Nocturnin (*NOCT*). They encode proteins that constitute complex positive/negative regulatory loops. Positive elements activate transcription of downstream clock-controlled genes to promote rhythmic gene expression, playing important regulatory roles in organismal physiology, behavior, and even psychology (Schibler, 2005; Zvonic et al., 2007; Guo et al., 2009; An et al., 2020).

2.1 Core circadian genes *BMAL1* and *CLOCK* and their biological functions

BMAL1 and *CLOCK* are positive regulators in the circadian rhythm system. *BMAL1*, a gene essential for the generation of 24-h periodic behavior, is predominantly located in the nucleus, where it encodes proteins with distinct circadian oscillations (Ray et al., 2020; Yang et al., 2020). A two-branch regulation model of circadian clock genes has been proposed, in which the *BMAL1*-centered circadian clock regulatory network in the body is divided into an autonomous response branch and a memory branch. When there is no clock dependent on *BMAL1*, the circadian clock is guided by light, but the body will rely on a *BMAL1*-dependent clock to "remember" time in the absence of light (Welz et al., 2019). BMAL1 often forms dimers with proteins encoded by other circadian clock genes (e.g., BMAL1-CLOCK, BMAL1-NPAS2), which bind to E-box or D-box elements located in the promoter of clock-controlled genes to activate their transcription (DeBruyne et al., 2007). CLOCK usually functions as a dimerization partner of BMAL1 by binding to BMAL1 (Menet et al., 2014). There are two known circadian rhythm complexes located in the nucleus: the BMAL1-CLOCK complex and the PER complex. The BMAL1-CLOCK positively drives the circadian rhythm system (Aryal et al., 2017). Studies suggested that *BMAL1* and *CLOCK* in mammals such as mice and humans are involved in the regulation of various biological processes such as circadian rhythms, immunometabolism, hormone production, and cellular activities, as positive regulators of circadian clocks (Beker et al., 2019; Nagao et al., 2019; Alexander et al., 2020).

2.2 Core circadian genes *CRY* and *PER* and their biological functions

CRY (*CRY1*, *CRY2*) and *PER* (*PER1*, *PER2*, and *PER3*) are negative regulators of the circadian rhythm

system. CRY and PER are phosphorylated by casein kinase 1δ (CK1δ) when their proteins accumulate to a critical level. They subsequently enter the nucleus to bind directly to the BMAL1-CLOCK protein dimer to form a complex that inhibits the transcription of CRY and PER themselves. This complex, called the PER complex, is another complex in the nucleus. It begins to accumulate at the onset of negative feedback and takes about 7 h to reach saturation and then negatively regulate circadian rhythms (Fogle et al., 2011; Aryal et al., 2017; Cao et al., 2017). *CRY* is considered as a blue photoreceptor in *Drosophila* due to its sensitivity to light, while its photoreceptor function in mammals has not been determined (Fogle et al., 2011). *PER* is circadian clock gene and is also thought to be tumor suppressor gene (Yang et al., 2009). Numerous studies have confirmed that *CRY* and *PER* regulate circadian rhythm and autoimmunity genes like *BMAL1* and *CLOCK*. They also play important roles in hormone production, hypoxic response, and thermoregulation as negative regulators of the circadian clock (Chappuis et al., 2013; Cao et al., 2017; Kobayashi et al., 2017; Patke et al., 2017).

2.3 Peripheral circadian clock genes and their biological functions

In addition to several core circadian clock genes, some other circadian clock genes play an integral role in the regulatory loop. We collectively refer to these other genes as peripheral circadian clock genes. Peripheral circadian clock genes include mainly *REV-ERBα* (*NR1D1*), *ROR*, *TIM*, *DEC*, *DBP*, and *NOCT*. Apart from the regulation of circadian rhythm, some of these genes have other functional commonalities due to their tissue specificity. For example, both *REV-ERBα* and *RORα* are involved in the development of airway inflammatory diseases and cancers (Gaertner et al., 2019; Durrington et al., 2020). However, their functions are not identical, as *REV-ERBα* plays a broad regulatory role in pathological situations of heart failure and cancer (Wang et al., 2020; Yue J et al., 2020), while *RORα* is currently considered a regulator of emotional disorders such as anxiety and depression (Guissart et al., 2018). Furthermore, these peripheral circadian clock genes can interact with core circadian clock genes and participate in the regulation of immune response, hypoxic response, energy metabolism, cell proliferation, and DNA replication (Lin et al., 2002; Liu et al., 2016, 2018; Sato et al., 2018; Kurien et al., 2019; Rageul et al., 2020). The expression of some peripheral circadian clock genes has been shown to serve as a reliable biomarker for the diagnosis and prognosis of certain diseases (Bianco et al., 2019; Rageul et al., 2020). Furthermore, some targeted drugs related to peripheral circadian clock genes have been developed to effectively treat some specific diseases in animals (Sulli et al., 2018). The peripheral clock circadian genes are not redundant in the circadian clock regulation system. On the contrary, they cooperate with the core circadian clock genes to greatly enrich the network of the circadian clock to regulate the metabolism of life activities. The cycle of the circadian clock system can be simply divided into three loops (Ruan et al., 2021), as shown in Fig. 1.

The relationship between circadian rhythm disorders and human diseases has received much attention. Many studies have focused on the mechanism of action of circadian clock genes in relation to cardiovascular diseases and tumor-related phenotypes, in which circadian clock genes were generally described as tumor suppressor genes (Jiang et al., 2016; Shi et al., 2022). Earlier studies pointed out that mice with defective circadian clock genes show reduced activity and their bones undergo pathological changes as they age. This showed that circadian clock genes not only regulate behavioral activities, but also play an important role in body metabolism, and obviously bone metabolism is one area of interest (Bunger et al., 2005; Jiang et al., 2016).

3 Regulation of bone metabolism by circadian clock genes

3.1 Bone metabolism

Bone metabolism is a dynamic and balanced process in which osteoclasts and osteoblasts coordinate with each other to break down and resorb aging bone and synthesize new bone to maintain normal bone mass and strength. In addition, bone mesenchymal stem cells (BMSCs) play an important role in bone metabolism because of their ability to differentiate into both osteoblasts and chondrocytes, while chondrocytes can be transformed into part of bone tissue. Therefore, as observed at the cellular level, cells involved in bone metabolism include osteoblasts,

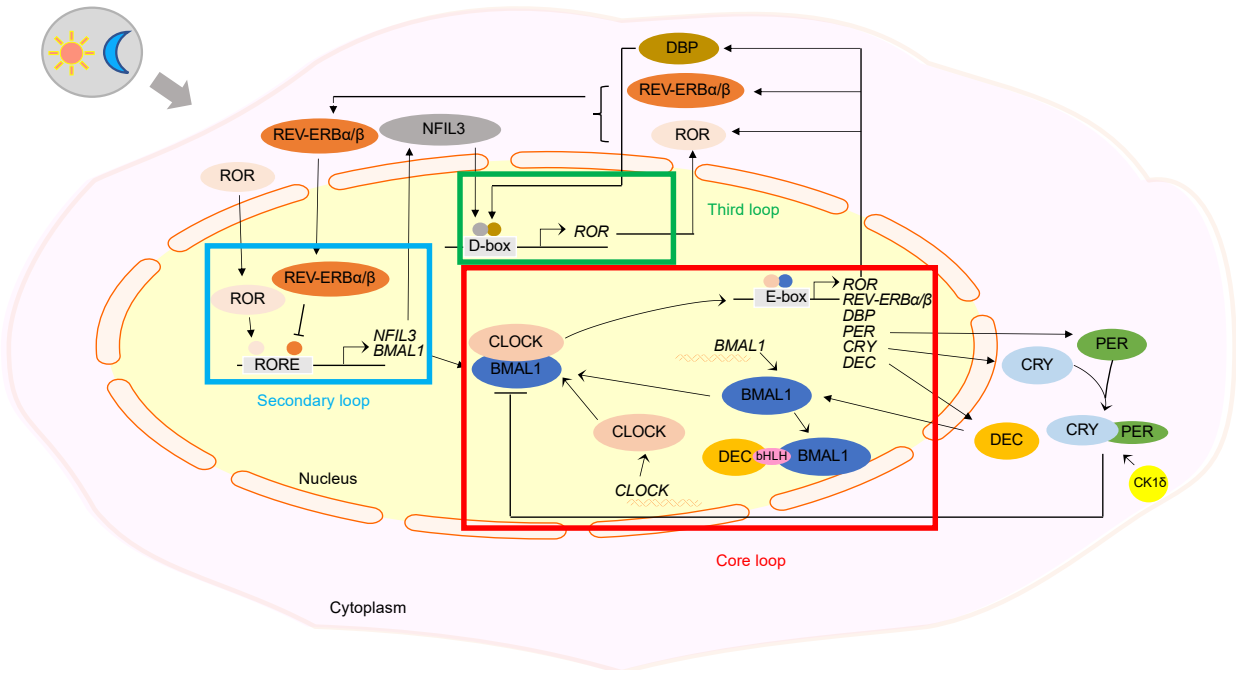

Fig. 1 A model for the positive and negative feedback loops formed by the interaction of circadian clock genes (modified from the study of Ruan et al. (2021)). bHLH: basic helix-loop-helix; BMAL1: brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1; CK1δ: casein kinase 1δ; CLOCK: circadian locomotor output cycles kaput; CRY: cryptochrome; DBP: Duffy-binding protein; DEC: differentiated embryo chondrocyte; NFIL3: nuclear factor interleukin 3; PER: period; REV-ERBα/β: nuclear receptor subfamily 1 group D 1/2 (NR1D1/2); ROR: retinoid-related orphan receptor; RORE: ROR/REV-ERB-response element.

osteoclasts, BMSCs, and osteocytes (Khosla, 2013). At the molecular level, bone formation or bone resorption is generally determined by the expression of specific genes or proteins. The identified osteogenic markers include alkaline phosphatase (ALP), Runt-related transcription factor 2 (Runx2), osteocalcin (OCN), osteopontin (OPN), osteoprotegerin (OPG), collagen I (Coll I), and bone sialoprotein (BSP). The osteoclastic markers include tartrate-resistant acid phosphatase (TRACP) and nuclear factor of activated T cell cytoplasmic 1 (NFATc1). They regulate bone metabolism by mediating various signaling pathways such as Wnt/β-catenin, bone morphogenetic protein (BMP), OPG/receptor activator of nuclear factor-κB (NF-κB) (RANK)/RANK ligand (RANKL), and Notch. Bone metabolism is also affected by many other factors, including mechanical factors (such as mechanical stimulation caused by exercise) and biological factors (such as hormones and cytokines), and indirectly affected by drugs used to treat other systemic diseases on bone metabolism (Luan et al., 2019; Tong et al., 2019; Wang et al., 2021). A growing number of studies in recent years have shown that the balance of bone metabolism is closely related to circadian clock genes (Chan et al., 2021), so circadian clock genes are also one of the factors that affect bone metabolism.

3.2 Circadian clock genes in bone metabolism

Various types of bone cells have been shown to express circadian clock genes. The temporal programming of the expression of circadian clock genes is driven by timekeeping mechanisms that lead to the activity of rhythmic clock protein transcription factors (Feeney et al., 2016). At the cellular level, changes in the expression of various circadian clock genes caused by circadian rhythm disorders mainly cause the function or activity of bone cells to change the turnover of bone, rather than the quantity. The expression of genes in osteoclasts was found to be more rhythmic than that of osteoblasts in the regular alternation of light and dark throughout the day. However, osteoblasts and other bone cells share the same circadian oscillations in gene expression. This suggests that circadian rhythms exist in the activity of all types of bone cells and collectively promote rhythmic bone remodeling (Fujihara et al., 2014; Schilperoort et al.,

2020). At the molecular level, the expression of some biomarkers related to bone formation and bone resorption maintains a characteristic oscillatory pattern with respect to the expression of circadian clock genes. For example, the expression of *Bmal1* and *Clock* in mice tends to increase during the day and decrease at night, while *Per* and *Cry* show a decrease in expression during the day and an increase at night. Correspondingly, the expression of OPG rises during the day and falls during the night, while RANKL and cathepsin K (CTSK) show a daily decline and a nightly rise (Schilperoort et al., 2020). There may be a correlation between the expression of bone metabolic factors such as OPG and the expression of circadian clock genes in mice. In addition, a clinical study showed that serum levels of type 1 procollagen N-terminal propeptide (P1NP), an indicator of bone formation, were reduced after circadian rhythm disturbances (Swanson et al., 2017). Therefore, the evidence demonstrates that there is an inextricable link between changes in the expression of circadian clock genes caused by altered circadian rhythms and bone metabolism (Fig. S1).

3.2.1 Core circadian genes *BMAL1* and *CLOCK* in bone metabolism

BMAL1 is expressed in bone and affects bone metabolic processes. Its abnormal expression is associated with skeletal diseases such as skeletal dysplasia, osteoarthritis, and osteoporosis (Chen et al., 2020). Recently, it has been shown that osteoclast-specific knockdown of *Bmal1* in 10-week-old mice results in a phenotype with higher bone mass due to reduced osteoclast differentiation after two weeks of diurnal reversal (Samsa et al., 2016; Xu et al., 2016). In contrast, Zhou et al. (2018) suggested that *Bmal1* deficiency accelerates osteoclast differentiation. This was demonstrated by a decrease in *Bmal1* in the mandible of patients with skeletal mandibular hypoplasia (SMH) and a simultaneous decrease in OPG expression, which inhibits osteoclast differentiation. In addition, reduced mandibular bone mass and reduced OPG expression were observed in both a circadian disorder mouse model established by advancing the daily light time by 6 h for 4–8 weeks and the *Bmal1*$^{-/-}$ mouse. The mechanism suggests that BMAL1 inhibits osteoclast differentiation by directly binding to the promoter of OPG and upregulating OPG expression, suggesting that *Bmal1* deficiency accelerates osteoclast differentiation (Zhou et al., 2018). However, Tsang et al. (2019) suggest that bone mass in mice may be controlled to a greater extent by the intrinsic circadian clock in BMSCs rather than in osteoclasts. This is because knockout of *Bmal1* in osteoclasts did not alter bone mass or trabecular architecture, and osteoclast differentiation was not affected in vitro compared to the knockout of *Bmal1* in BMSCs. Several studies on *Bmal1* and osteoclast differentiation have reached different conclusions. Differences in the age and sex of the mice selected, the manner in which the dysbiosis was modeled, the duration of the intervention, or the type of cells conditionally knocked out may be the reason. In addition, the indirect effect of BMSCs on osteoblast activity and the crosstalk between osteoblasts and osteoclasts may have been overlooked. For example, the expression of *Bmal1* is suppressed in the BMSCs of type 2 diabetes mellitus (T2DM) rats. The ratio of RANKL/OPG and the expression of inhibitor κB (IκB), phosphorylated p65 (p-p65), and caspase-3 phosphorylated IκB (p-IκB) are increased. Thus, overall osteogenic capacity was reduced in T2DM rats compared to healthy rats. When *Bmal1* is overexpressed, it restores the osteogenic capacity of BMSCs and inhibits osteoclast production and function by inhibiting the NF-κB signaling pathway (Li et al., 2018). This has been verified in the study of Mao et al. (2020) and provides a new direction for further exploring the mechanism of *Bmal1* in diabetic osteoporosis. Overexpression of *Bmal1* upregulates the expression of osteogenic genes including *BMP2*, *Runx2*, *ALP*, and *OCN* in BMSCs. When the effect of *Bmal1* on osteogenic differentiation of BMSCs was analyzed alone, *Bmal1* could promote osteogenic differentiation of BMSCs through the BMP2 signaling pathway (Huang ZF et al., 2020). The conclusion that *Bmal1* can promote osteogenic differentiation by regulating the expression of *BMP2* is also supported in osteoblasts and MC3T3-E1 (Min et al., 2016). The lack of *Bmal1* in osteoblasts accelerates osteoclast-mediated bone resorption because of the interaction between osteoblasts and osteoclasts (Takarada et al., 2017). Apparently, there is also evidence indicating that *Bmal1* in chondrocytes can regulate their activity and function, but the mechanism is unclear (Fu et al., 2019; Li et al., 2020).

Knockdown of *CLOCK*, a dimerization partner of BMAL1, in human BMSCs induced downregulation

of *BMAL1* and a reduction in the amplitude of oscillations without altering the cyclic oscillations. It also caused differentiation of BMSC to adipocytes and mildly suppressed expression of *OCN*, but this is not enough to conclude that knockdown of *CLOCK* significantly inhibits the osteogenic differentiation of BMSCs (Boucher et al., 2016). Knocking out the *CLOCK* alone may not lead to a dramatic effect on bone metabolism. An animal study with conditional deletion of *Clock* showed that protein disulfide isomerase family A member 3 (*PDIA3*), a circadian clock gene that encodes a protein for the 1,25-dihydroxyvitamin D3 (1α,25(OH)2D3) receptor, can promote the differentiation of osteoblasts in mice by acting as a transcriptional activator of PDIA3 to further activate 1α,25(OH)2D3 and the downstream PKC signaling pathway (Yuan GS et al., 2017). However, the effect of *CLOCK* on human bone metabolism has not been directly investigated. Knockdown of *CLOCK* in human ovarian granulosa-like cell line, KNG cells, resulted in reduced levels of estradiol. The BMP system is also involved and synergistically regulates ovarian steroid hormone production (Nagao et al., 2019). Since estrogen levels are closely related to bone homeostasis, this pathway in which *CLOCK* regulates estrogen expression and then regulates bone metabolism may be a novel mechanism to investigate.

3.2.2 Core circadian clock genes *CRY* and *PER* in bone metabolism

CRY is light-sensitive circadian regulator in invertebrates, but whether it has light-sensitive effects in vertebrates is still being explored. Laser irradiation induces translocation of CRY1 from the cytoplasm to the nucleus and subsequently regulates extracellular calcification in mouse BMSCs. This promotes osteogenic differentiation and reduces lipogenic differentiation of BMSCs (Kushibiki and Awazu, 2009). Activating transcription factor 4 (ATF4) is a major regulator of osteoblast differentiation in growing mice. The expression of *Cry1* is mediated by ATF4, which affects bone metabolism and bone strength in mice with chronic kidney disease (Pawlak et al., 2017). Further study of the mechanism revealed that *Cry1* regulates cell proliferation and osteogenic differentiation in a way that is dependent on protein kinase B (PKB/AKT) and extracellular signal-regulated kinase (ERK) signaling pathways. This finding was based on the results of a cell counting kit-8 (CCK8) assay and mRNA expression of some osteogenic factors such as ALP after inhibiting the expression of *Cry1* in C3H10 (Zhou et al., 2019). In pathological conditions such as arthritis, both mRNA and protein levels of CRY1 are significantly downregulated. After melatonin treatment of anti-type II collagen antibody-induced arthritis in mice, this downregulation is accompanied by degeneration of articular cartilage and bone structures, suggesting that *Cry1* may be involved in the progression of melatonin-treated arthritis (Bang et al., 2012). The latest study found that the expression of *CRY2*, rather than *CRY1*, was significantly reduced in the cartilage of humans and mice with arthritis. Also, the knockout of *Cry2* in mice showed a significant increase in the severity of pathological changes in cartilage, subchondral bone, and synovial tissue compared with the knockout of *Cry1* (Bekki et al., 2020). Moreover, this study suggested that *Cry2* may play an active role in maintaining dynamic homeostasis of the extrachondral matrix (ECM). Fifty-three differentially expressed genes, including *Nr1d1*, *Nr1d2*, *DBP*, and thyrotroph embryonic factor (*Tef*), which are all target genes of *Cry2*, were identified from RNA sequencing of knee cartilage in wild-type and *Cry2*$^{-/-}$ mice. Gene Ontology (GO) and search tool for the retrival of interacting genes/proteins (STRING) databases were used to analyze the pathways and mechanisms by which these differentially expressed genes regulate osteoarthritis. A key protein, platelet-derived growth factor receptor α (PDGFRA), was found to belong to the angiogenic pathway, the circadian rhythm pathway and the ECM pathway (Bekki et al., 2020).

Previous studies have shown that mice exhibit high bone mass if they lack *Per* in their osteoblasts. Normal expression of *Per* in osteoblasts mediates leptin-dependent sympathetic inhibition of bone formation through inhibition of osteoblast proliferation and the expression of *cyclin D1* (Fu et al., 2005). This suggests that *Per* negatively regulates bone formation. The speculation that *Per* does not directly regulate bone metabolism by altering gene expression in osteoblasts was corroborated in later studies in which *Per2* mutant mice showed significantly higher bone formation rates compared to wild-type mice, but the parameters of osteogenic or osteolytic-specific genes did not differ. Therefore, *Per2* mutation may lead to alterations in some other factors that can regulate

osteoblast activity, and different levels of systemic and cell-autonomous regulatory mechanisms appear to regulate osteoblast activity (Maronde et al., 2010). Recently, functional studies of *Per2* in BMSCs and osteoblasts derived from mice have shown that *Per2* negatively regulates both osteogenic differentiation of BMSCs and the proliferative capacity of osteoblasts (Zhuo et al., 2018; Abe et al., 2019). In contrast, transfection experiments in human BMSCs revealed that knockdown of *PER2* significantly promoted the lipogenic differentiation ability of BMSCs and mildly inhibited their osteogenic differentiation ability but had little effect, as did knockdown of *CLOCK*. The difference is that knockout of *PER2* can also affect the activity of stem cells and reduce the cell migration ability of BMSCs (Boucher et al., 2016). Thus, although *CRY* and *PER* are both negative regulators of circadian rhythms, they play their respective roles in bone anabolism and catabolism, with *CRY* having mainly osteogenic effects and *PER* negatively regulating bone formation.

3.2.3 Peripheral circadian clock genes in bone metabolism

A functional study has shown that *Rev-erbα* affects the ability of BMSCs to proliferate and differentiate into osteoblasts, and that its agonist has the ability to inhibit osteoclastogenesis and prevent bone loss caused by ovariectomy (Song et al., 2018). Mechanistic studies revealed that the expression of some markers of osteoclastic differentiation and osteogenic differentiation was strongly influenced by the level of *Rev-erbα* (He et al., 2015; Kim et al., 2020). Inhibiting the expression of *Rev-erbα* in osteoclast and osteoblast precursor cells caused simultaneous elevated expression of osteoclastic factors such as *TRACP* and *NFATc1*, and osteogenic factors such as *ALP* and *BSP*. Overexpression of *Rev-erbα* in osteoblast and osteoblast precursor cells resulted in a simultaneous enhancement of osteoclastic and osteogenic differentiation, accompanied by the activation of the p38 mitogen-activated protein kinase (MAPK) signaling pathway. Therefore, it was inferred that *Rev-erbα* may negatively affect osteoclast and osteoblast differentiation through the p38 MAPK signaling pathway (He et al., 2015; Kim et al., 2020). Another gene, *RORα*, which has been poorly studied in relation to bone metabolism, is involved in the metabolic activity of osteoblasts. It stimulates the expression of osteogenic factors such as ALP and OCN in MG-63 cells or suppresses the inflammatory response (Benderdour et al., 2011). There is still a lack of evidence that *RORα* directly regulates bone metabolism. The expression of *RORα* showed some association with the Wnt/β-catenin signaling pathway in a mouse model of acute respiratory infection (Li et al., 2019). The Wnt/β-catenin signaling pathway is one of the most classical signaling pathways in bone metabolism. Therefore, whether *RORα* regulates bone metabolism through the Wnt/β-catenin signaling pathway might be investigated in the future. The relationship of another peripheral circadian clock gene, *TIM*, with bone metabolism has not been studied. While *DEC* is affected by BMP2, parathyroid hormone (PTH), and PTH-related protein (PTHrp) because of its tissue specificity, it is upregulated in both chondrocytes and BMSCs and regulates cell differentiation (Kato et al., 2014). There are many peripheral circadian clock genes that are less well studied and therefore less understood, such as nuclear factor interleukin 3 (*Nfil3*) and *Noct*. *Nfil3* is expressed in osteoblasts in response to adrenergic receptor signaling and regulates the expression of BMP4, which in turn affects bone metabolism. *Noct* is regulated by the central circadian clock gene, although it is not part of the central core circadian clock complex. Its deletion promotes bone formation and rescues rosiglitazone-induced bone loss (Guntur et al., 2011; Hirai, 2018). In conclusion, functional and mechanistic studies have well demonstrated the regulatory role of peripheral circadian clock genes in bone metabolism, and different peripheral circadian clock genes may exert effects at different stages of bone metabolism.

The process of circadian clock genetic regulation of bone metabolism is very complex. On the one hand, circadian rhythm disturbances (e.g., shift work, sleep deprivation, fasting, or gene knockouts) are likely to cause bone and muscle dysfunction, but bone metabolism is affected to a greater extent by the presence of muscle–bone crosstalk. On the other hand, circadian rhythm disorders lead to fluctuations in the levels of nutritional factors, hormones, and cytokines in the body, which can also have an impact on bone metabolism (Feskanich et al., 2009; Quevedo and Zuniga, 2010; Booth et al., 2013). In addition, the passage of the circadian temporal phase is often accompanied by the interaction of various endocrine hormones,

cytokines, and non-coding RNAs such as miRNAs with bone metabolism. Therefore, the regulation of bone metabolism by circadian clock genes is not a direct and unidirectional process. Integrating new research on the contribution of miRNAs in studies of the influence of circadian rhythms on bone metabolism will bring new understanding of the mechanisms regulating bone metabolism.

4 Regulation of bone metabolism by miRNAs

4.1 Biological functions of miRNAs

MiRNAs are endogenous non-coding small RNAs about 22 nucleotides in length. They form RNA-induced silencing complexes (RISCs) when combined with ribonucleoprotein (RNP) complexes, and are capable of regulating the expression of downstream genes (Mohr and Mott, 2015). The main way by which miRNAs regulate gene expression is to degrade the mRNA of the target gene by guiding the Argonaute protein to the complementary site in mRNA. Another way is to inhibit the process of protein translation (Schirle et al., 2014). The expression of miRNAs themselves is influenced by many factors at the transcriptional, processing, and functional levels. The expression of target mRNAs is similarly regulated by miRNAs by means of epigenetic effects, promoter regulation, RNA processing and stability, and translation (Mohr and Mott, 2015). Ferrari et al. (2016) analyzed previous studies and found that miRNAs regulate physiological processes such as heart rate, vascular tone, and immune response by modulating purinergic receptors. Under pathological conditions, such as inflammatory responses in animal models, miR-147 was found to have a regulatory role (Kim et al., 2021). miR-122 was found to be involved in the regulation of ischemic responses in both human tissues and mouse models of liver ischemia (Ju et al., 2021). Numerous studies have also identified miRNAs that show great potential in the treatment of cancers and organ damages (Neudecker et al., 2016; Lee et al., 2020; Zhang et al., 2021). For example, delivery of miR-34a to various cancer models can exert significant inhibitory effects on tumor growth and survival (Liu et al., 2011; Kasinski et al., 2015). An increasing number of miRNAs have also gradually entered clinical studies in relation to different systemic diseases, but these studies are still in their early stages (Neudecker et al., 2016, 2017). Overall, miRNAs are widely involved in biological processes such as organism development, angiogenesis, cell proliferation, differentiation, and apoptosis as endogenous molecular regulators (Bartel, 2018).

4.2 MiRNAs in bone metabolism

Studies have confirmed that miRNAs play important roles in the differentiation and function of osteoblasts and osteoclasts (Table S1). Abnormal expression of miRNA leads to the development and progression of skeletal metabolic diseases (Sun YQ et al., 2019; Yuan et al., 2019). MiRNAs can regulate osteogenesis positively or negatively. miR-181a/b-1, miR-19b, and miR-96 promote osteogenesis and bone formation through the phosphatase and tensin homolog (PTEN)/phosphatidylinositide 3-kinase (PI3K)/AKT, PTEN/phospho-AKT (pAKT)/Runx2, and Wnt pathways, respectively. In addition, miR-378, miR-148b-3p, and many other miRNAs play active roles as positive regulators of osteogenesis in metabolic bone diseases such as osteoporosis, femoral head necrosis, and ectopic ossification (Zhang et al., 2018; Ma et al., 2019; Mollazadeh et al., 2019; Zheng et al., 2019; Sun et al., 2020). Other miRNAs play a negative regulatory role in osteogenesis or play a positive regulatory role in bone resorption. These include miR-23a and miR-1297, which inhibit osteogenic differentiation of periodontal ligament stem cells (PDLSCs) and BMSCs by targeting BMP, Wnt, and some other signaling pathways (Yuan Y et al., 2017, 2019; Wang et al., 2019; Zhang et al., 2019). Also, miR-155 can promote activation of osteoclast and bone resorption through both the AMPK and Wnt signaling pathways (Mao et al., 2019). However, a single miRNA can regulate multiple target genes, and can play different roles in the regulation of bone metabolism through different pathways. For example, knockdown of miR-223 can both reduce osteoclastogenesis and promote differentiation of osteoblasts, while overexpression of miR-223 has the dual role of both promoting and inhibiting the differentiation of osteoclasts (Xie et al., 2015).

Bone tissue is known to be sufficiently responsive to mechanical stimulation to enhance osteogenesis. It has been demonstrated that mechanical stress can mediate the involvement of miRNAs in the regulation

of bone metabolism. For example, miR-214 can attenuate the osteogenic effects produced by mechanical stimulation. miR-103a also acts as a mechanosensitive miRNA directly targeting Runx2 to regulate osteogenic differentiation (Yuan Y et al., 2017, 2019). In recent years, in the field of bone metabolism research, there has been increasing interest in transorgan regulation, in which kidney–bone crosstalk has been particularly popular (Hruska et al., 2017). MiRNAs, including miR-223, have been found to be the mediators of renal–bone crosstalk (Colbert et al., 2017; Ulbing et al., 2017). Moreover, there is crosstalk between osteoblasts and osteoclasts, and bone formation and angiogenesis are coupled. Some miRNAs, referred to as "coupled miRNAs," are involved (Li et al., 2016; Zhu et al., 2018).

5 Co-regulation of bone metabolism by circadian clock genes and miRNAs

5.1 Interaction between circadian clock genes and miRNAs

Circadian clock genes can regulate a variety of behavioral activities and physiological and biochemical metabolic processes, but other regulatory factors may also be involved. For example, miRNAs have been shown to participate in the regulation of circadian rhythms (Xue and Zhang, 2018). With the continuous enrichment and improvement of various bioinformatics platforms, we can easily obtain the targeting relationship between miRNAs and circadian clock genes. For example, predictions from the latest version of TargetScanHuman (https://www.targetscan.org/vert_80) revealed that although no miRNAs with highly conserved binding sites that might target *CRY1*, *DEC1*, or *NOCT* were temporarily identified in human, mouse, or rat, and no miRNAs with highly conserved binding sites that might target *Tim* were identified in rats, there are still an enormous number of miRNAs that may target other circadian clock genes.

Many miRNAs that can target circadian clock genes have been experimentally confirmed. It was verified that *BMAL1* can be directly targeted and regulated by miR-223, miR-219, miR-155, and miR-135b, and *CLOCK* can be targeted by miR-17-5p (Cheng et al., 2007; Curtis et al., 2015; Gao et al., 2016; Lou et al., 2017; Jiang et al., 2018). The relationships between *PER*, *CRY*, and miRNAs, on the other hand, often need to be explained by their interactions with *BMAL1*. For example, miR-219 is thought to be a target of BMAL1-CLOCK and it collaborates with miR-132 to activate transcription of *PER* through the BMAL1-CLOCK complex (Cheng et al., 2007). Surprisingly, studies of the interaction between peripheral circadian clock genes and miRNAs seem to have been more comprehensive and in-depth than those of core circadian clock genes. *Rev-erbα* was identified as the main circadian regulator of transcription of miR-122, and miR-122 was later found to regulate the rhythmic expression of *Noct* and *RORα* (Gatfield et al., 2009; Kojima et al., 2010). This indicates that there is a bidirectional pattern of regulation between circadian clock genes and miRNAs. Some other miRNAs have also been shown to target *RORα*. These include miR-1246, miR-7, miR-503-5p, and miR-137, which act synergistically to exert a potent inhibitory effect on *RORα* and thereby control the pathological development of diseases such as nonalcoholic steatohepatitis, hepatocellular carcinoma, and inflammation of brain tissue (Chai et al., 2020; Huang JL et al., 2020; Yue DX et al., 2020).

MiRNAs and circadian clock genes are increasingly being recognized as diagnostic and prognostic markers for some diseases. For example, miR-137 is considered a diagnostic candidate marker for autism spectrum disorders because it targets *RORα* in a specific way and affects the expression of autism-specific genes downstream of *RORα* (Devanna and Vernes, 2014). β-Catenin is an important factor in the Wnt signaling pathway. *RORα* can downregulate the expression of β-catenin to inhibit tumors, while miR-652 can activate the Wnt/β-catenin signaling pathway. Therefore, there is an antagonistic relationship between miR-652 and *RORα* to a large extent. Expression of miR-652 is closely associated with the tumor, node, metastasis (TNM) stage, so miR-652 is considered a possible prognostic marker for patients with gastric cancer (Sun et al., 2018; Li and Zou, 2019). *RORα* can also be considered a therapeutic target for diseases because it is targeted by specific miRNAs of some diseases if the function of *RORα* itself was analyzed from the perspective of the interaction between *RORα* and miRNAs. For example, a mechanism study found that miR-195 and lncRNA FGD5 antisense RNA 1 (FGD5-AS1) act as competitive endogenous RNA

(ceRNA) to regulate the expression of *RORα*. In addition, miR-18a can target *RORα* and activate the NF-κB signaling pathway. miR-195 and miR-18a are miRNAs specific to patients with glioma and acute myocardial infarction. Therefore, *RORα* is considered a therapeutic target for these diseases (Cai et al., 2020; Jiang et al., 2020).

MiRNAs and circadian clock genes can also be simultaneously involved as mediators in the regulation of some biological processes. For example, exosomes derived from hypoxia-induced glioma are rich in miR-10a. Mechanistic studies have also revealed that hypoxia activates myeloid-derived suppressor cells (MDSCs) through the miR-10a/RORα/IκBα/NF-κB signaling pathway to exert a powerful immunosuppressive effect (Guo et al., 2018). Like *RORα*, *TIM* is related to the occurrence and development of cancer, and its interaction with miRNAs also shows advantages for anti-cancer treatments. The study by Zou et al. (2020) of miRNAs and *TIM* in breast cancer led to the identification of a very complex regulatory pathway: miR-5188 directly targets forkhead box protein O1 (FOXO1) and interacts with β-catenin in the cytoplasm to stimulate the Wnt signaling pathway and activate key regulators of the cancer system. *TIM* can induce the expression of miR-5188 by promoting c-Jun-mediated transcription, and then interacts with Sp1/c-Jun. Therefore, *TIM* regulates the progression of breast cancer through miR-5188-FOXO1/β-catenin-c-Jun, which provides a theoretical basis for the use of miR-5188 antagonists in the clinical treatment of breast cancer. Although it can be determined that *TIM* is regulated by miRNAs, it is important to note that *TIM* has several isoforms. The mRNA expression of *TIM-cold* is controlled by miRNAs when it encodes TIM protein, while *TIM-short and cold* (*TIM-sc*) does not seem to be affected by miRNAs. In addition, miRNA sequencing suggests that miR-969 may be an enhanced regulator of *TIM* (Chen and Rosbash, 2016; Anduaga et al., 2019). However, further experiments are needed to confirm this. To sum up, circadian clock genes and miRNAs interact with each other and regulate many biological processes. Both can be candidate biomarkers for diagnosis and prognosis of many diseases.

5.2 Therapeutic potential of circadian clock genes and miRNAs in bone diseases

Both circadian genes and miRNAs can be used as diagnostic or prognostic markers for various diseases. The use of a small number of circadian clock genes and miRNAs as specific therapeutic targets for certain diseases has been discussed in a previous subsection. However, the main focus of most studies on the effects of circadian clock genes and miRNAs on bone metabolism has been to discern whether their regulatory effects are positive or negative. Their therapeutic effects on bone metabolic diseases are not yet clear enough, but their therapeutic potential should not be underestimated (Wu et al., 2019). An example is the concept of chronotherapeutics, induced by circadian rhythms, in which the concept of chronotherapy is used to tailor the dosing regimen to the patient's circadian rhythm in search of maximum efficacy and minimal side effects (Selfridge et al., 2016). Initial studies in a population of women with osteoporosis found that different timing of dosing affected the circadian rhythm of bone resorption markers (Luchavova et al., 2011). In the treatment of rheumatoid arthritis, the therapeutic effect of bedtime dosing was significantly enhanced compared to morning dosing (Arvidson et al., 1997; Buttgereit et al., 2013). MiRNA-elicited targeted therapies have shown initial success in the clinical treatment of cancer and cardiovascular disease. Studies related to such targeted therapies for bone disease are still being tested in animals, but are constrained by the lack of a safe and effective delivery system. However, the positive effects of miRNAs in animal experiments are also indicative of the therapeutic potential of miRNAs for bone disease (Sun X et al., 2019; Hu et al., 2020). Attention has been focused on the possible combined effects of chronotherapy and targeted therapies, and it has been proposed that targeting circadian genes using small molecule drugs to achieve therapeutic effects on bone diseases is worthy of study (Wu et al., 2019).

5.3 Regulation of bone metabolism by miRNAs through circadian clock genes

Smith et al. (2016) have demonstrated that miR-433 targets Runx2 to regulate bone metabolism, while predictions from miRanda suggest that it may also target binding to the core circadian clock gene *PER2*. The temporal phase of the peak expression of the core circadian clock gene *PER2* is consistent with the peak mRNA and protein expression levels of Runx2, suggesting that Runx2 may be under the control of the circadian clock (Reale et al., 2013). However, luciferase reporter analysis showed that miR-433 was not directly

targeted to *PER2*. Nevertheless, it cannot be inferred that there is no relationship between miR-433 and *PER2* based on the above results alone. *PER2* may be indirectly involved in the regulation of osteogenic gene expression by miR-433 through dual specificity phosphatase 1 (*DUSP1*) and glucocorticoid signaling. miR-433 can regulate the expression of the osteogenic gene *Runx2* by regulating glucocorticoid signaling, and the glucocorticoid receptor can activate the expression of *PER2* through the glucocorticoid response element in its intron (Smith et al., 2016). In addition, the peripheral circadian clock gene *Rev-erbα*, as mentioned above, has been identified as a major circadian regulator of miR-122 transcription, and miR-122 is able to regulate the rhythmic expression of *Noct* (Gatfield et al., 2009; Kojima et al., 2010). Combined with the relationship between *Noct* and bone metabolism, it is suggested that the *Rev-erbα*/miR-122/*Noct* pathway may also be one of the mechanisms by which circadian clock genes and miRNAs regulate bone metabolism.

All the above evidence suggests that circadian clock genes may be involved in the regulation of bone metabolism by miRNAs, and indicates that the regulatory mechanism of the miRNA/circadian clock gene/bone metabolism axis in bone metabolism has started to receive attention in the field of bone metabolism. However, the above studies have not been able to prove that circadian clock genes are the target genes of miRNAs to directly regulate bone metabolism. With the improvement of bioinformatics platforms and experimental techniques, recent studies have focused on the targeting relationship between miRNAs and circadian clock genes to deeply explore the regulatory mechanisms involved. It is surprising to find that knockdown of *CRY2* is able to upregulate the expression of *ALP*, *Runx2*, collagen 1 (*Colla1*), *OCN*, and *OPN* to promote osteoblast differentiation in C3H10T1/2 cell lines. The target gene prediction website shows that miR-7-5p may target *CRY2*, which has been verified by luciferase reporter analysis. Overexpression of miR-7-5p is shown to significantly repress the expression of *CRY2*, and inhibition of *CRY2* reduces its binding to CLOCK-BMAL1. This results in the release of a large amount of CLOCK-BMAL1 which then binds to the E-box in the P300 promoter region and stimulates transcription of P300. Finally, P300 promotes acetylation of histone 3 and forms a transcriptional complex with Runx2 to enhance osteogenesis. Thus, miR-7-5p targets *CRY2* and promotes osteogenic differentiation through CLOCK/BMAL1/P300 signaling (Tang et al., 2020). Unfortunately, there have been few studies on the mechanisms involving circadian clock genes, miRNAs, and bone metabolism. The known mechanisms are shown in Fig. 2.

6 Summary and outlook

In conclusion, bone metabolism is a very complex physiological metabolic activity that is influenced by multiple factors. Various functional and mechanistic studies have shown that core circadian clock genes such as *BMAL1* and *CLOCK*, and peripheral circadian clock genes such as *REV-ERB*, play different roles in bone catabolism and anabolism. Specifically, *BMAL1*, *CLOCK*, and *CRY* play active roles in bone anabolism, while *PER* and *NOCT* are more dominant in bone catabolism. Some other circadian clock genes need further research to determine their roles in bone metabolism. The abnormal expression of circadian clock genes affects bone metabolism, and conversely, abnormal bone metabolism disrupts the expression of circadian clock genes. Moreover, the passage of the circadian phase is often accompanied by the interaction of various hormones, cytokines, and non-coding RNAs with bone metabolism, so the regulation of bone metabolism by circadian clock genes is not a direct and unidirectional process.

The latest research has focused on the circadian clock gene as a therapeutic target to treat autoimmune diseases. However, there has been no relevant research on whether the circadian clock gene can be targeted to treat bone metabolic diseases. Nowadays, there are more and more people working in shifts, and the susceptibility to bone metabolic diseases is enhanced by working around the clock. Therefore, we should firstly clarify the metabolic characteristics of various types of bone diseases, and then take the time of administration into account when using targeted therapy based on the mechanism of miRNA-mediated circadian clock genes to regulate the pathophysiological metabolism of bone. Combining chronotherapy and targeted therapy to achieve timely prevention or maximize the efficacy of treatments of bone metabolic diseases is a direction worthy of further study.

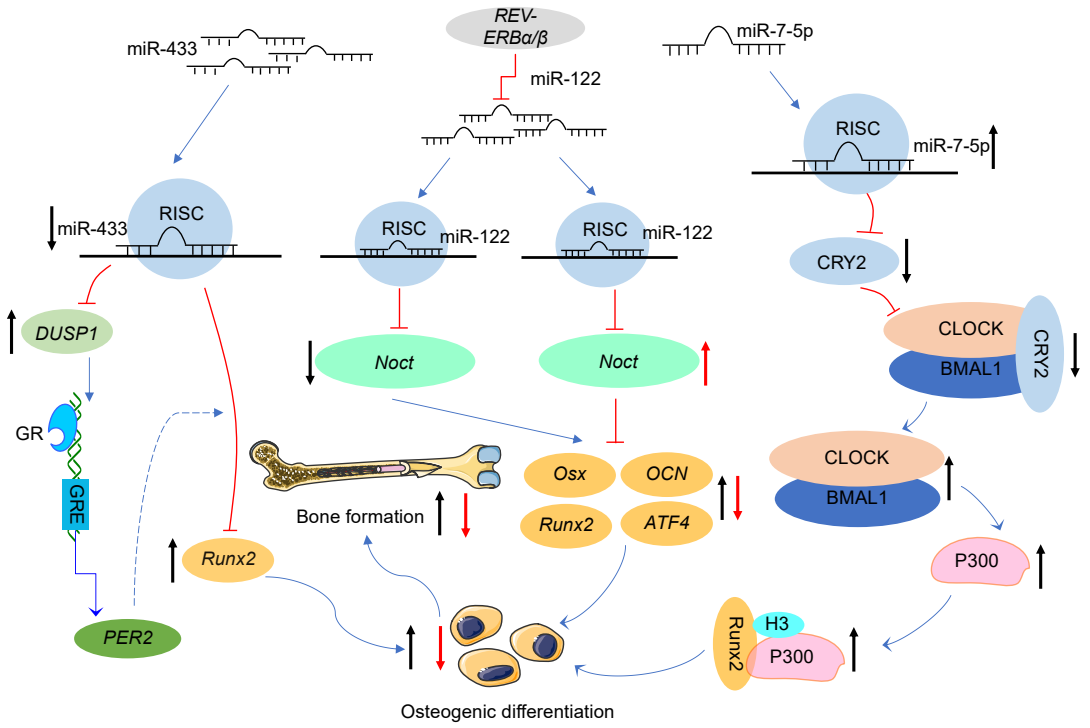

Fig. 2 Pathways of circadian clock genes and miRNAs regulating bone metabolism, modified from the study of Smith et al. (2016) and Tang et al. (2020). *ATF4*: activating transcription factor 4; BMAL1: brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like 1; CLOCK: circadian locomotor output cycles kaput; CRY2: cryptochrome 2; *DUSP1*: dual specificity phosphatase 1; GR: glucocorticoid receptor; GRE: glucocorticoid receptor element; *Noct*: Nocturnin; *Osx*: osterix; *OCN*: osteocalcin; PER2: period 2; *REV-ERBα/β*: nuclear receptor subfamily 1 group D 1/2 (*NR1D1/2*); RISC: RNA-induced silencing complex; Runx2: Runt-related transcription factor 2. ↑↑: up-regulated; ↓↓: down-regulated.

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Compliance with ethics guidelines

Tingting LI, Shihua ZHANG, Yuxuan YANG, Lingli ZHANG, Yu YUAN, and Jun ZOU declare that they have no conflicts of interest.

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Supplementary information

Fig. S1; Table S1