Special Report

International Journal of Clinical Rheumatology

# Circadian rhythm and joint stiffness/destruction in rheumatoid arthritis

In patients with rheumatoid arthritis, joint stiffness and pain levels reach peak in the morning, which is a characteristic symptom with diurnal fluctuation. In human, several types of hormone secretion, cytokine and antibody production are accelerated during night time as compared with those of day time, and these immune systems are partially controlled by circadian clock genes that regulate circadian rhythm. In this review, we focus on the interaction between circadian clock genes, immune system and the pathogenesis of rheumatoid arthritis.

**Keywords:** circadian rhythm • clock gene • inflammation • morning stiffness • rheumatoid arthritis

Kohsuke Yoshida[1], Teppei Hashimoto[2], Yoshitada Sakai[3] & Akira Hashiramoto*[,1,4]

[1]Faculty of Health Sciences, Kobe University School of Medicine, Kobe 654-0142, Japan
[2]Department of General Internal Medicine, Kobe University School of Medicine, Kobe 650-0017, Japan
[3]Division of Rehabilitation Medicine, Kobe University Graduate School of Medicine, Kobe 650-0017, Japan
[4]Department of Clinical Immunology, Kobe University Graduate School of Health Sciences, Kobe 654-0142, Japan
*Author for correspondence
Tel.: +81 78 796 4544
Fax: +81 78 796 4509
hash@kobe-u.ac.jp

Circadian rhythm is involved in a number of physiological functions and behavior of the life on Earth, including sleep/awakening, body temperature regulation, hormone secretion, division and proliferation of cells, and gastrointestinal function, that keep a period of approximately 24 h in an environment with no external constraints. Since this rhythm is slightly longer than 24 h in humankind [1,2], we need to correct our biological clock daily using external cues, in other words light stimulus. The center for rhythm oscillation in mammals is the hypothalamic suprachiasmatic nucleus (SCN), which is located at the bottom of the brain in front of the pituitary gland and acts as a master clock of the whole body [3]. However, subsequent studies found that the liver and lung cells maintain their own rhythm *in vitro*; the environment where input from the SCN does not exist. Thus, it has become apparent that tissues and cells can provide their own peripheral rhythm, similar but independent to that provided by the brain, and the clock genes orchestrate both central and peripheral oscillations [4,5].

The clock genes manage rhythm and time in a dual and hierarchical manner [6]. The rhythm signals propagated from SCN are subject to a feedback loop provided by clock genes including *Clock*, *Bmal1*, *Per*, *Cry*, *Rev-erb* α (also known as *Nr1d1*), *Ror*-α, *Dbp* and *E4bp4*. The circadian expression of these genes are regulated through E/E′ boxes, REV-ERB/ROR response element (RRE) and DBP/E4BP4 binding element (D box) in their promoter regions [7]. In addition, post-transcriptional machinery, such as phosphorylation, ubiquitination and chromatin remodeling, also mediate to generate rhythmicity of gene expression [8–10].

Rheumatoid arthritis (RA) is affected approximately 1% of adults worldwide and a chronic polyarthritis condition that goes through repeated relapse and remission as the disease progression. These cycles of repeated inflammation cause joint destruction and deformation, results in irreversible dysfunction. Once the inflammation is caused, the mesenchymal cells (synovial cells) lining the joint space begin to proliferate, and inflammatory granulation tissue, called pannus, invades into the bone and cartilage, leading to joint destruction. Growth factors, angiogenic factors and inflammatory lymphocytes work together to promote pannus formation, and joint destruction follows by the secretion of matrix metalloproteinase (MMP) and migration of synovial cells [11,12]. Therefore,

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chronic inflammation could lead to joint dysfunction through activation of immune cells.

## 'Morning stiffness,' a characteristic symptom with diurnal fluctuation in RA

'Joint stiffness in the morning' is the most common complaint and one of the crucial indicators of the condition in patients with RA [13], which seems to reflect the circadian nature of disease manifestation. Then, why the pain and stiffness level reach peak in the morning? The answer could be that several human immune responses are activated during night time (rest phase) than day time (active phase). For example, melatonin, a hormone that adjusts circadian rhythm, is produced by the pineal gland at night. Serum melatonin levels are undetectable in the daytime but are significantly higher during the night, even in the absence of optical stimulation [14–17]. Inflammatory cytokines including IFN-γ, IL-1 and IL-6 are all secreted from human peripheral blood mononuclear cells in response to melatonin stimulation, and in fact, melatonin is detected in RA synovium tissue macrophages and joint fluid [18]. As compared with healthy subjects, melatonin secretion at midnight is significantly increased in RA patients [19], and serum melatonin levels in the morning are higher in RA patients with shorter disease duration [20]. These studies seem to suggest that melatonin have an adverse effect in arthritis, on the other hand, melatonin inhibits the catalytic activity of MMP-9, which is involved in joint destruction in RA patients [21], and serum melatonin levels of RA patients is not correlated with the disease activity [20]. Thus, further study is needed to determine the effects of melatonin on joint destruction.

In addition, T-cell numbers and its reactivity were stable during day time, whereas a significant increase was observed in the late evening and early morning *ex vivo* [22], and the tyrosine kinase ZAP70, which acts at the downstream of T-cell receptor (TCR) and is crucial for T-cell activation, exhibited rhythmic protein expression [23]. Among them, number of $CD4^+CD25^+$ natural Tregs (nTregs) were increased during night time, and proliferation of $CD4^+CD25^-$ responder T cell was almost lost in the morning (7:00 AM) by nTreg-dependent manner [24]. The population of $CD4^+CD25^{high}$ matured nTreg cells in thymocytes, but not $CD4^+CD25^{low}$ unmatured nTreg, was higher in the evening (7:00–8:00 PM) as compared with the morning (7:00–8:00 AM) [25]. Further, $CD4^+CD25^-$ responder T-cell secreted cytokines, including IL-2, IFN-γ and TNF-α, which reached peak at 2:00 AM and were significantly suppressed by nTreg [26]. These results suggest that function and development of nTreg cells are activated during the night, and diurnal rhythm of nTreg could control function of responder T cells. Since an evidence suggests that patients with RA have defects in the function of Treg cells [27], this defects could lead to overproduction of inflammatory cytokine from responder T cells. Indeed, TNF-α and IL-6 are elevated in sera of patients with RA reaching the peak early in the morning [28,29].

Likewise, serum levels of IgA/IgM rheumatoid factor (RF) and immune complexes in patients with RA exhibit a rhythmic pattern with a peak in the morning [28], partially because a circadian rhythm exists in B cells and regulates these functions such as T-cell independent and dependent antibody production [30]. The administration of these antibodies into collagen-induced arthritis (CIA) mice markedly enhanced the clinical score and paw swelling [31]. As reported, recent cohort study showed that the patients positive for both RF and anticitrullinated protein antibodies (ACPA) have increased mortality compared with those of single-positive or seronegative [32]. Thus, antibodies related to RA also appeared to be ruled by circadian rhythm.

## Sleep disorders in RA

RA patients often exhibit sleep disorders classified as a nocturnal awakening type, characterized by a significant reduction in sleep efficiency and a significant increment in waking periods after sleep onset. Questionnaire studies of patients with sleep disorders report a 'Decline in the quality of sleep in patients with RA' as quantified by the Pittsburgh Sleep Quality Index [33,34], and 'Excessive somnolence trend during the day in patients with RA' by the Epworth Sleepiness Scale [35]. Further, level of sleep disorders seem to be correlated with RA disease activity, and this correlation is somewhat stronger in women and is mitigated by age [36,37]. Moreover, patients with nonapnea sleep disorder were associated with a risk for developing autoimmune diseases including RA [38]. These findings suggest that sleep disorders not only impacts on arthritic symptoms, but is also involved in the pathogenesis of RA [6,17].

Is sleep disorders involved in secretion of inflammatory mediators? Experimental sleep deprivation induces a significant increase in blood-circulating IL-6, TNF-α and C-reactive protein (CRP) [39,40], possibly due to activation of NF-κB that plays a key role in controlling the expression of proinflammatory genes [41]. Conversely, when stimulated with LPS (lipopolysaccharide), a major component of the outer membrane of Gram-negative bacteria, TNF-producing monocytes are increased at night during normal sleep as compared with continuous wakefulness [42]. In addition, sleep deprivation reduces secretion of CCL2 (also known as Mcp-1) and granulocyte-macrophage

colony-stimulating factor, but not changes those of melatonin [43].

## Circadian clock genes regulate immune response and inflammatory mediators

CD4 positive helper T cells play essential roles in several immune responses such as antibody production, cytokine secretion and antigen-presentation. Among them, IL-17-producing CD4$^+$ helper T (Th17) cells are proinflammatory immune cells that against bacterial and fungal infections at mucosal surface, and their lineage specification is regulated by RORγt [44]. *E4bp4*, also known as *Nfil3*, is a basic leucine zipper (bZIP) transcription factor, suppresses Th17 cell development by directly binding to *Rorγt* promoter. In addition, REV-ERBα directly represses *E4bp4* transcription by binding to a consensus sequence in their gene locus. Indeed, intestinal Th17 cell frequencies, but not Th1 cell, were reduced in *Rev-erbα* deficient mice [45]. Recently, it has been reported that *E4bp4* is also crucial for development of natural killer (NK) cells and innate lymphoid cells [46–49]. Further, gene expression of TLR9, which recognizes bacterial and viral DNA, has a circadian oscillation and is controlled by CLOCK:BMAL1 heterodimer [50]. As described above, clock genes have their specific tasks for the self-defense in both adaptive and innate immunity.

How does the circadian clock gene modify an inflammatory reaction? Several studies have shown the effect of core clock gene on expression of inflammatory mediators (Table 1). For example, bronchiolar cells-specific *Bmal1* knockout mice lead to enhance mRNA expression of inflammatory chemokines, including *Cxcl5*, *Ccl20* and *Ccl8* in lung tissue after LPS challenge [51]. In addition, myeloid-specific *Bmal1* knockout mice also exhibit a higher concentration of serum IL-1β, IL-6, IFN-γ and CCL2 after infection with Listeria monocytogenes, and a higher expression of *Ccl8* and *S100a8* mRNA in blood monocytes. Since the promoter region of these chemokines contained E-box motif, BMAL1:CLOCK heterodimer might recruit a repressor complex to silence chemokine gene expression [52]. Plasma levels of adipokines such as leptin and adiponectin, which have a proinflammatory and anti-inflammatory effect, respectively, are also elevated in *Bmal1* null mice compared with wild-type mice [53]. Unlike *Bmal1* gene, *Clock* mutant mice showed a significant repression of *IL-1*β, *IL-6*, *TNF*-α and *Ccl2* mRNA expression in bone marrow-derived macrophages [54], however, serum levels of leptin are increased in these mice [55].

*Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice are increased in the number of activated CD3$^+$ CD69$^+$ T cells and in serum levels of TNF-α [56]. In addition, the absence of *Cry* leads to constitutive activation of protein kinase A, results in phosphorylation of p65, thereby ultimately induces NF-κB activation. Interestingly, LPS-challenge induces higher amount of serum IL-6 as well as TNF-α, after bone marrow transplantation from *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice into immunosuppressive mice which lack T, B, NK cells and macrophages [57]. Likewise, *Per1* knockdown-rat spinal astrocytes are increased, whereas *Per1* overexpressed cells are decreased in production of IL-6 and CCL2, which is regulated by p38, JNK and NF-κB activation [58]. In *Per2*$^{-/-}$ mice, IL-6 and TNF-α protein levels are increased as compared with wild-type mice during myocardial inflammation [59]. Further, serum levels of leptin are elevated in *Per1*$^{-/-}$*Per2*$^{m/m}$ mice compared with wild type mice [60]

Using transfection or agonist treatment strategies, Rev-erbα represses *Ccl2* gene expression directly via RRE motif in their promoter region, which subsequently suppressed CCL2-dependent phosphorylation of ERK and p38 in murine macrophage RAW264 cells [61]. This is consistent with the previous result using human macrophage cells that REV-ERB ligand repressed mRNA transcription of *IL-6*, *Cxcl6*, *Cxcl11* and *IL-19* as well as *Ccl2* after LPS stimulation [62]. Recently, it has been reported that Rev-erbs regulate their target genes by inhibiting the functions of distal enhancers that are selected by macrophage-lineage-determining factors, thereby establishing a macrophage-specific program of repression [63].

Thus, clock genes directly or indirectly regulate production of inflammatory cytokines/chemokines/adipokines, and play a role of anti-inflammatory effect in host. These results suggest that diurnal rhythm of immune system, induced by clock gene oscillation, counteracts infection and increases resistance to pathogens. This concept is also supported by following result that the host response to antigens and pathogens differ between daytime and nighttime [23,54].

## Linkage between circadian clock genes & RA

RA is a chronic inflammatory disease with polyarthritis condition. Then, how does the circadian clock gene influence the etiology of RA? We clarified this proposition at first time using *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice [56]. Splenic T lymphocytes from *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice were found to be constitutively activated *in vivo*, and stimulation of splenocytes by anti-CD3/CD28 antibodies induced higher amounts of TNF-α production *in vitro*. In addition, G2/M cell cycle control factor Wee1 kinase and proto-oncogene c-Fos protein were overexpressed in the spleens of *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice [56,64]. Likewise, G1/S phase transition regulator cyclin D1 and AP-1 genes including c-Fos are overexpressed in osteoblasts of *Per1*$^{-/-}$*Per2*$^{m/m}$ mice [60], suggesting that clock genes regulate cell division and

Table 1. Effect of core clock genes on expression of inflammatory mediators.

| Clock gene | Animal or cell types | Materials | Inflammatory mediators | Induction | Ref. |
|---|---|---|---|---|---|
| *Bmal1* | Bronchiolar cells-specific knockout mice | mRNA (in lung tissue) | Up: Cxcl5, Ccl20, Ccl8 | LPS challenge | [51] |
| | Myeloid-specific knockout mice | Serum | Up: IL-1β, IL-6, IFN-γ, Ccl2 | Infection with Listeria monocytogenes | [52] |
| | | mRNA (in blood monocytes) | Up: Ccl8, S100a8 | – | [52] |
| | Null mice | Plasma | Up: leptin, adiponectin | – | [53] |
| *Clock* | Mutant mice | mRNA (in BMDMs) | Down: IL-1β, IL-6, TNF-α, Ccl2 | LPS stimulation | [54] |
| | | Serum | Up: leptin | – | [55] |
| *Cry1*/*Cry2* | Knockout mice | Serum | Up: TNF-α | – | [56] |
| | BMT experiments using knockout mice | Serum | Up: IL-6, TNF-α | LPS challenge | [57] |
| *Per1* | Knockdown cells | mRNA (in cultured rat spinal astrocytes) | Up: Ccl2, IL-6 | – | [58] |
| | Transfected cells | | Down: Ccl2, IL-6 | – | |
| *Per2* | Knockout mice | Supernatants (of homogenated heart tissue) | Up: IL-6, TNF-α | Myocardial inflammation | [59] |
| *Per1*/*Per2* | Deficient mice | Serum | Up: leptin | – | [60] |
| *Rev-erb* α | Transfected cells or agonist treatment | mRNA (in murine macrophage RAW264 cells) | Down: Ccl2 | LPS stimulation | [61] |
| | Agonist treatment | mRNA(in human MDMs) | Down: Ccl2, IL-6, Cxcl6, Cxcl11, IL-19 | LPS stimulation | [62] |

BMT: Bone marrow transplantation; BMDM: Bone marrow-derived macrophage; MDM: Monocyte-derived macrophage.

proliferation through cell cycle regulators. Interestingly, synovial cell proliferation was also enhanced through the suppression of p21 and overexpression of c-Fos [65], while its mitotic activity was inhibited through Wee1 kinase in patients with RA [66]. This is a characteristic feature of the synovial cells representing 'tumor cell-like proliferation', and then, we speculated that *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice are ready or primed for arthritis-onset. Accordingly, in *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice, arthritis of the limbs was strongly induced by Type II collagen cocktail and serum levels of TNF-α, IL-1β, IL-6 and MMP-3 was increased as compared with wild-type mice. This arthritis was suppressed by anti-TNF-α antibodies. Finally, mutual regulation between TNF-α and Cry genes was demonstrated using luciferase reporter assays [56]. These results are consistent with a subsequent report that expression of *Cry1* was markedly decreased by administration of melatonin and aggravated in mouse anti-type II collagen antibody-induced arthritis [67].

Do clock genes oscillate in patients with RA? First, in mice model of arthritis, PER2 and CRY1 protein were highly expressed through the day whereas they were expressed only in the night time in normal controls. In addition, the phase of *Per1*/*2* mRNA expression in spleen lymphocytes was shifted back approximately 6 h, and *Bmal1* and *Per1*/*2* mRNA expression levels were reduced in arthritic mice as compared with control mice [56]. Second, in human fibroblast-like synovial cells (FLS), rhythmicity of clock genes including *Bmal1*, *Clock* and *Per1* were present, however, the phase and amplitude of these genes were different between osteoarthritis (OA) and RA [68]. Conversely, Haas S *et al.* showed the absent of rhythmicity in OA-FLS and RA-FLS, although protein and mRNA levels of clock genes are detected in both synovial tissues [69]. Finally, in peripheral blood mononuclear cells from patients with RA, mRNA expression of *E4bp4* were higher, while those of *Dbp*, *Hlf* and *Tef* were lower as compared with

healthy controls (Figure 1). Interestingly, such composition of gene expressions was cancelled by infusion of anti-TNF antibodies (Infliximab), possibly because the changing of TNF-α concentration both in sera and synovial fluid during the entire observation period [70]. Indeed, these results were confirmed *in vitro* study that TNF-α enhanced RNA expression of *E4bp4*, whereas suppressed those of *Dbp*, *Hlf* and *Tef* [71]. However, mRNA expression of *Per2* were continuously higher in RA-peripheral blood mononuclear cells as compared with controls, although TNF-α strongly reduced the expression level of *Per2* in RA-FLS. This discrepancy is presumably due to glucocorticoid drugs, such as prednisone/prednisolone and dexamethasone, known as a strong inducer of *Per* gene oscillation [72,73]. In addition, recent study showed that circadian rhythms of the clock genes were disturbed in peripheral blood from patients with RA, especially in monocyte in which rhythms of *Per2/3* expression were lost [74]. Taken together, clock genes seem to modulate pathogenesis of RA via inflammation and cell proliferation, and *vice versa*.

## Chronotherapy in RA

How do we treat for patients with RA whose symptoms exhibit severe diurnal variations? For example, glucocorticoids have an anti-inflammatory and immunosuppressive effects, and diurnal rhythm of endogenous cortisol levels reach peak in the morning, but shift toward several hours in patients with RA compared with healthy controls. These cortisol levels are also increased in RA

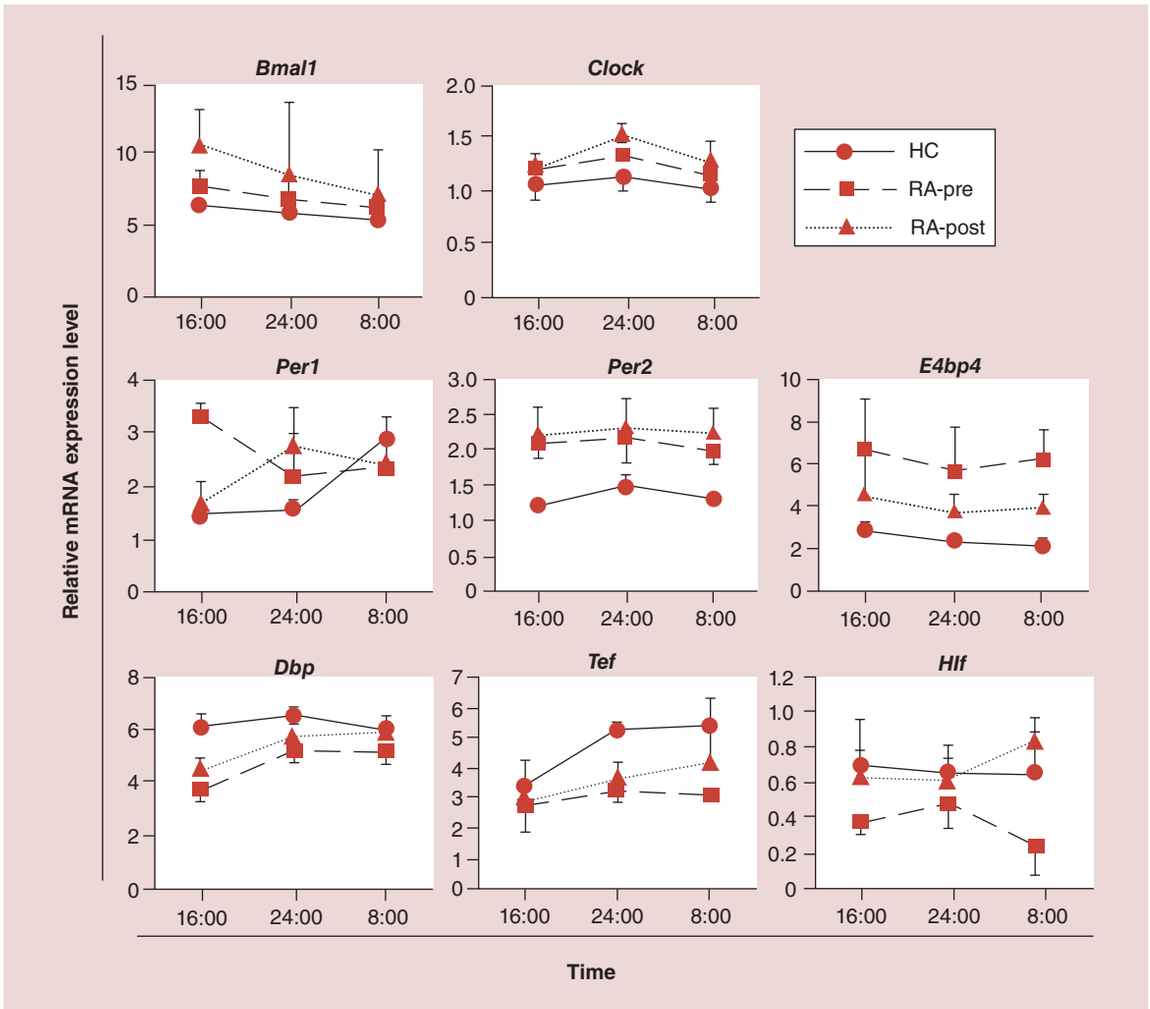


**Figure 1. Circadian clock gene mRNA expression in peripheral blood mononuclear cells from healthy controls (circle, n = 5) and patients with rheumatoid arthritis before and after infusion of infliximab (rheumatoid arthritis-before; square and rheumatoid arthritis-after; triangle, respectively, n = 4).** Total RNA were extracted from peripheral blood mononuclear cells in healthy donors and RA patients every 8 h, and subsequently, clock gene expression were analyzed by real-time PCR using TaqMan probe. Each gene expression levels were normalized to *Tbp*. Values shown are means ± SEM (standard error of the mean).
HC: Healthy controls; RA: Rheumatoid arthritis.

patients, however, this increase seems to be insufficient in view of the arthritis activity [75]. Thus, low-dose prednisone, a synthetic glucocorticoids, is used for the long-term treatment of chronic inflammatory diseases including RA [76]. Interestingly, it has been reported that oral intake of low-dose prednisone shows an excellent effect when given in the evening but not in the morning [77,78], indicating that chronotherapy could be a reasonable approach for treatment of RA. Recently, modified release prednisone (MR prednisone) was newly developed for the treatment of RA particularly for the purpose to recover morning stiffness [79]. Since MR prednisone releases its ingredients approximately 4 h after ingestion, MR prednisone taken at bedtime (released about 2:00 AM) could be more effective than conventional prednisone, immediate-release prednisone (IR prednisone), taken in the morning. Indeed, the prednisone chronotherapy with low-dose MR prednisone provided significant benefits over IR prednisone for the treatment of RA which are maintained for up to 12 months (CAPRA-1 study); not only in reduction of morning stiffness as expected but also in ameliorating the entire disease activity such as DAS28 (disease activity score 28) and VAS (visual analog scale), although adverse events involved in treatment with MR prednisone did not differ from the known profile of conventional low-dose prednisone [79,80]. This efficacy was confirmed in randomized, placebo-controlled study (CAPRA-2 study) [81], and was also observed when IR prednisone was switched to MR prednisone, given at bedtime [82]. Chronotherapy has been reported in case of methotrexate [83,84], and it could bring economic benefits for the patients to delay the introduction of biologics.

## Conclusion

In patients with RA, the symptoms show a diurnal rhythm such as morning stiffness, due to activation of immune response during the night, which is controlled partially by circadian clock gene oscillation. Accordingly, disruption of clock gene oscillation could modulate the host immune response, and cause host to the inflammatory condition though higher production of inflammatory mediators. This disruption also could lead to upregulation of cell cycle regulators such as Wee-1, which promote increased synovial cell proliferation, and result in joint stiffness and destruction in patients with RA. Conversely, the pathogenesis of arthritis can affect circadian clock gene expression profiles (Figure 2). Thus, circadian rhythm and pathogenesis of RA are closely interacted with each other, and the chronotherapy, currently in development based on these findings, might provide physical, mental and economic benefits for the patients.

## Future perspective

Recently, a small molecule KL001, screened from library of human osteosarcoma cell line, has been developed to prevent ubiquitin dependent degradation of CRY, resulting in lengthening of the circadian period. This small molecule may provide a novel therapeutic approach for diabetes since CRY protein associates with gluconeogenesis, particularly in hepatocytes [85]. A vast undeveloped field still remain in con-

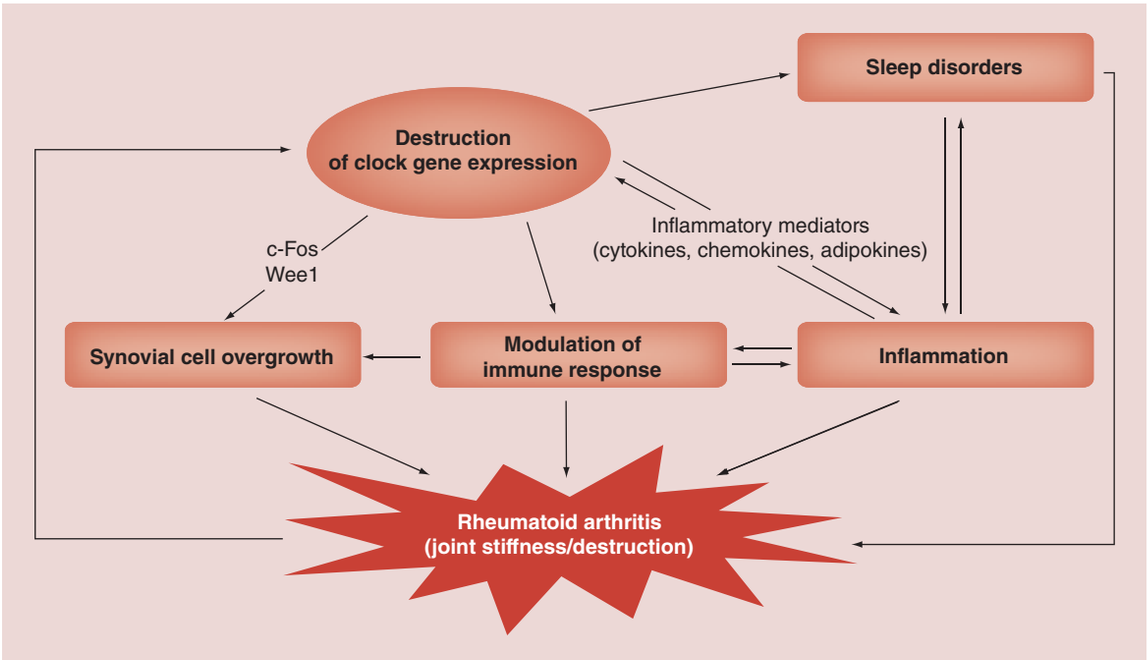


**Figure 2. Role of the clock genes in pathogenesis of rheumatoid arthritis.**

trolling the interaction between circadian clock genes and autoimmune disease including RA.

### Financial & competing interests disclosure

This work was supported in part by Grants-in-Aid for Scientific Research (KAKENHI) 26860751 to K Yoshida. The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

No writing assistance was utilized in the production of this manuscript.

## Executive summary

**'Morning stiffness,' a characteristic symptom with diurnal fluctuation in rheumatoid arthritis**

- The reason for morning stiffness in RA patients could be that activity of human immune responses including hormone secretion, cytokine and antibody production is accelerated during night time (rest phase) than day time (active phase).

**Sleep disorders in rheumatoid arthritis**

- Sleep disorders not only impacts on arthritic symptoms, but is also involved in the pathogenesis of rheumatoid arthritis (RA).
- However, further studies need to be determined the effects of sleep disturbance on inflammation.

**Circadian clock genes regulate immune response & inflammatory mediators**

- Clock genes directly or indirectly regulate the production of inflammatory cytokines/chemokines/adipokines, and play a role of anti-inflammatory effect in host.

**Linkage between circadian clock genes & RA**

- Clock genes modulate pathogenesis of RA through immune response such as inflammation and cell proliferation, and *vice versa*.

**Chronotherapy for RA**

- Chronotherapy for RA patients may provide benefits such as reduction in morning stiffness as compared with conventional treatment.

**Conclusion**

- Circadian rhythm is associated with joint stiffness and destruction in patients with RA.

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