

REVIEW SERIES

Circadian clock and the onset of cardiovascular events

Norihiko Takeda¹ and Koji Maemura²

The onset of cardiovascular diseases often shows time-of-day variation. Acute myocardial infarction or ventricular arrhythmia such as ventricular tachycardia occurs mainly in the early morning. Multiple biochemical and physiological parameters show circadian rhythm, which may account for the diurnal variation of cardiovascular events. These include the variations in blood pressure, activity of the autonomic nervous system and renin–angiotensin axis, coagulation cascade, vascular tone and the intracellular metabolism of cardiomyocytes. Importantly, the molecular clock system seems to underlie the circadian variation of these parameters. The center of the biological clock, also known as the central clock, exists in the suprachiasmatic nucleus. In contrast, the molecular clock system is also activated in each cell of the peripheral organs and constitute the peripheral clock. The biological clock system is currently considered to have a beneficial role in maintaining the homeostasis of each organ. Discoordination, however, between the peripheral clock and external environment could potentially underlie the development of cardiovascular events. Therefore, understanding the molecular and cellular pathways by which cardiovascular events occur in a diurnal oscillatory pattern will help the establishment of a novel therapeutic approach to the management of cardiovascular disorders.

Hypertension Research (2016) 39, 383–390; doi:10.1038/hr.2016.9; published online 18 February 2016

Keywords: acute myocardial infarction; cardiovascular event; circadian rhythm; molecular clock; vascular dysfunction

INTRODUCTION

Multiple systemic circulatory parameters, such as blood pressure (BP), heart rate (HR) and activity of the autonomic nervous system or renin–angiotensin cascade, exhibit time-of-day variation.^{1–3} BP mostly shows a circadian variation with a peak in the early morning, and dysregulation of systemic BP rhythm is independently associated with the poor prognosis of patients with acute myocardial infarction (AMI).⁴ The link between BP variation and the onset of cardiovascular diseases has been extensively studied.⁵ Moreover, the occurrence of cardiovascular events tends to show time-of-day variation.^{6–8} Therefore, elucidation of the link between the internal clock and cardiovascular function will help in the establishment of a novel therapeutic approach to cardiovascular diseases.

CIRCADIAN ONSET OF CARDIOVASCULAR DISEASES

Circadian variation in the onset of cardiovascular diseases has been observed in stroke,⁹ unstable angina¹⁰ and sudden cardiac death.¹¹ AMI has also been reported to mostly occur in the early morning.⁷ The rise in BP, HR, coagulation activity and vascular tone in the early morning underlies the frequent onset of AMI at this time of day (Figure 1). Ventricular arrhythmias such as ventricular tachycardia or ventricular fibrillation also tend to develop in the morning.^{6,12–14} Meanwhile, the onset of takotsubo cardiomyopathy, an acute but reversible left ventricular dysfunction disorder that is usually triggered by stressful events, also exhibits circadian variation with a peak in the

afternoon and nadir from 0000 to 0400 hours.¹⁵ The Mayo Clinic Percutaneous Coronary Intervention Registry investigated the medical records of patients ($n=124$) who were diagnosed with stent thrombosis and showed that the onset of stent thrombosis also exhibits circadian oscillation with a peak incidence around 0700 hours.¹⁶

In addition to thrombotic events, the occurrence of acute aortic dissection (AAD) also exhibits a time-of-day variation. The onset time of AAD has two peaks, consisting of a primary morning peak (0800–1100 hours) and a secondary evening peak (1700–1900 hours).¹⁷ Intriguingly, the diurnal BP pattern may affect the onset time of AAD, as illustrated by the observation that non-dipper or riser patients had a higher incidence of AAD during nighttime compared with dipper patients.¹⁸

It is important to note that the circadian variation of acute coronary syndrome (ACS) is not observed in patients receiving β -blocker treatment.⁷ The presence of chronic kidney disease (CKD) may also affect the onset time of ACS, as reflected by the finding that the onset of ACS showed circadian variation in non-CKD patients but not in CKD patients with an estimated glomerular filtration rate $<60 \text{ ml min}^{-1} 1.73 \text{ m}^{-2}$.¹⁹

Transition to or from daylight saving time occurs in spring and autumn, leading to the occurrence of a mismatch between the external and internal clocks during these transition periods. Compared with regular periods, there seems to be around a 30% increase in the

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Received 13 December 2015; revised 11 January 2016; accepted 12 January 2016; published online 18 February 2016

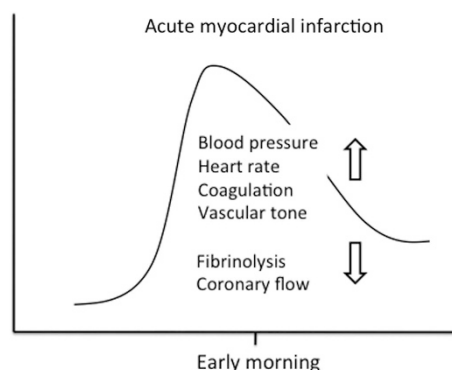


Figure 1 Acute myocardial infarction mostly occurs in the early morning. Morning rises in blood pressure, heart rate, coagulation cascade and coronary artery tone underlie the circadian onset of acute coronary syndrome.

incidence of ACS during the first 4 workdays of the transition period.²⁰ These results imply that the trigger of cardiovascular events is strongly affected by the coordination between the internal clock and external environment.

MOLECULAR CLOCK IN MAMMALIAN CELLS

In addition to the external environment, the internal clock also has a major role in physiological and biochemical functions. The center of the biological clock exists in the suprachiasmatic nucleus (SCN) of the hypothalamus and is referred to as the central clock. The intrinsic circadian rhythm of the central clock is elicited by the negative feedback loop formed by several core clock genes. Furthermore, the central clock receives external signals, such as light or physiological parameters, and adjusts the phase of its circadian clock accordingly, a phenomenon known as entrainment.²¹ The molecular processes of the biological clock have been summarized previously.^{5,22} The circadian expression of clock genes can also be observed in each cell or tissue, which composes the peripheral clock. The peripheral clock, together with the rhythms of the systemic autonomic nervous system and endocrine system, are coordinated by the central clock. Although the peripheral clock seems to underlie the diurnal variation of these fluctuations, its precise molecular mechanisms are still unclear.

A transcriptional–translational negative feedback loop composed of the core clock genes constitutes the internal clock. Core clock genes include *Clock*, *Npas2*, *Bmal1/2* and *Period (Per)1/2/3*, which belong to a group of helix-loop-helix/*Per-Arnt-Sim* domain-containing transcription factors. CLOCK/NPAS2 forms a heterodimer with BMAL1/2, and this CLOCK/BMAL heterodimer binds to the E-box upstream of *Per1/2/3* and *Cryptochrome1/2 (Cry)*. PER1/2/3 or CRY1/2 proteins are phosphorylated by casein kinase (CK)-1 ϵ and degraded through an ubiquitin-mediated proteasomal pathway. Under normal circadian conditions, however, PER1/2/3 and CRY1/2 gradually accumulate in amount and inhibit CLOCK/BMAL-mediated transactivation processes, thereby establishing a negative feedback loop (Figure 2). This feedback loop takes approximately 24 h, thus accounting for the diurnal rhythm. The fundamental role of *Bmal1* in the clock system is demonstrated by the fact that *Bmal1*-deficient mice (*Bmal1*-KO) exhibit arrhythmic behavior.²³

The CLOCK/BMAL heterodimer also binds to the E-box and induces the circadian expression levels of the following genes, *arginine vasopressin* and *Wee1*. Genes induced by core clock genes are termed clock-controlled genes (CCGs). CLOCK/BMAL also binds to the promoter of proline-acid-rich (PAR)-domain basic leucine zipper

transcription factors, D-element binding proteins (*Dbp*), hepatic leukemia factor (*Hlf*) and thyrotrophic embryonic factor (*Tef*). DBP, HLF, TEF and E4 promoter-binding Protein 4 (E4BP4) bind to D-box, which is also related to the molecular clock and involved in the diurnal expression of CCGs. In addition, the REV-ERB/ROR-binding element (RRE) acts to maintain the molecular clock system as well. The CLOCK/BMAL heterodimer induces the expression of *Rev-erba/β* and *Rora* genes, resulting in the circadian mobilization of REV-ERB α/β and ROR α to the RRE upstream of other CCGs.

In addition to the central clock in the SCN, clock genes are also expressed in each organ with diurnal variation and make up the peripheral clock.²⁴ With the exception of some specific differences, most of the molecular clock system of the peripheral clock seems to be similar to that of the central clock. We previously identified *Clif/Bmal2* as one of the clock genes in vascular endothelial cells.²⁵ CLIF/BMAL2 may function as a tissue-specific clock gene as circadian expression of *Clif/Bmal2* is observed in the liver but not in the colon.²⁶ Although the precise role of each peripheral clock still remains to be elucidated, the intrinsic clock in each organ is now considered to have a beneficial role in maintaining tissue homeostasis (Figure 3).²⁷

CIRCADIAN REGULATION OF BP

It has been reported that BP in human individuals exhibits time-of-day variation with a rise in the morning and a decline at night time.^{28,29} Fluctuations in the activities of the autonomic nervous system, renin–angiotensin–aldosterone axis and plasma cortisol level underlie the diurnal variation of BP. In addition, nighttime BP is inversely associated with urinary melatonin secretion in elderly population.³⁰

Accumulating evidence has demonstrated that BP is also affected by the internal clock. Variation in *Cry1* gene seems to be associated with arterial hypertension.³¹ In addition, circadian variation in BP is abolished in mice deficient in the *Bmal1* gene or mutation in the *Clock* gene.³² Circadian variation of BP does not seem to depend on the internal clock in vascular endothelial cells, as its oscillation was not disrupted in endothelial cell-specific *Bmal1*-KO mice.³³ Molecular clocks, however, in vascular smooth muscle cells, adrenal gland or kidney contribute to the variation in BP. The nuclear transcription factor, *peroxisome proliferator-activated receptor γ (PPARγ)*, directly induces *Bmal1* mRNA expression. Notably, BP fluctuation was disrupted in mice deficient in PPAR γ in vascular smooth muscle cells.³⁴ Meanwhile, core clock genes *Cry1* and *Cry2* repressed the expression of the adrenal enzyme, *type IV 3 β -hydroxy-steroid dehydrogenase (Hsd3b6)*.³⁵ Aldosterone synthesis is thus enhanced in *Cry1/2*-deficient mice, resulting in the development of salt-sensitive hypertension. Moreover, renal collecting duct cells also have a critical role in BP fluctuation through the regulation of sodium channel expression.³⁶ PER1 acts downstream of aldosterone and directly activates the expression of the α -subunit of the renal epithelial sodium channel (α ENaC) in a circadian manner.³⁷ In addition, endothelin-1 expression is increased in *Per1*-deficient mice, which suppresses the activity of sodium channel.³⁶ Consistent with this hypothesis, systemic BP is decreased in *Per1*-deficient mice. The expression of α ENaC is also decreased in *Clock*-knockout mice, thereby resulting in hypotension.³⁸

DIURNAL VARIATION IN THE COAGULATION SYSTEM

Of the several systemic parameters that affect the circadian onset of cardiovascular disorders, the coagulation cascade is also another factor that contributes to this diurnal variation. Systemic coagulation activity is determined by the balance between coagulation and fibrinolytic

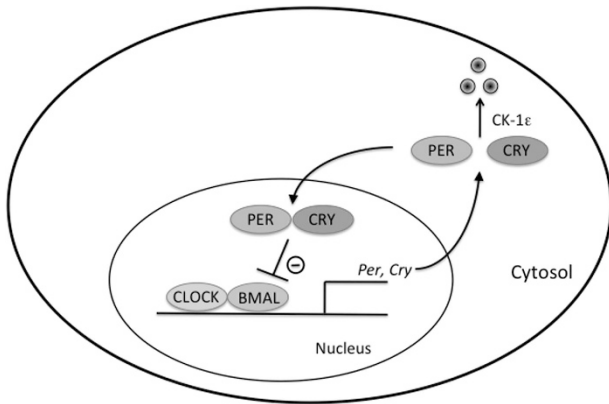


Figure 2 The heterodimer of CLOCK and BMAL bind to the E-box upstream of *per* and *cry* genes and activates their transcription. PER and CRY proteins are phosphorylated by casein kinase 1 epsilon (CK-1ε) and degraded through the proteasomal pathway. PER and CRY proteins, however, gradually accumulate in amount and inhibit CLOCK/BMAL-mediated transcription of *per* and *cry* genes. A full color version of this figure is available at the *Hypertension Research* journal online.

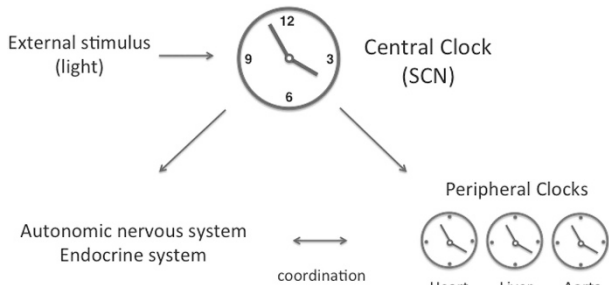


Figure 3 The central clock exists in the suprachiasmatic nucleus (SCN) in the hypothalamus. In addition, clock genes expressed in a circadian manner in each organ, thus called as peripheral clock. Coordination between the peripheral clock and autonomic nervous system or endocrine system has an integral role in maintaining the tissue homeostasis.

activities. In particular, serpin peptidase inhibitor/plasminogen activator inhibitor-1 (PAI-1) regulates the activity of tissue-type plasminogen activator (t-PA), thereby controlling systemic fibrinolytic activity. Importantly, PAI-1 mRNA and protein levels show a clear circadian variation.^{39,40} Consistent with this result, plasma levels of the plasmin–plasmin inhibitor complex decreases in the morning.⁴¹ These results imply that the mismatch between coagulation and fibrinolytic activity may elicit the onset of hemostatic disorders. The coagulation cascade is further dysregulated in patients with obstructive sleep apnea syndrome (OSAS). In OSAS patients who suffer intermittent hypoxia, plasma PAI-1 activity was increased, whereas the activity of t-PA was decreased. Augmentation of the mismatch between coagulation and anticoagulation cascades may therefore account for the higher incidence of cardiovascular events in OSAS patients.⁴²

Several studies have examined the molecular processes by which *Pai-1* expression is induced in a circadian manner. Forced disruption of the behavioral cycle did not alter the diurnal oscillation of plasma PAI-1 levels in 12 healthy volunteers, suggesting that the morning peak in human plasma PAI-1 level is elicited by the internal clock.⁴³ As an underlying mechanism, we and other groups identified that the heterodimer of CLOCK/BMAL1/2 binds to the E-box upstream of the *Pai-1* gene, resulting in the circadian activation of its transcription.^{25,44}

ROLES OF THE INTERNAL CLOCK IN VASCULAR FUNCTION

Consistent with the observation that BP or HR tends to increase in the early morning,⁴⁵ vascular function also exhibits diurnal oscillation. In particular, endothelium-dependent vasodilatory activity shows time-of-day variation not only in *in vitro* cultured rat vascular endothelial cells but also in human subjects.^{46,47} Importantly, vasodilatory function decreases in the early morning,⁴⁸ resulting in a mismatch between oxygen consumption and supply during this time period.

Rho and its effector, Rho-associated protein kinase (ROCK), phosphorylates myosin light chain in vascular smooth muscle cells and thus acts as a major regulator of vascular contraction. Intriguingly, activation of ROCK2 shows diurnal variation, leading to the circadian phosphorylation of myosin light chain in cultured vascular smooth muscle cells.⁴⁹ As an underlying molecular mechanism, *RORα*, a clock gene, enhances the expression of *ROCK2* gene in a circadian manner. Notably, dysregulation of the Rho-kinase pathway has a major role in the pathogenesis of vasospastic angina (VSA), supported by the finding that inhibition of Rho-kinase seems to be beneficial in alleviating the VSA attack in both animal model and human VSA patients.⁵⁰ Intriguingly, VSA patients exhibit enhanced Rho-kinase activity not only in vascular smooth muscle cells but also in circulating leukocytes.⁵¹ Moreover, Rho-kinase activity in circulating leukocytes also exhibits circadian variation.⁵² Although these results demonstrate the existence of a pathological process that activates Rho-kinase in a time-of-day-dependent manner, the molecular mechanisms by which Rho-kinase activity is enhanced in VSA patients still remain to be elucidated.

Similar to how it is in the central clock in the SCN, core clock genes are also expressed in vascular endothelial cells or smooth muscle cells.^{53–55} We previously identified thrombomodulin as one of the downstream CCGs in vascular endothelial cells, a factor that exerts anticoagulant activity on the membranes of vascular endothelial cells.⁵⁵ In addition to the coagulation system, the expression levels of tissue inhibitor of metalloproteinase 1 and 3, collagen 3a1, transgelin1 (sm22α) and calponin1 also exhibit diurnal variation in vascular smooth muscle cells.⁵⁶

Genetically engineered mice with mutations in core clock genes help to understand the roles of each peripheral clock in the pathogenesis of vascular disorders. Mouse carotid artery ligation was performed to induce vascular remodeling and thickening of the vessel walls. Intriguingly, carotid artery ligation of *Bmal1*-KO resulted in altered vascular remodeling with dilated vascular lumen and thrombus formation. Although *Clock*^{mut} mice behave normally in a light/dark (L/D) cycle environment, it becomes arrhythmic in constantly dark conditions. The extent of vascular remodeling in *Clock*^{mut} mice that have undergone carotid artery ligation was similar to control mice when the mice were kept in a L/D cycle. However, vascular remodeling was significantly augmented in *Clock*^{mut} mice when they were kept in constant darkness, indicating the roles of intrinsic circadian rhythm in the development of vascular pathology. Importantly, the expression of *Pai-1* gene in vascular endothelium or liver is increased in *Bmal1*-KO mice. Moreover, aortic ring prepared from *Bmal1*-KO mice showed reduced response to acetylcholine, highlighting the impaired endothelial function in *Bmal1*-KO mice. Supporting this hypothesis is the observation that endothelial Akt signaling is also blunted in *Bmal1*-KO mice.⁵⁷

Although endothelial nitric oxide (NO) has an integral role in vascular integrity, its activity shows diurnal variation. The abundance of endothelial NO synthase (eNOS) mRNA or protein has time-of-day variation.⁵⁸ Rat mesenteric artery exhibits time-of-day variation in its contractility. Intriguingly, treatment with nitro-L-arginine methyl

ester, a NOS inhibitor, abolished its fluctuation. Core clock genes also induce the circadian expression of dihydrofolate reductase, which regulates the bioavailability of an eNOS cofactor, bipterin.⁵⁹ Aorta from *Bmal1*-KO mice showed a reduction in tetrahydrobiopterin, and an increase in dihydrobiopterin, resulting in an impaired coupling of eNOS. Importantly, vascular signaling such as NO or angiotensin II could in turn affect the internal clock.^{54,60} It has been reported that the amplitude of circadian clock decreases with age. Treatment with NO donor significantly upregulates the expression of *Per2* gene, resulting in a restoration of the biological clock.⁶⁰

Photochemical injury of the femoral artery was performed as a murine experimental thrombosis disease model. Time to thrombotic vascular occlusion (TTVO) after photochemical injury was measured at zeitgeber time (ZT) 2, 8, 14 and 20. Time-of-day variation in TTVO was observed in wild-type mice with the longest TTVO at ZT 8. Importantly, circadian oscillation of TTVO is disrupted when photochemical injury was performed in *Clock*^{mut} mice, suggesting that the intrinsic molecular clock system contributes to the onset of vascular occlusion. Multiple factors contribute to thrombosis formation, including endothelial function, BP and coagulation activity. To elucidate the role of the peripheral clock in vascular endothelial cells, the photochemical injury model was carried out in endothelial cell-specific *Bmal1*-KO mice. Intriguingly, diurnal oscillation of TTVO disappeared in endothelial *Bmal1*-KO mice, whereas diurnal oscillation of BP or coagulation activity was not affected. These results thus showed that internal clock processes in vascular endothelial cells have an integral role in the development of vascular thrombosis.³³

Biological clock system also regulates the tissue remodeling or angiogenesis. In response to a tissue injury, CD34⁺/CD133⁺ progenitor cells (PCs) or endothelial PCs (EPCs) were mobilized and promotes tissue repair or angiogenic processes. The number of human peripheral blood PCs shows diurnal oscillation with a peak at 2000 hours and nadir at 0800 hours.⁶¹ Intriguingly, *Per2* is highly expressed in EPCs.⁶² EPC from *Per2*-deficient mice had reduced potential in proliferation, migration or tube formation. Supporting this hypothesis, mice transplanted with *Per2*-deficient EPC showed decreased left ventricular function after myocardial infarction compared with wild-type EPC-transplanted mice. Clock genes also have an integral role in angiogenic processes of zebrafish.⁶³ Morpholino to *Bmal1* reduced the expression of vascular endothelial growth factor, resulting in the impairment of developmental angiogenesis. Inhibition of *Per2* in turn accelerates the angiogenesis, implying the link between clock genes and developmental angiogenesis.

The internal clock in smooth muscle cells also contributes to the time-of-day variations of BP. Deletion of *Bmal1* in smooth muscle cells (SM-*Bmal1*-KO) showed decreased oscillation of clock genes in mesenteric arteries. Although the contractile responses of mesenteric arteries to phenylephrine or serotonin show time-of-day variation, it was significantly attenuated in SM-*Bmal1*-KO mice. As an underlying molecular mechanism, BMAL1 transactivates *Rock2* gene expression and leads to the oscillation of myosin light chain phosphorylation. Consistent with this hypothesis, diurnal oscillation of BP is abolished in SM-*Bmal1*-KO mice, illuminating the roles of the internal clock in BP regulation.⁶⁴

Peripheral clock in the vasculature also contributes to the homeostasis of transplanted vessels. Development of atherosclerosis and inflammatory cell accumulation were evaluated after isograft transplantation of the carotid artery. Compared with the isograft obtained from wild-type mice, those from *Bmal1*-KO or *Per* triple knockout mice showed severe atherosclerotic lesions.⁶⁵

MOLECULAR CLOCK IN THE HEART

In addition to the peripheral clock in the vasculature, cardiomyocytes also have an intrinsic molecular clock where the core clock genes exhibit diurnal variation.²⁵ Similar to the serum shock of cultured fibroblasts, the phase of each peripheral clock in cardiomyocytes can be synchronized using norepinephrine.⁶⁶ A comprehensive analysis of the heart or liver for genes that exhibit circadian expression revealed 8–10% of all genes in each organ to be CCGs.⁶⁷ Intriguingly, most of the CCGs are organ specific, suggesting that each organ has its own specific peripheral clock. Several CCGs in the heart have been identified, including *pyruvate dehydrogenase kinase isozyme-4* (*Pdk-4*), *solute carrier family 2* (facilitated glucose transporter), member 1 (*Slc2a1*), also known as *glucose transporter 1* (*Glut-1*), *Slc2a4/Glut-4* and potassium channels *Kv1.5* and *Kv4.2*.^{68–70}

The peripheral clock in each organ may have a beneficial role in maintaining organ homeostasis. Natriuretic peptide A (NPPA/ANP) is known to act as a cardioprotective agent. Importantly, the expression of *anp* shows circadian oscillation. As BP rises in a time-of-day-dependent manner, simultaneous induction of ANP in cardiomyocytes may offset an increase in afterload on cardiomyocytes.

In addition to the circadian onset of AMI, myocardial infarction tolerance also exhibits diurnal oscillation. In a mice model of ischemia/reperfusion, hearts subjected to ischemia at ZT 12 showed significantly greater infarct size compared with hearts subjected to ischemia at ZT 0. Importantly, this time-of-day dependence of myocardial damage is abolished in cardiomyocyte-specific circadian *Clock*-mutant mice (CCM), thereby highlighting the roles of endogenous clock rhythm in ischemia tolerance.⁷¹ Ischemia tolerance also exhibits circadian oscillation in human subjects. A retrospective study investigated the medical records of patients with ST elevation myocardial infarction (STEMI). Using peak serum creatine kinase (CK) levels as a surrogate marker of infarct size, the study demonstrated a significant association between time of STEMI onset and peak CK level. Peak CK level was maximum when the STEMI developed at 0100 hours. Consistent with peak CK levels, the extent of decrease in left ventricular ejection fraction also depended on the time of STEMI onset.⁷²

Importantly, the phase or amplitude of the peripheral clock in the heart seems to be affected when cardiac disease is present. In an animal model of pressure overload-induced cardiac hypertrophy, circadian expression levels of PAR transcription factors (*Dbp*, *Hlf*) and *Anp* were significantly dampened.⁶⁹ As E4BP4 acts to antagonize the function of PAR transcription factors, attenuation of the peripheral clock of the heart is seen in myocardial ischemia/reperfusion owing to significant increase in E4BP4 expression under such conditions.⁷³ Disruption of the peripheral clock in the heart is also observed in diabetic hearts.⁷⁴

Desynchronization between the internal clock and external environment may elicit the onset of cardiovascular disorders. Continuous changes in the daily L/D cycle shortens the survival of cardiomyopathic hamsters.⁷⁵ Transverse aortic constriction procedure is used to acutely increase the afterload of the left ventricle (LV), leading to LV hypertrophy. Rhythm disruption from a 24-h (12-h/12-h L/D) to a 20-h (10-h/10-h) cycle significantly impaired the LV systolic function of mice that were subjected to the transverse aortic constriction model.⁷⁶ Moreover, even a short-term rhythm disruption augments the severity of LV remodeling after myocardial infarction.⁷⁷

In contrast, adjusting the circadian rhythm with restrictive feeding may improve cardiac performance. *Drosophila*, a fruit fly, also has an internal circadian rhythm, and its cardiac performance can be

evaluated by high-speed video imaging as fractional shortening. Time-restricted feeding (TRF) is a condition in which food access of a fly is limited to daytime 12 h every day. TRF did not affect the food consumption or locomotor activity of flies. Although fractional shortening in *Drosophila* usually decreases with age, TRF significantly improved cardiac performance in aged flies, indicating the beneficial effect of TRF on cardiac function.⁷⁸ Moreover, synchronization of the rhythm between the internal clock and external environment seems to be beneficial for shift-workers. The properties of human circadian rhythm, namely chronotypes, are highly individualistic and range from early, intermediate to late chronotypes. Intriguingly, both daily rhythm and sleep state were significantly improved when a work-shift schedule is designed based on the chronotype of each working individual.⁷⁹ These findings also support the hypothesis that the peripheral clock in each organ has a beneficial role by promoting the coordination between the internal clock and the external environment.

To elucidate the roles of the peripheral clock in the heart, genetically engineered mice were used for the cardiovascular disease models. Although *Bmal1*-KO mice exhibit arrhythmic behavior, it also develops several organ dysfunctions such as infertility,⁸⁰ structural alteration in skeletal muscle⁸¹ and dilated cardiomyopathy.⁸² To further elucidate the roles of clock genes in cardiomyocytes, the CCM transgenic mouse line was established in which mutant CLOCK protein is overexpressed specifically in cardiomyocytes. When the CCM mice were maintained in a repeated phase shift (12-h phase shift biweekly) environment, a significant rise in cardiac hypertrophy marker gene expression was observed.⁸³ Studies using CCM mice also revealed that intracellular metabolism of cardiomyocytes, including triglycerides and glycogen stores, show circadian variation in a peripheral clock-dependent manner.^{84,85} The roles of the peripheral clock in cardiomyocytes were also evaluated using cardiomyocyte-specific *Bmal1*-KO mice.^{82,86} Although glucose metabolism was repressed, fatty acid oxidation was activated in cardiomyocyte-specific *Bmal1*-KO mice. Importantly, decreased ejection fraction and subsequent development of heart failure was observed in aged cardiomyocyte-specific *Bmal1*-KO mice. Phosphoinositide-3-kinase, regulatory subunit 1 (Pik3r1) is a subunit of PI3K and regulates its intracellular signaling. Intriguingly, the *Pik3r1* gene is identified as one of the CCGs in cardiomyocytes. Taken together, these results indicate that the peripheral clock in cardiomyocytes is involved in growth factor signaling and fatty acid oxidation.

CIRCADIAN CLOCK AND THE ONSET OF CARDIAC ARRHYTHMIA

Diurnal variation of the autonomic nervous system and peripheral clock induces the time-of-day variation in the basic electrophysiological parameters of cardiomyocytes. Atrioventricular nodal function, QT interval and ventricular refractory period display circadian variation.^{87–89} Of the numerous CCGs identified, those involved in cellular electrophysiological function include two voltage gated K channels, *Kv1.5* and *Kv4.2*,⁶⁸ and *sodium channel, voltage-gated, type V, alpha subunit* (*Scn5a*).⁹⁰ Intriguingly, BMAL1 directly binds and induces the circadian expression of *Scn5a* gene in cardiomyocytes, resulting in HR variability. Supporting this hypothesis, mice with a cardiomyocyte-specific deletion of the *Bmal1* gene have slower HR and prolonged RR intervals, resulting in a higher susceptibility to the development of arrhythmia during electromechanical stimulations.

The onset of ventricular arrhythmias also shows time-of-day variation, with a first peak in the morning (0700–1100 hours) and followed by a second peak in the afternoon.^{6,13} The short ventricular refractory period underlies the onset of ventricular tachycardia or

ventricular fibrillation in the morning hours.¹⁴ Kv channel-interacting protein 2 (KChIP2) contributes to the transient outward potassium flow in cardiomyocytes, and its transcript level shows circadian variation. Intriguingly, BMAL1-mediated induction of a transcription factor, *kruppel-like factor 15* (*Klf15*), elicits the diurnal variation in *KChip2* expression,⁹¹ resulting in the time-of-day variation in QT interval and subsequent onset of ventricular arrhythmia.

It should be noted that the autonomic nervous system also seems to contribute to the time-of-day variation in ventricular arrhythmias. The Sudden Cardiac Death in Heart Failure Trial examined whether circadian patterns in ventricular arrhythmia is observed in patients with implantable cardioverter-defibrillator and found that the circadian onset of ventricular tachycardia/ventricular fibrillation disappeared in patients receiving beta-blocker therapy.⁹²

NOVEL FUNCTIONS OF CORE CLOCK GENES

It is important to be reminded that core clock genes may also have clock-independent functions. CRY, one of the core clock genes, seems to be involved in heart morphogenesis. Silencing *Drosophila cryptochrome* (*dCRY*) slowed the HR and reduced heart size in flies, implicating the developmental functions of core clock genes.⁹³

The core clock gene *Bmal1* also has an anti-inflammatory effect. BMAL1 affects the epigenetic modification and suppresses the expression of *chemokine (C-C motif) ligand 2* (*Ccl2*), a chemokine that mostly recruits pro-inflammatory monocytes (Ly6C^{hi}) through the interaction with its receptor, CCR2.⁹⁴ Supporting this hypothesis, the number of circulating Ly6C^{hi} monocytes exhibits circadian oscillation, and the disease severity of *Listeria monocytogenes* infection was augmented when the pathogen was inoculated at ZT 8. In addition, REV-ERB α also suppresses the expression of *Ccl2*.⁹⁵ Recruitment of pro-inflammatory monocytes/macrophages was increased in *Rev-erba*-deficient mice, resulting in an augmentation of atheroma formation in a vascular injury model.⁹⁶

Clock genes also directly affect intracellular metabolism. The nuclear transcription factor PPAR γ has an essential role in adipocyte differentiation through its binding to the PPAR response elements (PPREs). PER2 directly inhibits the function of PPAR γ by suppressing the binding of PPAR γ to PPRE.⁹⁷ Importantly, fatty acid oxidation was activated but triacylglycerol accumulation was decreased in *Per2*-deficient cells. Moreover, *Per2*-deficient fibroblasts exhibit higher differentiation potential to adipocytes. Intriguingly, a clinical study in diabetic patients showed that gene polymorphisms of *Cliff/Bmal2* are significantly associated with the presence of type 2 diabetes, suggesting a role for *Cliff/Bmal2* in intracellular metabolism.⁹⁸

Signaling involving adenosine and the adenosine receptor, ADORA2B, has an essential role in ischemia preconditioning of the heart. Importantly, the adenosine signal stabilizes the glycolytic transcription factor, hypoxia inducible factor (HIF)-1 α , through a PER2-dependent mechanism.⁹⁹ HIF-1 α subsequently activates the expression levels of glycolytic genes, resulting in the adaptation of cardiomyocytes to ischemia. HIF-1 α also induces the expression of *Per1* gene.¹⁰⁰ Circadian oscillation of *Per1* promoter activity is dampened in chronic hypoxia, illuminating an interplay between circadian rhythm and hypoxia signaling.

CONCLUSION

The internal clock system exists not only in the SCN as the central clock but also in each cell of the cardiovascular organs. The peripheral clock in each organ or cell seems to have a beneficial role by anticipating the changes in external environments. Therefore, disruption between the clock system in each organ and the external

environment tends to augment the severity of the organ dysfunction. Elucidation of the precise roles of the molecular clock in each cardiovascular organ will help us establish a novel therapeutic approach for cardiovascular disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

This study was supported by a Grant-in-Aid for Scientific Research from the Japan Society for the Promotion of Science, Japan (to KM and NT, 25461113).

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