

# Biological clocks: a cardiovascular perspective

**Georgios K. Paschos, PhD; Garret A. FitzGerald, MD**

*Institute for Translational Medicine and Therapeutics - University of Pennsylvania School of Medicine - Philadelphia - Pennsylvania - USA*

*Many aspects of cardiovascular physiology are subject to diurnal variation, and serious adverse cardiovascular events, including myocardial infarction, sudden cardiac death, and stroke, occur with a frequency conditioned by the time of day. Over the last decades, growing evidence has revealed the existence of an endogenous oscillator intrinsic to the cell—called the molecular clock—that orchestrates the physiology of such time-dependent oscillatory patterns of events. In the present review, we first provide a short overview of the molecular basis of the oscillator. Then, we explain the organization of the clock at the level of the organism. We discuss the current understanding of the role of clocks in cardiovascular physiology and summarize potential entraining signals of relevance to cardiovascular function. Finally, we consider the interaction between the clock and metabolic function, which can also impinge on the propensity to cardiovascular disease.*

## THE BIOLOGICAL CLOCK

Rotation of the earth involves diurnal changes in light and temperature. Organisms have evolved to adapt to diurnal environmental changes with an endogenous oscillator that allows tracking of time. This oscillator is intrinsic to most cells, persisting when tissues and cells are isolated or cultured *in vitro*.<sup>1</sup> The oscillator exists as a self-sustained transcriptional-translational loop creating a rhythm in gene expression with a period of approximately (“circa”) one day (“dies”), hence “circadian.” This rhythm in gene expression drives the circadian rhythms of physiology, which can adapt the physiology of an organism to its needs in an anticipatory manner. Organizing physiology appropriately to adapt to changes in the timing of recurring events (eg, sunrise, the time of food availability) lies in the ability of the circadian oscillator to synchronize its phase in response to external cues. Similar mechanisms seem likely to underline periodicities of different lengths (eg, ultradian rhythms), and there is some evidence for interaction between rhythms of differing periodicity,<sup>2</sup> but the molecular basis for these interactions is presently poorly understood.

### Molecular basis of the biological clock

While interest in diurnal variability in physiological functions is long-standing, the field was largely descriptive until Konopka and Benzer<sup>3</sup> identified a chromosomal region controlling the period of eclosion time in *Drosophila* in 1971. Since that time, the molecular basis of the endogenous oscillator has been identified. In mammals, the basic helix-loop-helix (bHLH) transcription factors Bmal1, Clock, and Npas2 act as activators and drive transcription through E-boxes located within the promoters of various target genes, including *Period* (*Per1-3*) and *Cryptochrome* (*Cry1-2*) genes. In a highly simplified model (*Figure 1, page 9*),<sup>4</sup> Clock:Bmal1 or Npas2:Bmal1 heterodimers bind to E-box consensus sequences in the *Per* and *Cry* genes. When *Per* and *Cry* are translated, they heterodimerize, translocate to the

**Keywords:** circadian clock; vascular smooth muscle cells; vascular endothelium; blood pressure; metabolism

**Address for correspondence:** Garret A. FitzGerald, Professor of Medicine and Pharmacology, McNeil Professor in Translational Medicine and Therapeutics, Institute for Translational Medicine and Therapeutics, University of Pennsylvania School of Medicine, 153 Johnson Pavilion, Philadelphia, Pennsylvania 19104, USA (e-mail: garret@upenn.edu)

*Dialogues Cardiovasc Med.* 2010;15:7-24

nucleus, and repress Clock/Bmal1-mediated transcriptional activity at E-boxes (Figure 1).<sup>4</sup> Increases in Per protein levels are delayed approximately 6 hours relative to their mRNA, and serine phosphorylation plays a critical role in regulating their ubiquitin-mediated degradation. Inhibition of *Clock/Bmal1* transcription by Per/Cry complexes ultimately decreases their own expression levels, resulting in relief of inhibition—thus restarting the cycle—approximately every 24 hours. Fbxl3, a component of a specific ubiquitin-ligase complex, was recently implicated in the proteasome-mediated decay of Cry proteins.<sup>5-7</sup> Additional interacting feedback loops involve bHLH domain-containing transcription factors Dec1, Dec2,<sup>8</sup> and Rev-erb  $\alpha$ .<sup>9</sup> Both *Dec1/2* and *Rev-erb  $\alpha$*  contain E-boxes in their promoters and are thus transcriptionally regulated by Clock/Bmal1 heterodimers. On translation of the corresponding proteins, Rev-erb  $\alpha$  specifically represses *Bmal1* transcription, whereas Dec1/2 are thought to impair *Clock/Bmal1* transactivational capacity.<sup>8</sup> *Bmal1* expression is positively regulated by Per2 and by retinoic acid-related orphan receptor  $\alpha$  (ROR- $\alpha$ ) acting through

ROR response element (RORE) sequences in the *Bmal1* promoter.<sup>10</sup> The overall result of these transcriptional/translational feedback loops is oscillatory expression of *Clock* genes and Clock proteins with a period at or near 24 hours, which is the molecular basis of the circadian clock. At the same time, Clock:Bmal1 and Npas2:Bmal1 heterodimers bind to E-box elements not only in *Per1-3*, *Cry1/2*, *Dec1/2*, and *Rev-erb  $\alpha$*  genes, but also in numerous genes not participating in the circadian clock (Figure 1).<sup>4</sup> Oscillating gene expression of these genes is then thought to occur, thus potentially impacting cell function in a rhythmic manner. It is now known that  $\approx$ 8% to 10% of the transcriptome is expressed in a circadian manner.<sup>11</sup>

Apart from the oscillating gene expression, posttranslational modifications act on Clock proteins to control finely circadian rhythms. Clock proteins undergo robust circadian changes in phosphorylation, which affect transcriptional activity, stability, and cellular localization.<sup>12</sup> Sumoylation of Bmal1 affects its rhythmic expression,<sup>13</sup> and dimerization of Bmal1 and Clock causes phosphorylation and directs nuclear accumulation of the complex.<sup>14</sup> Interestingly, the central tenet of the clockwork model—the dependence of rhythmic transcription of core clock genes—has been called into question in many nonmammalian organisms, indicating that rhythmic mRNA may not be necessary for oscillating protein levels.<sup>15</sup> These studies suggest that rhythmic protein phosphorylation is perhaps more fundamental to generating output oscillations in protein levels.

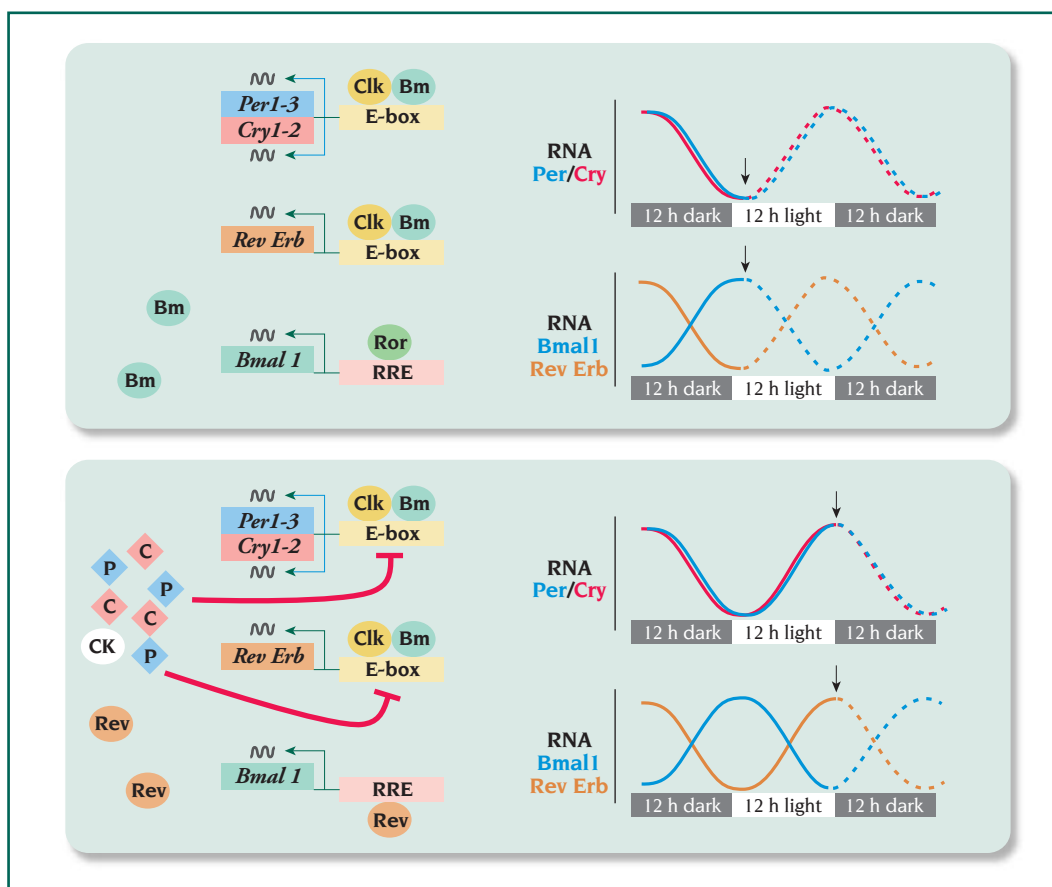
The components of the circadian clock are highly conserved among species. All clock genes described in mammals have been found in humans. Familial advanced sleep phase syndrome is a rare Mendelian inherited human syndrome of clock dysfunction, which is characterized by early sleep times and early morning awakening. A mutation in a phosphorylation site within the casein kinase I binding domain of *Per2* or the *Casein kinase I delta* gene, both cause a shortening of period length and give rise to the disease. This was the first human circadian rhythm variant to be well characterized. Evidence also links polymorphisms in clock genes with psychiatric disorders, such as seasonal affective disorder (SAD)<sup>16</sup> and alcohol consumption.<sup>17,18</sup>

### The biological clock at the organism level

At the organism level, the circadian system is largely organized in a hierarchical manner. Surgical ablation of the suprachiasmatic nucleus (SCN), a remarkable paired nuclear structure in the hypothalamus, ablates

#### SELECTED ABBREVIATIONS AND ACRONYMS

<b>ACTH</b>	adrenocorticotrophic hormone
<b>bHLH</b>	basic helix-loop-helix
<b>BP</b>	blood pressure
<b>CCG</b>	clock-controlled gene
<b>Cry</b>	cryptochrome
<b>EPI</b>	epinephrine
<b>GR</b>	glucocorticoid receptor
<b>KO</b>	knockout
<b>MBPS</b>	morning blood pressure surge
<b>NAD</b>	nicotinamide adenine dinucleotide
<b>NADH</b>	reduced form of NAD
<b>NADP</b>	nicotinamide adenine dinucleotide phosphate
<b>NADPH</b>	reduced form of NADP
<b>NE</b>	norepinephrine
<b>NO</b>	nitric oxide
<b>PAI-1</b>	plasminogen activator inhibitor 1
<b>Per</b>	period
<b>PPAR-<math>\alpha</math></b>	peroxisome proliferator-activated receptor $\alpha$
<b>RAS</b>	renin-angiotensin system
<b>SCN</b>	suprachiasmatic nucleus
<b>THR</b>	thyroid hormone receptor
<b>VSMC</b>	vascular smooth muscle cell



**Figure 1. Overview of transcriptional/translational feedback loops in the vascular circadian clock.**

Rhythmic molecular transcriptional/translational feedback loops form the basis of the circadian clock in many tissues including the vasculature. Clock (Clk) and Bmal1 (Bm) are 2 bHLH PAS transcription factors that heterodimerize and promote transcription through E-box motifs in many core clock component genes. Period 1 to 3 (Per) and Cryptochrome 1 to 2 (Cry) transcription/translation is induced in a rhythmic manner toward the beginning of the 12-hour light period. Rev-erb  $\alpha$  (Rev), an inhibitor of Bmal1 transcription, is similarly induced through E-box motifs by the Clock:Bmal1 heterodimer. Hence Bmal1 levels will decrease, whereas Per and Cry levels rise as the light phase progresses. At the end of the light phase, the levels of Per and Cry proteins are at their peak and Bmal1 is at a low level. The accumulating Per and Cry proteins (indicated by P and C diamond shapes) are phosphorylated by casein kinase (CK) and heterodimerize to shuttle back into the nucleus from the cytoplasm and prevent further Clock:Bmal1 driven transcription through E-box elements. Positive regulators of Bmal1 transcription, such as ROR- $\alpha$  (Ror), facilitate a subsequent rise in Bmal1 levels and increased Clock:Bmal1-mediated transcription, thus restarting the cycle.

**Abbreviations:** bHLH, basic helix-loop-helix; Bm, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like; C, Cry protein; CK, casein kinase; Clk, circadian locomotor output cycles kaput; Cry, cryptochrome; Npas, neuronal PAS domain-containing; P, Per protein; Per, period; Rev, Rev-erb  $\alpha$ ; Ror, retinoic acid-related orphan receptor  $\alpha$ .

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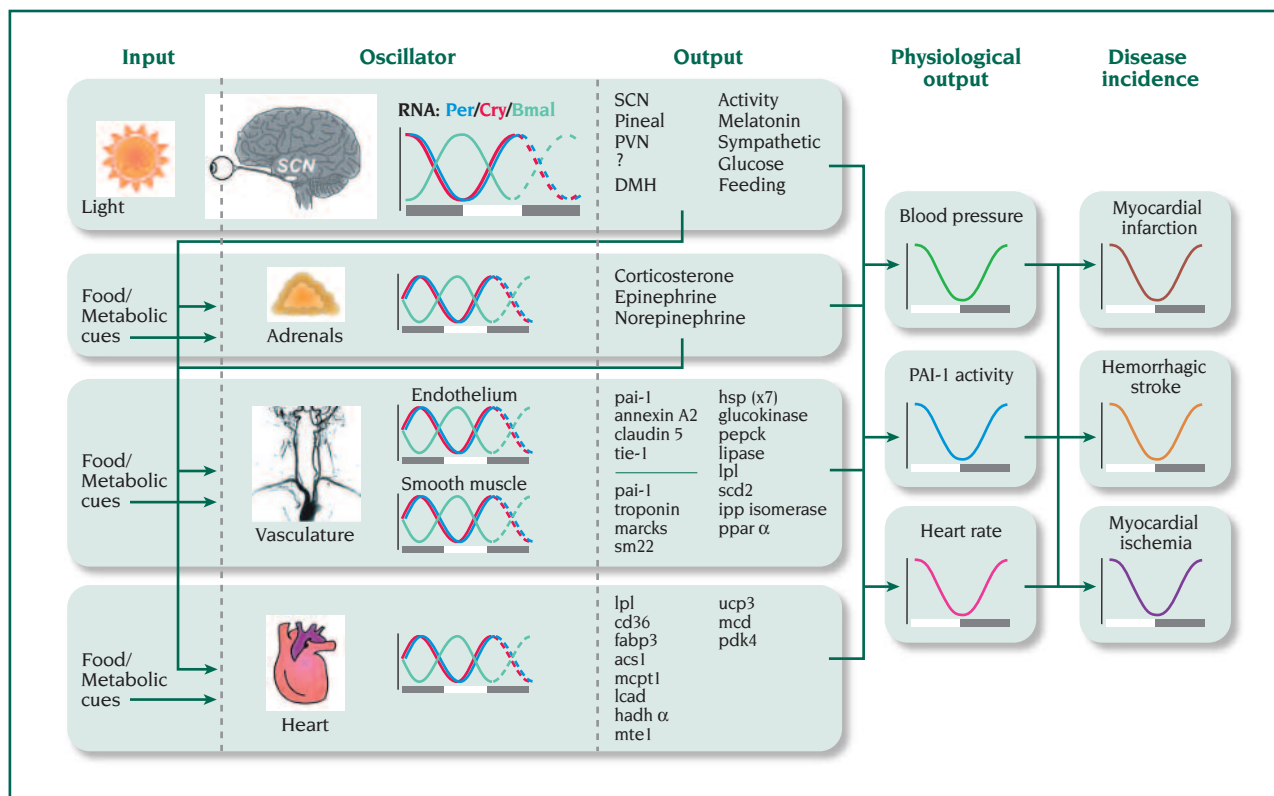
all hormonal and activity rhythms, suggesting the existence of a master circadian pacemaker located in the SCN.<sup>19</sup> The SCN receives photic input via the retino-hypothalamic tract to entrain the circadian clock to the light-dark cycle of the environment. Initially, the SCN was believed to be the sole site of the circadian clock, but as the genes comprising the core circadian clock were identified, their expression was detected outside the SCN.<sup>20</sup> Core circadian clock genes are expressed in non-SCN neural tissues as well as in peripheral tissues throughout the body, where most

exhibit an oscillating circadian pattern of expression. Indeed, even in culture, immortalized rat-1 fibroblasts and other cell lines show a circadian pattern of expression after treatment with serum,<sup>1,21</sup> suggesting the existence of peripheral clocks with the same molecular machinery found in the central clock in the SCN. The best known exception to this rule is the testis, which shows constant, rather than cyclic, expression of circadian clock genes in multiple animal species.<sup>22</sup> In contrast to the central clock in the SCN that is entrained by light, peripheral oscillators are synchronized

**Biological clocks: a cardiovascular perspective - Paschos and FitzGerald**

by various neurohumoral stimuli<sup>23</sup> and food or metabolic cues<sup>24</sup> (Figure 2).<sup>4</sup> The daily cycle of feeding and starvation, indirectly controlled by the SCN via activity rhythms, appears to be a dominant entrainment signal for peripheral clocks. When food is available *ad libitum*, food intake for mice is normally consolidated to the dark phase, corresponding to the activity phase for this nocturnal species. However, during a restricted-feeding regime, where food access is limited to the light phase, circadian gene expression in the periphery, but importantly, not in the SCN, is inverted by 12 hours.<sup>24,25</sup> Thus food intake, which may present as a

synchronous or asynchronous cue, is capable of entraining peripheral oscillators. Although restricted feeding appears to be a dominant entrainment signal for many tissues, the rate at which these various oscillators have the capacity to alter phase can vary. Metabolic signals from feeding (or perhaps, more importantly, starving) appear to be dominant cues for peripheral clocks. It is becoming increasingly apparent that daily metabolism and the circadian cycle are intertwined.<sup>26</sup> Circannual cycles in mammals (eg, hibernation) are similarly known to influence, and be influenced by, metabolism.<sup>27</sup>



**Figure 2. Organization of the central and peripheral molecular clocks.**

The suprachiasmatic nuclei synchronize peripheral oscillators including those within the vascular endothelium, smooth muscle, and the heart through a combination of autonomic, behavioral, endocrine, and food-related cues. Both the heart and the vasculature (endothelium and the smooth muscle compartments) rhythmically express the molecular components of the circadian clock. Tissue specific components, such as *Bmal2* (*Clif*), may help mediate tissue-specific circadian patterns of clock controlled gene expression eg, in the endothelium. Clock-controlled genes or “outputs,” such as plasminogen activator inhibitor 1 (*PAI-1*), *Dbp*, *Glut1*, *Glut4*, or *Pdk4*, can sustain local clock-dependent physiologies, such as the thrombolytic activity of tissue plasminogen activator, the 24-hour blood pressure pattern, or circadian variations in heart rate. Thus, the network of local circadian oscillators in vascular tissues could influence clock-dependent cardiovascular pathologies, including myocardial infarction, ischemia, and stroke. Vascular tissue-specific differences in molecular components, outputs, and their response to synchronizing factors could allow the design of more effective treatments for pathologies that carry a circadian bias.

**Abbreviations:** *acs*, acyl-coA synthetase; *Bmal*, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like; *Clock*, circadian locomotor output cycles kaput; *Cry*, cryptochrome; *dbp*, D site of albumin-binding protein; *DMH*, dorsomedial hypothalamus; *fabp3*, fatty acid-binding protein 3; *Glut*, glucose transporter; *hadh*, hydroxyacyl-coA dehydrogenase; *hsp*, heat shock protein; *ipp*, isopentenyl diphosphate; *lcad*, long-chain acyl-coA dehydrogenase; *lpl*, lipoprotein lipase; *marcks*, myristoylated alanine-rich C-kinase substrate; *mcd*, malonyl coA decarboxylase; *mcpt*, muscle carnitine palmitoyl transferase; *mte*, mitochondrial acyl-coA thioesterase; *pai-1*, plasminogen activator inhibitor 1; *pdk*, pyruvate dehydrogenase kinase; *pepck*, phosphoenolpyruvate carboxykinase; *Per*, period; *ppar alpha*, peroxisome proliferator-activated receptor alpha; *PVN*, paraventricular nucleus; *scd*, stearyl coenzyme A saturase; *SCN*, suprachiasmatic nucleus; *sm22*, transgelin; *tie*, angiotensin receptor; *ucp*, mitochondrial uncoupling protein.

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It is believed that the SCN can synchronize peripheral clocks through neurohumoral stimuli directly and indirectly (eg, by controlling feeding behavior). The SCN can signal the medial cortex around the SCN, the hypothalamic-pituitary-adrenal (HPA) axis, the pineal and adrenal glands, and peripheral sites through multisynaptic autonomic connections.<sup>28</sup> There are many candidate hormones that may act in a more subtle way to fine-tune peripheral rhythms. *Npas2*, a component of the molecular oscillator, heterodimerizes with retinoic acid receptors, and ligation of these receptors by retinoic acid phase shifts *Per2* rhythms in mouse aorta and heart, as we showed previously.<sup>21</sup> Others have shown that the glucocorticoid analog dexamethasone phase-shifts peripheral clocks in fibroblasts, liver, heart, and kidney, without influencing the SCN.<sup>24,29</sup> Glucocorticoid hormones have also been demonstrated to inhibit the uncoupling of central and peripheral oscillators that occurs during restricted feeding.<sup>30</sup> However, there is likely redundancy amongst such signals, as circadian phase in peripheral tissues remains unaffected both in glucocorticoid receptor (GR) deficiency in hepatocytes and in kidneys and livers of adrenalectomized mice. In peripheral tissues such as the liver, kidney, and heart, circadian rhythms in RNA abundance are apparent for each of the *Per* genes, although the phase of oscillation is delayed 3 to 9 hours relative to the oscillation in the SCN.<sup>20</sup> Oscillations in cultures of peripheral tissues were observed to dampen more rapidly than SCN cells in vitro, which sustain rhythms for weeks.<sup>23</sup> However, sophisticated luciferase reporter methods have revealed that peripheral oscillators are capable of self-sustained oscillations for more than 20 cycles in isolation,<sup>31</sup> and SCN ablation does not abolish this circadian oscillation. Phase dispersion of luciferase rhythms was observed in tissues explanted from SCN-lesioned mice. Hence, signals emanating from the SCN are now thought to synchronize, rather than sustain, rhythms in peripheral oscillators.<sup>31</sup> Taken together, all the above suggests that peripheral clocks have the potential to function autonomously, perhaps with periodic phase adjustment from the SCN.

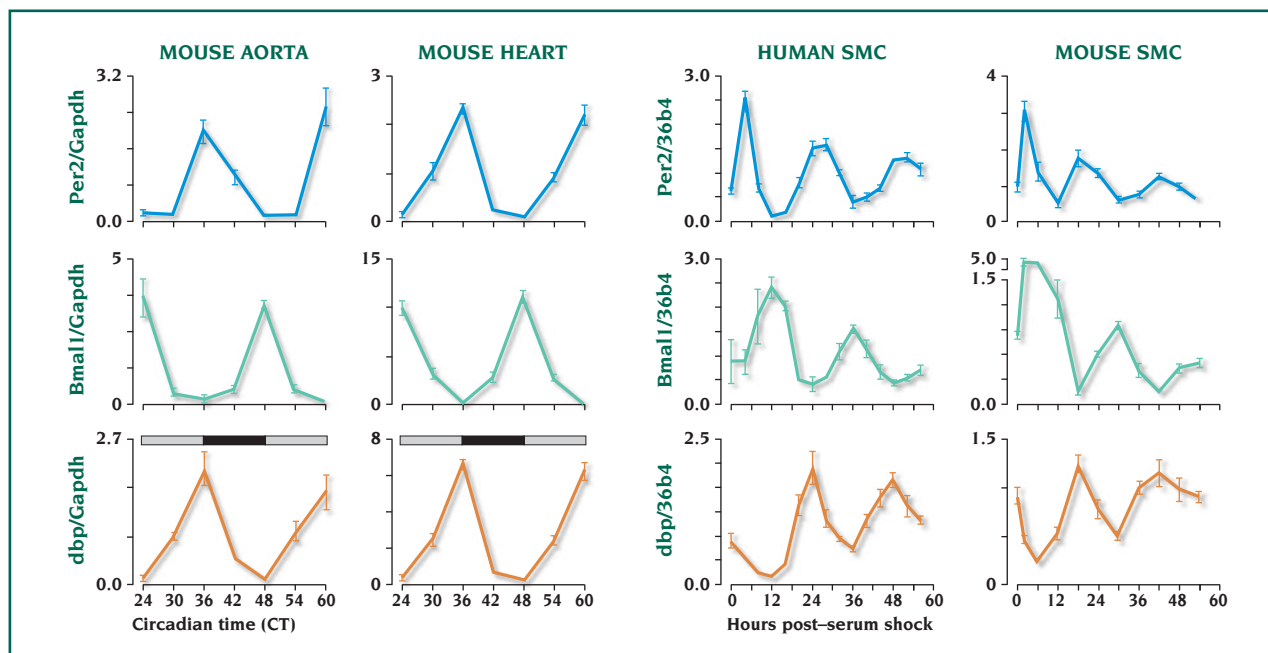
### Peripheral clocks

Circadian clock genes are expressed in many organs, and these peripheral oscillators may tailor circadian responses to tissue-specific alterations in physiological demands, such as might pertain during starving, feeding, or exercise. One potential function of a peripheral clock is to regenerate a weak or dampened SCN signal, thus amplifying the oscillation of the signal in each tissue. Another is to coordinate locally clock-controlled

gene expression in each tissue generating an amplified and synchronized rhythm in response to asynchronous blood-borne signals. Several microarray studies have helped elucidate that  $\approx 10\%$  of the transcriptome is under circadian control in the liver,<sup>11</sup> heart,<sup>32</sup> SCN,<sup>11,32</sup> and in the aorta.<sup>33</sup> Surprisingly, the complement of rhythmic genes between tissues differs substantially, with a very small percentage of clock-controlled genes common to any two tissues, suggesting a tissue-specific role of the peripheral clocks. Transcripts found to be oscillating in more than one tissue tend to be core clock components or transcription factors (*Dbp* and *Rev-erb  $\alpha$* ) close to the core loop that mediates circadian programming.<sup>34</sup> Recently, a previously unappreciated requirement for *Per1*, *Per2*, and *Cry1* to sustain cellular circadian rhythmicity was established.<sup>35</sup> It appears that intercellular coupling in the SCN acts not only to synchronize component cellular oscillators, but also to insulate against genetic perturbations. Peripheral oscillators also differ from the SCN in that they display tissue-specific molecular components of the transcriptional feedback loop that sustain rhythmicity. *Bmal2* (also known as *Mop9* or *Clif*) expressed in endothelial cell cultures dimerizes with *Clock* to drive clock-controlled genes (CCGs).<sup>36</sup> *Npas2* (also known as *Mop4*) is a paralog of *Clock*. It is expressed in the fore-brain and vasculature and can also dimerize with *Bmal1* and drive gene expression through E-box sequences.<sup>21</sup> Transcriptional coactivators and histone acetyltransferases, p300/CBP PCAF and ACTR, associate with *Npas2* and *Clock* to positively regulate clock gene expression.<sup>37</sup> Temporal coactivator recruitment and chromatin remodeling on the relevant promoters permits the mammalian clock to orchestrate circadian gene expression.<sup>38</sup> Indeed, a recent report suggests *Clock* has intrinsic histone acetyltransferase activity.<sup>39</sup> However, the relative importance of *Npas2* and *Clock* to the core loop in many tissues remains to be determined.

### DIURNAL VARIATION IN CARDIOVASCULAR FUNCTION AND DYSFUNCTION

Many common cardiovascular events, such as sudden cardiac death, myocardial infarction, unstable angina, ventricular tachycardia, and ischemic and hemorrhagic stroke, are subject to diurnal variation, peaking in the early morning hours. For example, the risk of myocardial infarction increases by 40%,<sup>40</sup> of sudden cardiac death by 29%,<sup>40</sup> and of the onset of a stroke by 49% for the period 6:00 AM to 12:00 PM when compared with 24-hour averages.<sup>41</sup> These cardiovascular events also display seasonal variations<sup>42</sup> (circumannual rhythms), which correlate with higher plasma fibrinogen



**Figure 3. Accumulation of circadian clock gene transcripts in aortic smooth muscle cells and mouse aorta and heart.**

In the mouse aorta and heart, the Clock gene mRNA transcripts *Per2*, *Bmal1*, and *Dbp* display circadian oscillations. Clock and *Bmal1* heterodimers drive transcription of *Per2* and *Dbp* by binding to E-box consensus sequences in their promoters. The peak in *Per2* and *Dbp* expression is observed around circadian time (CT) 36 corresponding to the transition between the light and dark period. *Bmal1* expression is driven by *Ror- $\alpha$*  acting at RORE sequences and subsequently repressed by *Rev-erb  $\alpha$* . The peak in *Bmal1* expression is observed at CT24 corresponding to the transition from the dark phase to the light phase. Mouse tissues were harvested in constant darkness. Clock gene mRNA transcripts similarly display circadian rhythmicity in human and mouse aortic smooth muscle cells after treatment with 50% serum. Oscillation of the clock output gene *Dbp* in smooth muscle cells is in phase with *Per2* with a peak at  $t=24$  hour post-serum shock in human cells. *Per2* and *Dbp* peak slightly earlier (close to  $t=18$  hours) in mouse smooth muscle cells. Expression of *Per2*, *Bmal1*, and *Dbp* was monitored by qPCR.

**Abbreviations:** 36b4, 60S acidic ribosomal protein P0; *Bmal*, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like; *Clock*, circadian locomotor output cycles kaput; *Cry*, cryptochrome; CT, circadian time; *dbp*, D site of albumin-binding protein; *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase; *Per*, period; qPCR, quantitative real-time polymerase chain reaction; *Ror- $\alpha$* , retinoic acid-related orphan receptor  $\alpha$ ; RORE, Ror response element; SCN, suprachiasmatic nucleus; SMC, smooth muscle cell.

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levels in the colder winter months. Disruption of normal circadian rhythms via nighttime shift work or prolonged air travel may also increase the risk of cardiovascular events<sup>43</sup> and, in the former case, prompt the emergence of biomarkers of metabolic syndrome.<sup>44</sup>

## PERIPHERAL CLOCKS IN TISSUES RELEVANT TO THE CARDIOVASCULAR SYSTEM

### Circadian clock in the vasculature

The diurnal incidence of cardiovascular disease arises from a complex interplay among local oscillators in the heart, endothelium, and vascular smooth muscle, their endocrine interactions, and their regulation by SCN-dependent changes in autonomic tone, feeding, stress, and energetic demands. Dramatic oscillations in circadian clock components have been observed in

vascular smooth muscle cells and in mouse aorta isolated at different times throughout the 24-hour period (Figure 3).<sup>4</sup> Rhythmic accumulation of transcripts for almost all of the core clock components have been observed in murine vascular tissue.<sup>21,33,37,45,46</sup> To date, aortic vascular smooth muscle cells are the most well-characterized in vitro model of the vascular clock. Rhythms in *Per1*, *Per2*, *Cry1*, *Cry2*, *Bmal1*, *Clock*, *Npas2*, *Rev-erb  $\alpha$* , *Dbp*, and *E4bp4* have been observed in smooth muscle cells in vitro and aortic tissue ex vivo. Oscillations in *Per1/2*, *Cry1/2* mRNA levels in the mouse aorta peak during the early circadian night, whereas *Bmal1*, *Npas2*, and *Rev-erb  $\alpha$*  peak at the beginning of the circadian day. Smooth muscle cell models show a similar phase relationship to that seen in vivo. Rhythms in clock gene mRNA expression have not yet been observed in vivo in vascular tissues outside the aorta; however, using tissues cultured from a *Per1* luciferase transgenic rat, Davidson et al demonstrated rhythms



in *Per1* promoter activity in a wide variety of arteries and veins.<sup>47</sup> The aorta has been the most widely studied vascular tissue in terms of gene expression in a circadian context, in part because of its accessibility and relative ease of isolation; however, it is quite possible that molecular clocks within venous and arterial vascular beds may express differing clusters of circadian output genes. Indeed, within any single vessel, endothelial and smooth muscle cells and fibroblasts express widely differing transcriptomes. It is therefore possible that the complement of genes under circadian control may differ between these cell types. Oscillating clock or clock output gene expression has not, so far, been reported in isolated endothelial cells in vitro. Cultured NIH 3T3 fibroblasts harbor cell-autonomous and self-sustained circadian oscillators with a relatively wide distribution of period length. However, they can be synchronized transiently by a short treatment with substances that activate a wide variety of signaling pathways.<sup>48</sup> Single cell recordings and coculture experiments indicated that cultured fibroblasts do not influence each other's rhythms to any measurable degree. However, different tissue explants harvested from the same SCN-lesioned animal display circadian oscillations with different phases and period lengths,<sup>31</sup> hence intercellular communication within an organ may be lost in culture ex vivo. It is tempting to speculate that different cellular clocks within a blood vessel may communicate timing information via paracrine signaling.

Previously, the circadian pattern of gene expression in the thoracic aorta was examined using Affymetrix high density arrays.<sup>33</sup> Circadian expression of approximately 8000 probe sets was examined using U74A arrays. Some 307 genes exhibited a circadian pattern of oscillation in mouse aorta, including those intrinsic to the function of the molecular clock. In addition, many genes relevant to protein folding, protein degradation, glucose and lipid metabolism, adipocyte maturation, vascular integrity, and response to injury demonstrated profound circadian oscillation. It is poorly understood how oscillating gene expression in vascular tissues might contribute to the temporal pattern of cardiovascular disease. Assessing cardiovascular parameters throughout the circadian day in transgenic mouse models with disrupted circadian clocks has provided initial information in this regard. Both mean blood pressure (BP) and its variability over time are affected by disruption of both the positive (*Bmal1*, *Clock*, and *Npas2*)<sup>46</sup> and negative components (*Cry1* and *Cry2*) of the oscillator.<sup>49</sup> Recently, we showed that deletion of *Bmal1* specifically in the vascular endothelium results in loss of the temporal pattern of susceptibility to thrombotic

vascular occlusion, as assessed by a vessel injury model.<sup>50</sup> The depression of endothelial *Bmal1* reduced BP during the active phase of the day and increased heart rate for both rest and active phases without changes in plasma catecholamines, nitric oxide (NO) biosynthesis, or fibrinolytic efficiency. Further studies of mouse models with tissue-restricted deletion of transcription factors intrinsic to molecular clocks will elucidate the relative contribution of both systemic cues and these peripheral oscillators to physiology and disease.

The identification of a vascular clock implicates local tissue-specific events as potential contributors to the temporal patterning of cardiovascular disease and, as such, they may be amenable to local modulation. The circadian oscillatory mechanism throughout the tissues of the vasculature is potentially a target for tissue-specific therapy, independent of the SCN and other clock mechanisms. Factors that entrain timing or sustain rhythmicity within the peripheral vascular clock are potentially avenues of management.

### **Neurohumoral influences on the vascular clock**

The reasons for the morning increase in cardiovascular events have been long discussed. They include both a "clock-independent," but time-dependent variation in exposure to environmental stress and an exaggeration of "clock-dependent" oscillation in vasoactive hormones, several of which vary in their circulating levels according to the time of day.

Glucocorticoids, of which cortisol in humans<sup>51</sup> and corticosterone in rodents<sup>52</sup> are the most potent, have robust diurnal rhythms, reaching a peak in plasma from 4:00 AM to 8:00 AM and a nadir at 11:00 PM to 12:00 AM; there is a particular increase upon awakening. The cortisol rhythm only partially adapts to the night-oriented work schedule in shift workers.<sup>53</sup> Increases in cortisol in the morning hours could affect vascular smooth muscle cell (VSMC) tone by potentiating the arterial contractile sensitivity to catecholamines, such as norepinephrine (NE) and epinephrine (EPI), which themselves subservise a diurnal rhythm,<sup>54</sup> and vascular resistance, through GRs. Indeed, levels of the GR are also known to vary over the 24-hour cycle, both in tissues and leukocytes.<sup>55</sup> Glucocorticoids can suppress the induced production of vasodilators, such as prostacyclin and NO, in the endothelium.<sup>56</sup> However, glucocorticoids are also known to suppress inflammation and regulate vascular permeability through control on tight junction proteins.

The diurnal variation in cortisol and that of the awakening response may be regulated differentially. The cortisol response upon awakening in humans with hippocampal damage is abolished, whereas the remainder of the diurnal cycle is unaffected.<sup>57</sup> This suggests the existence of separate mechanisms to control the steroid response to an asynchronous cue—such as environmental stress—and their diurnal variation. In animal models, there is molecular evidence consistent with such discordance. For example, the corticosterone responses to both insulin-induced hypoglycemia<sup>52</sup> and restraint<sup>46</sup> are retained in *Bmal1* knockout (KO) animals, even though these animals display deficiencies in glucose homeostasis and adrenergic activation, respectively, in response to these manipulations.<sup>46,52</sup> Indeed, light activates the adrenal gland and causes glucocorticoid release via an adrenocorticotrophic hormone (ACTH)-independent route.<sup>58</sup> Therefore, regulation of corticosterone may be regulated through SCN-dependent and independent pathways, and may vary according to stimulus and time of day.

Similarly, both plasma NE and EPI are responsive both to time of day and environmental stress, in both humans<sup>59</sup> and mice,<sup>60</sup> issues of potential relevance to the incidence of cardiovascular events. Acutely, catecholamines might contribute to vasoconstriction, endothelial dysfunction, and platelet activation in such patients. More chronic elevation of basal levels and/or amplification of diurnal variation may influence atherogenesis, the response to vascular injury and cardiac pump function.<sup>61,62</sup> Animal models suggest that the positive components of the molecular clock can affect expression of key rate-limiting enzymes in catecholamine generation, such as tyrosine hydroxylase,<sup>63</sup> catechol-*O*-methyl transferase, phenylethanolamine *N*-methyltransferase, and monoamine oxidase B.<sup>46</sup> Furthermore, we have found that adrenergic signaling can affect clock gene expression in vitro: however, loss of both EPI and NE by deletion of dopamine-beta hydroxylase does not affect peripheral rhythms in vivo.<sup>64</sup> However, plasma dopamine is elevated in these mice and may substitute as an entraining agonist and regulate circadian function in the periphery.<sup>65</sup>

Angiotensin II plays a central role in the regulation of systemic BP and fluid homeostasis through its multiple effects on the vasculature, adrenal glands, kidneys, and brain. These pleiotropic actions are mediated by specific receptors (AT<sub>1</sub> and AT<sub>2</sub>).<sup>66</sup> In rats and mice, high concentrations of these receptors are found in the brain, including within the SCN. However, the significance of this latter observation is currently unknown.

Components of the renin-angiotensin system (RAS) exhibit considerable diurnal variations and, therefore, potentially influence BP circadian rhythms.<sup>67</sup> Nonaka et al<sup>45</sup> have shown that angiotensin II induces significant oscillations in *Bmal1*, *Per2*, and *Dbp* in VSMCs. Overexpression of the *Renin II* gene in the rat is associated with a phase delayed or inverted circadian rhythm of BP, attenuated circadian and photic induction of the *C-fos* gene in SCN neurons, and attenuated phase shifting of behavioral and cardiovascular rhythms in response to light.<sup>68,69</sup> Studies in AT<sub>2</sub> receptor KO mice revealed disrupted circadian rhythms in BP and heart rate compared with wild-type mice.<sup>70</sup> These findings raise the possibility that angiotensin II may contribute to regulation of cardiovascular circadian function, including integration of the SCN with the peripheral clock in the vasculature.

An elegant study by Guo et al used parabiosis between intact and SCN-lesioned mice to show that nonneuronal signals, either hormonal or behavioral, are capable of synchronizing some peripheral tissues, but not others.<sup>71</sup> Moreover, the rhythmic expression of *Per2* mRNA<sup>72</sup> and cell adhesion molecules<sup>73</sup> in circulating peripheral mononuclear leukocytes, which have no neuronal connections, provides further evidence for the existence of circulating phase shifters or inducers of peripheral clocks in vivo. Peripheral oscillations seem to be coordinated both by major entrainment cues as well as local phase shifting signals. It is likely that integration of diverse signals resulting from food ingestion, hormones (possibly SCN-driven), or energy homeostasis may contribute to the entrainment of peripheral clocks, and that these clocks may be entrained by the SCN through diverse mechanisms.

### **Circadian clock in the heart**

Oscillations in gene expression in the heart have been examined extensively in mouse models using both real-time polymerase chain reaction and expression array analysis.<sup>21,32,37,71,74,75</sup> Genes encoding both core clock components and CCGs relevant to cardiac function have demonstrated dramatic oscillations in heart tissue isolated at intervals throughout the circadian day. Included in these oscillating transcripts are genes relevant to carbohydrate utilization, mitochondrial function, and fatty acid metabolism.<sup>76</sup> While numerous studies have provided insight into rhythmic heart gene expression, we are only now beginning to understand the impact of the molecular oscillator on cardiac physiology.<sup>32,75-78</sup> The role of the circadian clock within the cardiomyocyte may allow for the anticipation of diurnal



nal variations in workload, substrate availability, or the energy supply-to-demand ratio. Circadian variation in cardiac function persists in isolated heart tissue, highlighting the cell-autonomous function of the cardiac clock. Indeed, rat hearts in vitro continue to display circadian variation in contractile function, sustained by a circadian variation in oxidative metabolism.<sup>76</sup> Diurnal variations in oxidative stress tolerance and lipid peroxidation have also been observed in isolated perfused rat hearts.<sup>79</sup> Similarly, myocytes isolated at different times of the day have displayed circadian variation in transient outward and steady state currents *ex vivo*.<sup>80</sup> The observation that rhythmic cardiac function persists in the absence of external cues (ie, *ex vivo*) highlights the importance of this autonomous clock within the heart in the regulation of rhythmic cardiac physiology. Other myocardial processes under circadian control likely include the responsiveness to sympathetic stimulation, electrical properties, calcium homeostasis, and antioxidant capacity.

Although the circadian clock within the heart drives cardiac physiology, function of this clock can be disrupted under pathological conditions. In a model of experimentally induced cardiac hypertrophy, the core molecular oscillator continues to cycle, but the amplitude of oscillations in transcription factors such as *Dbp* are blunted,<sup>77</sup> and the circadian cycle of metabolic gene expression is lost. Hence, the tissue would be less prepared to cope with routine increases in physiological demand, predisposing it to metabolic crisis. Streptozotocin-induced diabetes in the rat is another model of contractile dysfunction that alters clock gene expression in the heart; clock component oscillations show normal amplitude, but are phase-advanced by approximately 3 hours in this model.<sup>81</sup> Spontaneously hypertensive rats show a marked increase in the amplitude of daily cardiac mRNA rhythms of components of the RAS.<sup>82</sup> Thus, the impact of disease states on the cardiac circadian clock seems to be at the level of both circadian clock genes as well as clock-controlled output genes relevant to tissue-specific functions.

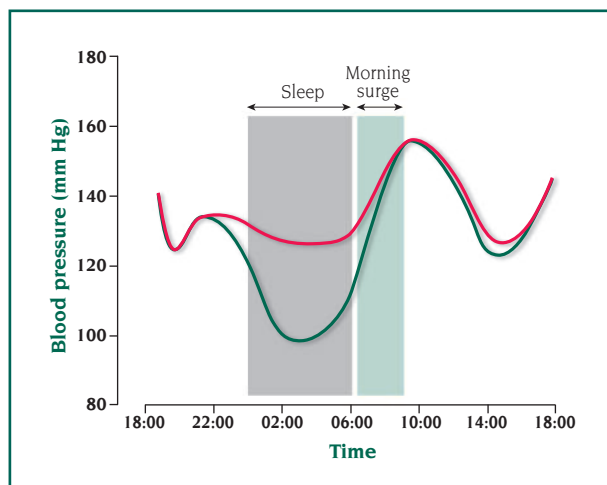
### **CIRCADIAN CLOCK AND CARDIOVASCULAR PHYSIOLOGY**

Chronobiologists have long struggled with the question of whether physiological processes such as the daily rhythms in cardiovascular parameters, energy metabolism, body temperature, and hormone release are gated simply by behavioral sleep/wake and fasting/feeding rhythms or are subject to independent control by a circadian oscillator. Locomotor activity rhythms

and circadian oscillations in circulating hormones (eg, NE, EPI) add a layer of complexity when trying to decipher the relative contribution of the central (SCN) and peripheral oscillators to circadian cardiovascular physiology. Furthermore, the interplay between metabolism/cell cycle and the circadian clock introduces both direct and indirect clock-related effects in the control of cardiovascular physiology. We now have good evidence in many species that sleep and wakefulness are affected by the circadian clock, but we are only beginning to decipher to what extent rhythms in physiological functions are themselves directly influenced by the molecular clockwork or, rather, are merely reflective of behavioral rhythms. The existence of a circadian pattern of body temperature, BP, endothelial function, fibrinolytic function, and circulating hormones has long been known. This section will focus on the diurnal variation in BP and fibrinolytic efficiency and will also discuss the relationship of the circadian clock with metabolism and cell cycle.

### **Diurnal variation in blood pressure**

Circadian variations in BP and heart rate are the most intensely studied circadian rhythms in cardiovascular physiology. A variety of descriptive approaches to phenotypic segregation have been employed. These include quantitation of the rise in BP from its nadir to its maximum, “the morning blood pressure surge” (MBPS), and the presence or absence of a significant decline in BP during sleep, “dipping.” Peak BP levels in humans occur during the midmorning (at about 10:00 AM) then decrease progressively throughout the remainder of the day to reach a trough value the following morning at around 3:00 AM<sup>83-85</sup> (*Figure 4, page 16*). A slow-but-steady increase in BP is then observed over the early morning hours before awakening, with an abrupt and steep increase at approximately 6:00 AM, coincident with arousal and arising from overnight sleep. This MBPS from low nighttime levels to higher daytime levels continues for 4 to 6 hours after awakening, with a secondary dip around 2:00 PM, thus variations in BP in general tend to reflect the sleep activity cycle (*Figure 4*). Activation of the sympathetic nervous system is an important mediator of the morning surge. A study on patients with varying degrees of progressive autonomic failure (affecting sympathetic and parasympathetic function) due to familial amyloid polyneuropathy displayed progressive blunting of the circadian BP rhythm with disease progression.<sup>86</sup> The MBPS in community-dwelling Japanese subjects is an independent predictor of stroke in hypertensive patients, independent of their 24-hour BP levels, and is associated with cardiac hypertrophy,



**Figure 4. Graphic representation of the diurnal variation in mean arterial blood pressure.**

Blood pressure of healthy human individuals shows a trough during sleep followed by a rapid increase during the early morning hours (morning surge). Blood pressure remains high for about 4 hours to come to a second trough around early afternoon (green line). Individuals showing no dip in blood pressure during the night (red line) have increased risk for heart failure, stroke, myocardial infarction, and sudden death.

even when adjusted for physical activity.<sup>87,88</sup> Another study found that MBPS correlated more strongly with indices of target organ damage than the morning surge in systolic BP or daytime BP variability did.<sup>89</sup> Therefore, it appears that the morning surge in BP, irrespective of the presence or absence of hypertension, relates directly to the incidence and outcomes of cardiovascular events.

Although always assumed, evidence to support the direct role of the molecular clock in BP regulation has been limited. It has been shown that the rhythms of BP and heart rate are controlled by an endogenous circadian circulating system, in which the SCN plays an important role in rats.<sup>90</sup> However, it is unknown how circadian information from the SCN is modulated/processed to regulate the 24-hour rhythm of BP and heart rate. The amplitude of the diurnal variation in BP is increased in patients with hypertension and the oscillation again coincides with the temporal variability in their incidence of acute vascular events, such as myocardial infarction, sudden cardiac death, and stroke.<sup>91</sup> Most normotensive individuals have a greater than 10% reduction in nighttime systolic BP when compared with mean daytime values—"dipping." The circadian pattern of BP is maintained in hypertensive patients, although there is an upward shift to the BP curve throughout the entire 24-hour period compared with normotensive subjects, and the amplitude of the rhythm may be altered.<sup>92</sup> However, some patients do not exhibit

the nocturnal dip in BP and are at increased risk of developing hypertension<sup>93</sup> and consequent end-organ damage.<sup>94</sup> For example, the absence of a normal drop in systolic BP (subjects known as "nondippers") from day to night and absolute nighttime diastolic BP are both strong predictors of heart failure, stroke, myocardial infarction, as well as sudden death in elderly patients with hypertension.<sup>94-97</sup> Recently, Halberg and colleagues found that chronobiological analysis of human BP recordings interpreted in the light of reference values specified by gender and age predicts actual and proxy outcomes when dipping fails.<sup>98</sup>

Day to night differences in physical and mental activity are thought to be major determinants of BP rhythmicity.<sup>99</sup> Analysis of BP rhythms in shift workers revealed an almost complete resynchronization within the first 24 hours of the shift rotation. This may reflect variation in sympathetic activity consistent with the correlation between diurnal variation in plasma catecholamines and BP and heart rate. Sympathetic activity appears to integrate the major driving factors of temporal variability in BP, but evidence also indicates that the hypothalamic-pituitary-adrenal, hypothalamic-pituitary-thyroid, opioid, renin-angiotensin-aldosterone, and endothelial vasoregulatory systems as well as other vasoactive peptides play a role. Many hormones with established actions on the cardiovascular system, such as arginine vasopressin, vasoactive intestinal peptide, melatonin, somatotropin, insulin, steroids, serotonin, corticotropin-releasing factor, corticotropin (ACTH), thyrotropin-releasing hormone, and endogenous opioids, show diurnal variations.<sup>100-106</sup> Physical, mental, and pathologic stimuli, which may drive activation or inhibition of these neuroendocrine effectors of biologic rhythmicity, may also interfere with the temporal BP structure. However, the time-dependent responsiveness of cardiovascular tissues to such stimuli may be just as important. The recent use of telemetry systems suitable for small animals allows for recording of systolic BP, diastolic BP, heart rate, pulse pressure, and activity in unrestrained animals. The circadian BP pattern was examined in mouse models with disruption of the positive (*Bmal1*, *Clock*, and *Npas2*)<sup>46</sup> or negative components (*Cry1* and *Cry2*) of the oscillator.<sup>49</sup> It was found that genes that subserve core functions in the molecular clock regulate differentially enzymes relevant to the synthesis and disposition of catecholamines. As we already mentioned when discussing the circadian clock in the vasculature, this resulted in alterations in BP, plasma NE and EPI, their diurnal variation, and, surprisingly, their response to immobilization stress.<sup>46</sup> Indeed, the absolute level of BP and the baroreflex



response to hypotension are differentially affected by deletion of *Bmal1* or *Npas2* disruption, on the one hand, versus deletion of both *Cry1* and *Cry2*, on the other.<sup>49</sup> It appears that the circadian clock may influence the vascular response to stress indirectly by controlling the underlying rhythm of BP, on which asynchronous cues are imposed, but also directly by modulating pressor response, irrespective of timing. Both effects reflect the observed influence of the clock on sympathoadrenal function, which is activated in the integrated arousal response and many of its discrete elements, such as assumption of an upright posture, exercise, and emotional stress. Another study revealed that disruption of the baroreflex in rats results in loss of circadian variation in mean arterial pressure.<sup>107</sup> We have previously reported that the baroreflex response, along with BP, is subject to diurnal variation in humans.<sup>108</sup> Finally, clock-dependent effects on BP may also interact with diurnal variation of hemostatic variables, such as plasminogen activator inhibitor 1 (PAI-1),<sup>109</sup> to determine the diurnal influence of cardiovascular events.

### Diurnal variation in fibrinolytic efficiency

Platelet aggregation and resultant thrombus formation subsequent to plaque rupture is fundamental to vascular occlusions and resultant tissue infarction and ischemia. Platelets are anucleate cells with a very limited capacity for de novo protein synthesis<sup>110</sup> and therefore are unlikely to contain endogenous oscillators. Although there have been reports of diurnal variation in platelet aggregability ex vivo,<sup>111</sup> the relationship of these observations (prone as they are to artifact ex vivo, attributable to diurnal variation in extracellular volume in vivo) to actual platelet activation in vivo is unknown. However, factors external to the platelet—such as PAI-1 and tissue plasminogen activator, which do indeed oscillate—may influence platelet activation in vivo. Other mediators of the hemostatic system display diurnal variation, including coagulation factors (II, VII, X, and tissue factor pathway inhibitor).<sup>112-114</sup> The morning onset of myocardial infarction may partly result from circadian variation of fibrinolytic activity. Fibrinogen, the circulating precursor of fibrin (a clot-stabilizing protein), displays circadian variation in humans,<sup>115</sup> with a peak in the early morning, whereas the expression of the fibrinogen gene in mouse liver displays two peaks, the first of which seems to be regulated by the molecular clock.<sup>116</sup> Although this issue has not been addressed rigorously, there are some suggestions of clinical relevance. For example, platelets become more aggregable by conventional agonists in

vitro when they are loaded with cholesterol. Circulating cholesterol, platelet number, and thromboxane generation peak in vivo in the late afternoon<sup>115</sup> and may contribute to the smaller secondary peak in cardiovascular events that occurs at this time.<sup>117</sup>

*Pai1* gene expression is predominantly regulated by the molecular clock. The *Pai1* gene contains two consensus E-boxes in its promoter, and is driven by the endothelial specific circadian heterodimer *Bmal2*: Clock,<sup>118</sup> and also *Bmal1*:Clock.<sup>119</sup> Circadian expression of *Pai1* was severely blunted in hearts of *Clock* mutant mice.<sup>120</sup> Interestingly, the efficacy of thrombolytic therapy decreases, coincident with peak levels of PAI-1 in the early morning hours.<sup>121</sup>

### Circadian rhythms and metabolism

It has now been well established, by using restricted feeding paradigms, that food/metabolic signals can have a profound influence on peripheral clock timing at the molecular level. Although this phenomenon is not yet fully understood, it serves to highlight the co-regulation of circadian rhythms and metabolism. The SCN controls the phase of peripheral tissues mainly by imposing a rest/activity cycle, which in turn determines a daily feeding cycle. In addition, many neuroendocrine and metabolic systems are subject to strong circadian control. Metabolic genes have featured extensively as rhythmic transcripts in many tissues examined.<sup>11,32,33,122</sup> One such examination of the mouse aorta<sup>33</sup> showed circadian variations in genes of relevance to lipid metabolism, energy balance, adipocyte maturation, maintenance of vascular integrity, and vascular response to injury. Twenty-two genes relevant to glycolysis, gluconeogenesis, fatty acid synthesis and degradation, triglyceride mobilization and storage, and cholesterol biosynthesis exhibited pathway-specific coordination subject to circadian variation. Indeed, several pathways have been identified that might link circadian networks with both gluconeogenic and lipogenic pathways. Important metabolic nuclear hormone receptors and transcription factors, including SREBP 1a, SREBP 1c, ROR- $\alpha$ , Rev-erb  $\alpha$ , thyroid hormone receptor (THR), and peroxisome proliferator-activated receptor  $\alpha$  (PPAR- $\alpha$ ), show significant circadian expression in numerous tissues. SREBP 1a and 1c regulate hepatic lipogenesis; ROR- $\alpha$  has been shown to regulate lipid flux, lipogenesis, and lipid storage in skeletal muscle; and increased Rev-erb  $\alpha$  and THR expression is observed during adipogenesis, whereas PPAR- $\alpha$  is involved in the regulation of numerous metabolic processes. Regulation of clock genes by the redox

state of the cofactors nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP) has been demonstrated in a human neuroblastoma cell culture system.<sup>123</sup> The reduced forms of these cofactors, NADH and NADPH, strongly enhance DNA binding activity of the Clock:Bmal1 and Clock:Npas2 heterodimers. In contrast, the oxidized form of these redox factors inhibits DNA binding by these heterodimers. The regulation by NADPH in vitro implies that oscillations in metabolic flux participate in feedback loops with the clock genes. In particular, glycolytic flux results in cytosolic NADH production and depends on shuttle mechanisms for NADH transport into the mitochondria. A study by Young et al reported diurnal variations in myocardial metabolic flux and contractile function.<sup>76</sup> Contractile performance, carbohydrate oxidation, and oxygen consumption in isolated working rat hearts were greatest in the middle of the night, with little variation in fatty acid oxidation. In addition, they observed circadian rhythmicity in a variety of genes involved in carbohydrate and fatty acid metabolism. In the context of NAD/NADH enhancing Clock/Bmal1 binding to E-box elements, variations in carbohydrate flux demonstrated by Young et al in the intact heart could represent an interacting feedback mechanism for the clock. It is therefore a possibility that abnormalities in metabolic flux and NADH generation could disrupt or reset the peripheral clock in the heart.

In studies using *Clock* mutant and *Bmal1*<sup>-/-</sup> mice, a profound role for the clock in the recovery from insulin-induced hypoglycemia and, specifically, in gluconeogenesis was observed.<sup>52</sup> A high-fat diet amplified the diurnal variation in glucose tolerance and insulin sensitivity in a manner dependent on the molecular clock. Turek et al also reported abnormalities in adipogenesis and metabolism in *Clock* mutant mice, including a subtle decrease in energy expenditure, hyperphagia exaggerated in response to a high-fat diet, and dysregulation of neuropeptides (ghrelin, cart, and orexin) that regulate energy metabolism.<sup>26</sup> However, on an imprinting control region (ICR) background, *Clock* mutant mice develop lipid malabsorption and, hence, do not develop obesity.<sup>124</sup> The *Clock* mutant mouse, which was a triumph of forward genetics, is a dominant mutation that results in deletion of a putative activation domain and arrhythmicity in constant darkness. A recently generated *Clock*<sup>-/-</sup> mouse retains rhythmic activity, suggesting compensation by *Npas2*, although this remains to be addressed definitively.<sup>125</sup> Studies of metabolic phenotypes in *Bmal1*<sup>-/-</sup> mice are tempered by progressive arthropathy and an advanced aging

phenotype.<sup>126</sup> Indeed, the question of whether progressive dysfunction of the molecular clock contributes to biological aging is an interesting one and potentially pertinent to the syndrome of accelerated vascular and metabolic dysfunction that may complicate AIDS. No doubt emerging mouse models with tissue specific dysfunctional clocks will allow a greater understanding of the contribution of distinct molecular clocks to energy balance and metabolic homeostasis. How peripheral clocks in many tissues, including the cardiovascular system, impact the incidence of cardiovascular disease and metabolic syndrome remains a fertile area of investigation.

### **Circadian rhythms and the cell cycle**

Like many other biological processes relevant to the cardiovascular system, the cell cycle displays circadian rhythmicity. Key components of the cell cycle, including cyclin A, cyclin D1, Cdc2, c-Myc, and Wee1, have been shown to be under transcriptional control by the molecular clock.<sup>127,128</sup> More direct evidence for molecular clock regulation of the cell cycle in vivo stems from the work by Matsuo et al. Progression of the cell cycle through the G2/M transition, but not S-phase progression, is gated by the molecular clock to certain times of the day in a partial hepatectomy model of liver regeneration.<sup>129</sup> This circadian gating involves the clock-controlled gene *Wee1*, which inactivates the Cdc2-cyclin B complex required for G2/M transition. Mouse models of molecular clock dysfunction have provided further evidence for clock control of the cell cycle. Liver regeneration after partial hepatectomy is retarded in cryptochrome-deficient mice,<sup>129</sup> whereas mutation of *Clock* was recently shown to inhibit the proliferation of mouse fibroblasts in vitro.<sup>128</sup> Aberrant cell division in vivo has been observed in *Per2* mutant mice, which have an increased susceptibility to tumor development on challenge with gamma irradiation.<sup>127</sup> Although these data provide evidence for a role of the molecular clock in regulating the cell cycle, a functional oscillator is not absolutely required for cell division, as several mouse models of molecular clock dysfunction are viable. Nevertheless, the impact of the molecular clock as a modulator of cell division is evident.

Future work will likely shed light on the relative importance of the molecular clock on proliferative diseases of varying etiology, including cancer, atherosclerosis, arthritis, and others. Circadian time may also influence the outcome of coronary interventions such as angioplasty, where restenosis reflects a dysplastic response to injury.<sup>130</sup>



## CONCLUSIONS

Molecular deletion and mutation of transcription factors involved in the molecular clock have already revealed unexpected roles for this mechanism in discrete aspects of physiology—hemodynamics, coagulation, glucose and lipid metabolism, inflammation, and aging—which impact on cardiovascular disease. The appreciation of the existence of distinct molecular clocks, which have the capacity for entrained and autonomous functions in endothelium, vascular smooth muscle cells, perivascular fat, and cardiomyocytes, suggests a complex interrelationship by which oscillations in cardiovascular function might be determined. Added to this, the clock may influence, in a fundamental and similarly diverse way, drug response. Indeed, drug-metabolizing enzymes are amongst the most dramatically oscillating genes in the liver. Presently, we understand little of how central and peripheral clock function is integrated, how the distinct clocks in adjacent cardiovascular cells interact, and whether genetic variation in clock genes condition a predisposition to cardiovascular disease. However, the emergence of increasingly diverse rodent models of clock function and an emerging interest in clock genetics suggest that the time is ripe for this field to impact on translational medicine.

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