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Molecular components of the mammalian circadian clock

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Circadian rhythms are ~24-h oscillations in behavior and physiology, which are internally generated and function to anticipate the environmental changes associated with the solar day. A conserved transcriptional–translational autoregulatory loop generates molecular oscillations of 'clock genes' at the cellular level. In mammals, the circadian system is organized in a hierarchical manner, in which a master pacemaker in the suprachiasmatic nucleus (SCN) regulates downstream oscillators in peripheral tissues. Recent findings have revealed that the clock is cell-autonomous and self-sustained not only in a central pacemaker, the SCN, but also in peripheral tissues and in dissociated cultured cells. It is becoming evident that specific contribution of each clock component and interactions among the components vary in a tissue-specific manner. Here, we review the general mechanisms of the circadian clockwork, describe recent findings that elucidate tissue-specific expression patterns of the clock genes and address the importance of circadian regulation in peripheral tissues for an organism's overall well-being.

OVERVIEW OF THE CIRCADIAN MOLECULAR CLOCK

The circadian system is responsible for regulating a wide variety of physiological and behavioral rhythms (1,2). The mammalian circadian system is organized in a hierarchy of oscillators. At the top of this hierarchy is the suprachiasmatic nucleus (SCN) of the anterior hypothalamus. The SCN is responsible for coordinating independent peripheral oscillators so that a coherent rhythm is orchestrated at the organismal level (3,4). The clock mechanism in the SCN and the peripheral oscillators are known to be similar at the molecular level (5–8), which consists of a network of transcriptional–translational feedback loops that drive rhythmic, ~24-h expression patterns of core clock components (1,2). Core clock components are defined as genes whose protein products are necessary for the generation and regulation of circadian rhythms within individual cells throughout the organism (9).

In the primary feedback loop, the positive elements include members of the basic helix-loop-helix (bHLH)-PAS (*Period-Arnt-Single-minded*) transcription factor family, CLOCK and BMAL1. CLOCK and BMAL1 heterodimerize and initiate transcription of target genes containing E-box *cis*-regulatory enhancer sequences, including *Period* (in mice, *Per1, Per2* and *Per3*) and *Cryptochrome* (*Cry1* and *Cry2*) (10–14). Negative feedback is achieved by PER:CRY heterodimers that translocate back to the nucleus to repress their own transcription by acting on the CLOCK:BMAL1 complex (12,15–18).

Another regulatory loop is induced by CLOCK:BMAL1 heterodimers activating transcription of retinoic acid-related orphan nuclear receptors, *Rev-erb*α and *Ror*α (19–22). REV-ERBα and RORα subsequently compete to bind retinoic acid-related orphan receptor response elements (ROREs) present in *Bmal1* promoter. It has been shown that members of ROR (α, β and γ) and REV-ERB (α and β) are able to regulate *Bmal1* through ROREs (23). RORs activate transcription of *Bmal1* (20,22,23), whereas REV-ERBs repress the transcription process (19,23). Hence, the circadian oscillation of *Bmal1* is both positively and negatively regulated by RORs and REV-ERBs.

The autoregulatory feedback loops described (illustrated in Fig. 1) take ~24 h to complete a cycle and constitute a circadian molecular clock. This generation of the ~24-h molecular clock is governed by post-translational modifications such as phosphorylation and ubiquitination. These processes

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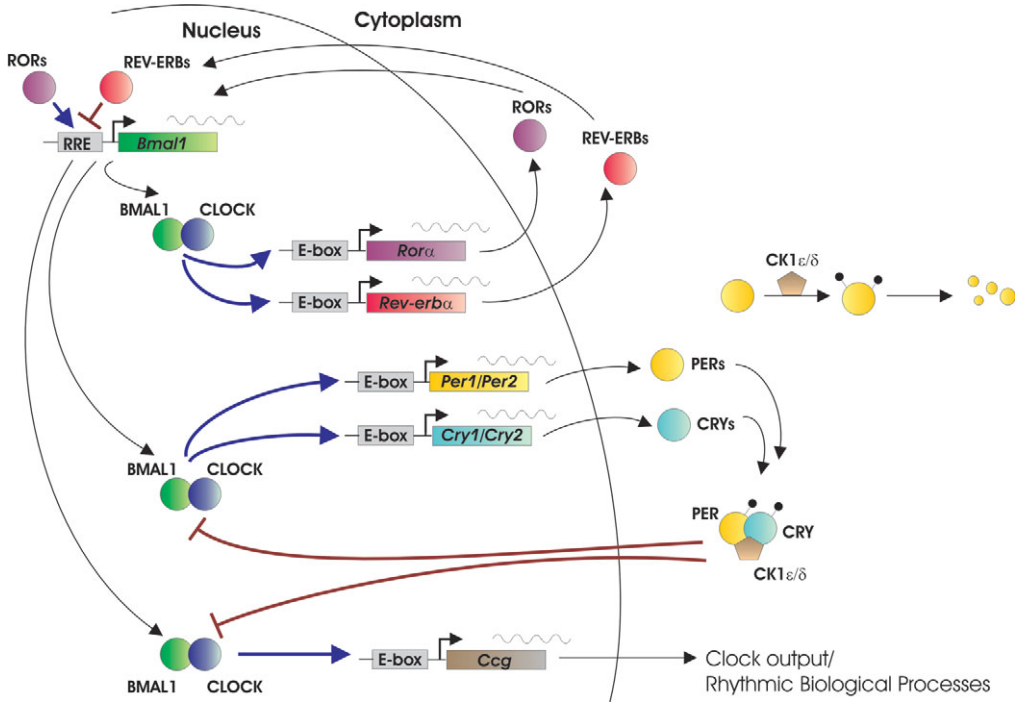

Figure 1. A network of transcriptional–translational feedback loops constitutes the mammalian circadian clock.

significantly contribute to the precision of the mammalian clock by affecting the stability and nuclear translocation of aforementioned core clock proteins (24–29). *Casein kinase 1 epsilon* and *Casein kinase 1 delta* (*CK1*ε and *CK1*δ) are critical factors that regulate the core circadian protein turnover in mammals (24,26,27,29). More recently, a small ubiquitin-related modifier protein modification of BMAL1 has also been proposed as another level of post-translational regulation (30). The importance of the post-translational regulation within the core mechanism of the circadian clock is supported by the fact that mutations in *CK1*ε and *CK1*δ can have dramatic effects on circadian period. Mutations in *CK1*ε and *CK1*δ result in altered kinase activities and cause shorter circadian periods in mammals (24,29,31,32). These mutations have become of particular interest in humans as they are implicated in familial advanced sleep phase syndrome (FASPS) (25,29).

PHENOTYPIC EFFECTS OF CIRCADIAN MUTATIONS

The molecular mechanism underlying the mammalian clockwork has been most extensively studied in the mouse. Experimental animals harboring naturally-occurring, chemically-induced or targeted mutations have been critical to understand the role of each clock component in overall functionality of the molecular clock. A current list of mammalian clock genes along with their properties and mutation phenotypes are described in Table 1.

The positive elements, CLOCK and BMAL1, produce rhythmic transcriptional activation that serves as a basic driving force behind the circadian clockwork (10,11,13,33). Mice carrying homozygous dominant-negative, antimorphic *Clock* allele mutation ($Clock^{\Delta 19/\Delta 19}$) display a long circadian period that becomes arrhythmic with prolonged exposure to constant darkness (34,35). The mutant CLOCK protein renders functionally defective CLOCK:BMAL1 heterodimers and, as a consequence, induces markedly blunted molecular rhythms (11,36,37). Furthermore, mice homozygous for a null allele of *Bmal1* have severely disrupted behavioral and molecular rhythms (13). These observations have suggested CLOCK and BMAL1 as critical components of the molecular clock. However, a recent study has reported that CLOCK-deficient mice are able to generate normal behavioral and molecular rhythms (38), challenging the long-standing idea that CLOCK and BMAL1 are at the heart of initiating and sustaining circadian rhythms. Neuronal PAS domain protein 2 (NPAS2), a close analog of CLOCK, has been implicated to substitute for CLOCK (38,39).

The idea of functional substitution or partial compensation has already been suggested in the negative elements of the molecular clock. The clock continues to oscillate when a single gene is mutated within PER or CRY family (14,40–44). However, disruption of *Per1* and *Per2* genes together (or *Cry1* and *Cry2* genes) causes behavioral and molecular arrhythmicity (14,40–42). It is important to note that individual mutation in PERs or CRYs results in aberrant circadian periodicity; hence, the role of each clock gene cannot be entirely compensated by the other components. $Per1^{-/-}$ mice show slightly shorter (~0.5–1 h) free-running periods than the wild-type mice (14,40,44). $Per2^{-/-}$ mice exhibit even shorter (~1.5 h) free-running periods and some animals can become arrhythmic in constant conditions (40,43). *Per3*

Table 1. Mouse circadian clock and clock-related genes

Gene	Average circadian time at peak transcript level		Allele	Mutant phenotype	References
	SCN	Periphery			
Bmal1 (Arntl)	15–21	22–02	$Bmal1^{-/-}$	Arrhythmic	(13)
Clock	Constitutive	21–03	$Clock^{\Delta 19/\Delta 19}$	4-h longer pd/arrhythmic	(34)
			$Clock^{-/-}$	0.5-h shorter pd	(38)
Per1	4–8	10–16	$Per1^{brdm1}$	1-h shorter pd	(14)
			$Per1^{ldc}$	0.5-h shorter pd/arrhythmic	(40)
			$Per1^{-/-}$	0.5-h shorter pd	(44)
Per2	6–12	14–18	$Per2^{brdm1}$	1.5-h shorter pd/arrhythmic	(43)
			$Per2^{ldc}$	Arrhythmic	(40)
Per3	4–9	10–14	$Per3^{-/-}$	0–0.5-h shorter pd	(45)
Cry1	8–14	14–18	$Cry1^{-/-}$ [a]	1-h shorter pd	(41,42)
Cry2	8–14	8–12	$Cry2^{-/-}$ [a]	1-h longer pd	(41,74)
Rev-erbα (Nr1d1)	2–6	4–10	$Rev\text{-}erb\alpha^{-/-}$	0.5-h shorter pd/disrupted photic entrainment	(19)
Rorα	6–10	Arrhythmic/various[b]	*staggerer*	0.5-h shorter pd/disrupted photic entrainment	(20)
Rorβ	4–8	18–22	$Ror\beta^{-/-}$	0.5-h longer pd	(75)
Rorγ	N/A[c]	16–20/various[b]	$Ror\gamma^{-/-}$	Unknown	
NPAS2	N/A[c]	0–4	$NPAS2^{-/-}$	0.2-h shorter pd	(76)
CK1ε (Csnk1ε)	Constitutive	Constitutive	$CK1\varepsilon^{tau}$ [d]	4-h shorter pd	(24)
CK1δ (Csnk1δ)	Constitutive	Constitutive	$Csnk1\delta^{-/+}$	0.5-h shorter pd	(29)

[a]Two independent groups generated *Cry1* and *Cry2* null mutants and the mice showed similar phenotypes.
[b]See references (22,23,77).
[c]Not detected in the SCN.
[d]Hanster mutation.

null mutant mice maintain molecular and behavioral rhythms and do not have a critical role in the feedback loops (45). $Cry1^{-/-}$ mice display ~1-h shorter and $Cry2^{-/-}$ mice display ~1-h longer free-running periods than the wild-type mice (41,42).

TISSUE-SPECIFIC EXPRESSION PATTERNS OF THE CLOCK GENES

Recent studies have revealed that the circadian clock is cell-autonomous and self-sustained not only in the SCN but also in peripheral tissues and in dissociated cultured cells (3–8). This finding has led to an increasing effort to better understand the circadian mechanisms in independent peripheral oscillators and to further elucidate the nature of the mammalian circadian system hierarchy. What has become clear recently is that specific contributions of each molecular clock component, and interactions among the clock components, may vary in a tissue-specific manner.

Most of the core components of the molecular clock maintain their rhythmicity in the SCN and in peripheral tissues. Some components, however, vary in their intrinsic rhythmic properties across the tissues. For example, *Clock* mRNA cycles in the peripheral tissues, but it is constitutively expressed in the SCN (1). In addition, members of the *Ror* family (α, β and γ) present strikingly different expression patterns across tissues with varying circadian peak times (22,23). *Rorα* display robust circadian rhythm in the SCN but only a slight oscillation is observed in peripheral tissues (20,22,23). *Ror γ*, however, does not express in the SCN, but shows rhythmic expression in the peripheral tissues and participates in the peripheral molecular clockwork (20,23). Mice lacking functional *Rorα, staggerer* (46), have normal clock gene rhythms in peripheral tissues including *Bmal1* mRNA rhythm; this suggests that ROR proteins (α, β and γ) may contribute differently to rhythmic *Bmal1* activation in a tissue-dependent manner (20,47). Tissue-specific regulation of *Bmal1* may be important to note because BMAL1-deficient ($Bmal1^{-/-}$) mice display a variety of phenotypes including loss of circadian rhythms, decreased body weight, infertility, progressive arthropathy and shortened life span (13,48–50). This suggests that *Bmal1* may play a role in a variety of functions depending on the tissue type in which it is expressed.

Specific contributions of each molecular clock component to other transcript oscillations may also depend on the tissue examined. For example, $Clock^{-/-}$ mice show altered circadian gene profiles in a gene-specific and tissue-specific manner. There is a modest effect of CLOCK deficiency on the amplitudes of the *Rev-erbα* mRNA oscillation in the SCN, while the amplitude of the *Rev-erbα* transcript oscillation is markedly reduced in the liver. On the other hand, *Per1* mRNA in CLOCK-deficient liver is robustly rhythmic with its absolute level considerably elevated in comparison to that of the wild-type liver. *Per1* level in the CLOCK-deficient SCN is more damped and lower in its absolute level compared with the wild-type. Therefore, it appears that the activity of the transcription factors promoting circadian gene expression patterns, in the absence of the CLOCK, are target gene-specific and tissue-specific (38). Furthermore, *Per2* mRNA rhythms also show tissue-dependent disruption by the $Clock^{\Delta 19}$ mutation (51,52). Circadian rhythmicity of *Per2* persists in the CLOCK mutant liver and muscle albeit with lower amplitude and delayed phase compared with the wild-type counterparts, however, the *Per2* transcript level is severely blunted in the CLOCK mutant kidney and heart (51).

Taken together, it is necessary to take a closer look at the role of each clock component within the molecular clockwork

Table 2. Circadian gene defects and the biological consequences

Disrupted gene	Physiological effects	References
Bmal1	Infertility Progressive arthropathy Abnormal gluconeogenesis Abnormal lipogenesis Altered sleep pattern	(13,48–50,57)
Clock[a]	Metabolic syndrome Abnormal gluconeogenesis Abnormal behavioral sensitization to psychostimulant Altered sleep pattern	(48,56,66,78)
Per1	Abnormal apoptosis/cancer development Abnormal behavioral sensitization to psychostimulant	(64,67,79)
Per2	Improper cell division/cancer development Abnormal behavioral sensitization to psychostimulant Improper alcohol intake FASPS(80)	(25,71, 79–81)
Per3	Associated with DSPS (80)	(54,55)
Cry1;Cry2	Altered sleep pattern	(58)
*Ror*α	Cerebellar ataxia Abnormal bone metabolism	(82–85)
*Ror*β	Locomotor difficulties Retinal degeneration/blind Male reproductive abnormality during first 6 months of age	(75)
*Ror*γ	Lack of lymphoid organ development Abnormal lymphocyte homeostasis	(86–88)
NPAS2	Altered sleep pattern Impaired memory	(76,89)
*CK1*ε/*CK1*δ	FASPS (80)	(25,29)

[a]$Clock^{\Delta 19/\Delta 19}$ mutation.

at the systems level. This is becoming particularly important as an increasing number of diseases (Table 2) are associated with circadian timing disruptions.

CIRCADIAN TIMING AND ITS EFFECTS ON PHYSIOLOGICAL PROCESSES

Circadian clocks influence nearly all aspects of physiology and behavior, including rest–wake cycle, cardiovascular activity, hormone secretion, body temperature and metabolism. Recently, a familial sleep disorder in humans has been linked to mutations in human circadian genes *Per2* and *CK1*δ (25,29). This behavioral trait is known as FASPS, and the patients exhibit early sleep onset followed by early-morning awakening (53). In contrast, delayed sleep phase syndrome (DSPS) patients show sleep-onset insomnia with an inability to awake at a conventional time in relation to the general public. Genetic studies suggest that DSPS is associated with a specific haplotype of human *Per3* gene (54,55). These findings indicate involvement of the clock genes in the susceptibility to sleep disorders, and altered sleep homeostasis has been observed in various circadian mutant mice. $Clock^{\Delta 19/\Delta 19}$ mice stay awake more and sleep less per day relative to wild-type mice, and show smaller increases in rapid eye movement (REM) sleep when recovering from sleep deprivation (56). $Bmal1^{-/-}$ mice show increases in total sleep time and sleep fragmentation with an attenuated rhythm of sleep–wakefulness cycle across the 24-h period (57). Mice deficient of CRY ($Cry1^{-/-}Cry2^{-/-}$) exhibit increases in baseline amounts of non-REM sleep and consolidation of non-REM sleep episodes relative to that of wild-type mice. They also lack the normal compensatory response in sleep amount following sleep deprivation (58). Other circadian mutant mice show more intact sleep patterns compared with the ones described thus far. For example, PER mutant mice ($Per1^{ldc/ldc}Per2^{ldc/ldc}$) maintain the total sleep time compared with wild-type mice. Nonetheless, the mutant animals exhibit altered phase of activity and body temperature rhythms relative to the light:dark cycle, suggesting that the 24-h distribution of sleep may be affected by the mutation (59).

Considerable insight into the role of circadian timing in biological processes has been gained from gene profiling studies. Microarray results from different tissues in the wild-type and mutant mice support the tissue-dependent circadian gene expression patterns (60–63). Circadian genes are expressed in a tissue-specific manner with only a minor overlap of cycling transcripts between tissues. For example, when the sets of cycling transcripts are compared between the SCN and liver, only ~10% are common to both (60,62). This is also seen in other comparisons of different tissues (61). Furthermore, a significant number of the transcripts that express in both tissues cycle in only one of the tissues and not in the other, and different circadian transcripts within one tissue can accumulate with varying phases.

The identification of the circadian transcripts has revealed that the transcriptional circadian regulation extends beyond core clock components to include various clock-controlled genes (CCGs), including key regulators for cell cycle and metabolism (60,61). Overall, circadian regulation in peripheral tissues is important to maintain normal cellular functions, and a disruption of core clock genes can be damaging to the organism's overall well-being (64–66).

Per genes (negative elements of the molecular clock) have been implicated to play an important role in cell growth and to function as tumor suppressors (64,67). Ectopic expression of *Per1* in human cancer cells led to significant growth reduction, and a reduced *Per1* transcript level was observed in human cancer patient samples (67). *Per* genes may be deregulated in breast cancer cells as well as in endometrial and pancreatic cancers (67–70). Interestingly, analysis of *Per2* levels in the lung and endometrial samples show a less profound difference between tumor and normal samples (67,69), whereas significantly reduced expression of *Per2* level is noted in lymphoma cell lines as well as in acute myeloid leukemia patient samples (71). On another note, it has been reported that wild-type and circadian mutant mice demonstrate striking differences in their responses to cancer therapy (72). The sensitivity of wild-type mice to chemotherapy varies depending on the daily timing of drug administration, however, the $Clock^{\Delta 19/\Delta 19}$ and $Bmal1^{-/-}$ mice remain highly sensitive to the treatment at all times of

the day. On the contrary, *Cry1*$^{-/-}$*Cry2*$^{-/-}$ mice are not as sensitive and show more resistance to the drug compared with the wild-type mice. This variation in response to chemotherapy has been attributed to the functional status of the CLOCK:BMAL1 transcription complex (72).

It has been found that many of the circadian transcripts also participate in common metabolic pathways (60,73). The link between metabolic activity and circadian rhythms has long been studied; however, recent studies have shown that the *Clock* and *Bmal1* genes may contribute to normal metabolic regulation (48,50,66). The *Clock*$^{\Delta 19/\Delta 19}$ mutant mice show altered patterns of food intake and develop symptoms of the metabolic syndrome including hepatic steatosis, hyperleptinemia, hyperglycemia and hypoinsulinemia (66). The *Clock*$^{\Delta 19}$ mutation can influence the levels of plasma glucose and triglycerides in mice, as well as the development of glucose intolerance and insulin resistance in response to high-fat diet (48,66). Similar metabolic phenotypes are observed in the *Bmal1*$^{-/-}$ mice (48). Furthermore, BMAL1-deficient embryonic fibroblast cells (MEFs) fail to differentiate into adipocytes. When BMAL1 is transfected back into BMAL1 knockout MEFs, the cells accumulate cellular lipids and induce adipocyte-related genes, such as peroxisome proliferators-activated receptor (PPAR)γ2 and adipocyte fatty acid binding protein 2 (aP2) (50).

CONCLUSION

The above findings emphasize significance of the molecular clock and its regulation of the rhythmic production of CCGs, which subsequently influence different biochemical pathways involved in pathophysiology. As described earlier, the members of ROR and REV-ERB families participate in the control of *Clock* and *Bmal1* expression (19,20,22,23). These components of the molecular clock have been described to vary in their expression patterns across the tissues (20,22,23,47), and the way these transcripts act in response to the clock disruption vary depending on a tissue (38). Circadian rhythmicity of other clock genes (e.g. *Per1* and *Per2*) also show tissue-specific disruption in the presence of a dysfunctional molecular clock (38,51,52). Overall, this raises the need for future experiments that will carefully examine the molecular details of clock operation in a tissue-specific manner. This work is in progress as more direct and definitive experimental tools are becoming available to examine the role of the clock components, i.e. tissue-specific conditional knockouts of the peripheral clocks will help elucidate not only the organizational hierarchy of the oscillators, but also the specific roles of peripheral clocks as well as the roles of the clock components within the peripheral clocks.

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Conflict of Interest statement. None declared.

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