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Time microscopy of circadian expression of Cardiac Clock Gene mRNA Transcription: Chronodiagnostic and Chrono-therapeutic implications

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Abstract

Background and Purpose. Molecular clocks present in organs and individual cells throughout the body are central for the temporal coordination of internal biological processes among themselves and with external environmental cycles. Relationships between circadian clocks and normal vs. abnormal organ physiology can have significant impact relevant to not only cardiovascular health, but also to the general treatment and prognosis of human disease. Chronobiological statistical procedures were applied to previously published circadian clock gene (CG) mRNA expression data which were described macroscopically, in order to establish rhythm probability and point and interval estimates for amplitudes and acrophases for 14 clock and clock-controlled genes in mouse heart. CGs in general and their importance to cardiovascular health, as well as to diagnosis and treatment of human disease, are reviewed.

Materials and Methods. Organs from male Balb/c mice were harvested every 4 h for 24-h on the 3rd day in constant darkness and analyzed by quantitative real-time reverse transcription-polymerase chain reaction for 14 CGs: mPer1, mPer2, mPer3, mCry1, mCry2, mBmal1, mCK1δ, mCK1ε, mClock, mDbp, mNpas2, mRev-erbα, mRev-erbβ, and mTim. Relative mRNA levels normalized to corresponding G3-PDH RNA levels were re-expressed as percent of the highest value for each CG and analyzed for circadian time effect by one-way ANOVA and for circadian rhythm characteristics by single cosinor.

Results. 12 CGs showed a significant time-effect at $p \leq 0.031$ by ANOVA and 13 CGs displayed a significant 24-h rhythm at $p \leq 0.011$ by cosinor analysis. Five CGs (mRev-erbα, mDbp, mPer1, mRev-erbβ, mPer3) reached their maxima late in the presumed resting span, 5 CGs (mPer2, mCry2, mCK1δ, mCK1ε, mCry1) reached their peak early in the presumed activity span, while 3 genes (mBmal1, mClock, mNpas2) reached their peak late in the presumed activity span.

Conclusions. Macroscopic inspection concluded a robust circadian rhythm in 8 CGs, while cosinor analysis detected significance in 13 of 14 CGs (the developmental gene mTim is usually not circadian rhythmic) and computed point and interval estimates for amplitudes and acrophases, useful in making future objective comparisons among organisms and conditions. Information on statistically-determined rhythm characteristics of the molecular clock presents new avenues for diagnosis and therapeutic intervention in conditions where disturbance of circadian CG expression is an important cause of morbidity in chronic illnesses and diseases with a strong circadian component, including coronary vascular disease. ***Clin Ter 2009; 160(2):e25-e34***

Key words: *biological clock, circadian, clock gene, heart, mouse, transcription*

Introduction

The rhythmic nature of life influences the very existence of organisms from bacteria to mammals, and as such is an integral part of health, disease and survival. As a whole, life moves in synchrony to the beats of photic and non-photic clocks and calendars, some outside the body and some within the very cells of all living things. It is now known that internal molecular circadian clocks are central for the temporal coordination of internal biological processes among themselves and with external environmental cycles. The molecular clock machinery in mammals consists of a number of clock genes (CGs) and their resultant proteins that form interlocking auto-regulatory transcription-translation feedback loops consisting of positive and negative elements that drive self-sustaining clock oscillations of approximately 24 hours in cells of both the suprachiasmatic nucleus (SCN) and peripheral organs. These loops generate and maintain the 24-h CG mRNA and protein oscillations and consequential biological and physiological rhythms (1). Some elements of the molecular clock have been shown to be partially functional in rat fetal SCN (2-4) and fully functional within days of birth, as well as in peripheral tissues, such as the rat heart (5) or liver (6).

Many features of the intracellular molecular clock machinery have been elucidated (7). Major positive components

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in the feedback loop are a pair of transcription factors, BMAL1 and CLOCK, which can form heterodimers to drive the rhythmic expression of three *Period* genes (*Per1*, *Per2*, *Per3*) and two *Cryptochrome* genes (*Cry1, Cry2*). The resultant PER:CRY protein dimers act as negative components and translocate back into the nucleus and inhibit CLOCK: BMAL1 activity, thereby completing the negative limb of the feedback loop. Posttranslational processes and some other secondary feedback loops involving clock-controlled genes (CCGs) also contribute to the precision of the cell clockwork machinery by playing important roles in fine-tuning, stabilization, and/or resetting the clock (8, 9). While synchronized by environmental cues such as photoperiod and feeding schedules, robust circadian CG oscillations can persist in the absence of external time cues, as has been shown for up to 32 days in *Per1*-luciferase (luc) expression in rat SCN (10), up to 20 or more cycles in *Per2*-luc expression in mouse SCN, liver and lung explants (11), during 3 to 12 cycles in *Per1*-luc expression in rat cardiovascular tissues (12), and up to 25 days in *Per2*-luc expression in mouse retinas in which photoreceptors had degenerated (13).

Self-sustaining rhythmic expressions of mammalian CGs are found not only in the central circadian pacemaker located in the suprachiasmatic nucleus (SCN) in the ventral part of the hypothalamus, but also in peripheral tissues, indicating the existence of both central and peripheral oscillators (8, 14). There are no reproducible data to show that peripheral tissues in mammals receive direct photic input; hence, a hierarchical model where the SCN provides an essential link as a primary coordinator or synchronizer between the outside world and internal autonomous circadian time-keeping mechanisms throughout the body has been indicated (15). Another potential synchronizer is timed or restricted feeding that can cause periodic availability of circulating macronutrients and signaling pathways, which may then influence the timing of peripheral clocks in a tissue-specific manner (16-18). Circadian oscillations in CGs have been documented in numerous peripheral tissues, including heart, lung, liver, kidney, stomach, spleen, pancreas, gut, thymus, oviduct, bone marrow, skin, oral mucosa, cornea, retina, submandibular (salivary) glands, blood, hypothalamus, pineal, and skeletal muscle in mammals such as mice (10, 11, 13, 18, 21-30), rats (31-39), hamsters (41-44) and humans (45-50).

CG output is thought to indirectly (via a transcriptional cascade) regulate the rhythmic expression of downstream genes within the same cell that are involved with the regulation of physiology and behavior (15, 51-54). For example, it has been estimated that ≥ 8-10% of all genes in mouse heart and up to 50% in liver exhibit high-amplitude circadian regulation, and perhaps a considerably greater prevalence of genes show circadian regulation with lower amplitudes, all with probable impact on diverse physiological processes (21, 55, 56). In mammals, functional CGs have been shown to influence nearly all aspects of behavior and physiology, including cardiovascular activity, endocrinology, hepatic metabolism, gastro-intestinal tract functions, cell cycle regulation, body temperature, and sleep-wake cycles (8, 57).

It is well-known that virtually all cardiovascular functions in humans, such as blood pressure, heart rate, coagulation parameters (e.g., fibrinolytic activity, viscosity, platelet aggregability), and myocardial metabolism (e.g., energy metabolism, contractile function), display a circadian rhythm in health (58). Circadian variation for cardiovascular morbidity and mortality (e.g., coronary artery thrombosis, acute myocardial infarction, angina pectoris, silent ischemia, ischemic stroke, sudden coronary death, etc.) has been also been well-documented, often with peak episodes occurring in the morning hours around arising (59-61). The circadian patterns of cardiac events often follow the natural fluctuations in endogenous physiological processes, with a vulnerable period consistently observed in the morning hours around awakening (62). Intracellular circadian clock mechanisms in vascular smooth muscle cells and cardiomyocytes have been identified that appear to regulate myocardial metabolism both directly and indirectly (63), thereby giving rise to the potential role of the circadian clock as a modulator of physiological and pathophysiological cardiovascular events (64-68). For example, a disruption of the circadian clock within the cardiomyocyte influences disruption in the 24-h heart rate rhythm, responsiveness of the heart to workload changes, myocardial metabolism, and changes in myocardial genes known to influence transport, transcription, signal transduction, protein turnover and metabolism (69).

Systematic analysis of the expression of CGs in peripheral tissues is underway in order to address how peripheral oscillators share similarly-timed molecular machinery and how changes in rhythm characteristics may be involved in or be a result of biological consequences, such as impaired CG function and/or disease. However, although the expression profiles of principal CGs have been characterized extensively in mammals and other species, analysis for time-effects and any rhythm features (i.e., period, amplitude, acrophase) in CGs has usually been described macroscopically (i.e., by eye) or by statistical comparisons of paired values (e.g., peak value vs. values at other times) and not quantitatively by chronobiological statistical analysis. Consequently, subjective observation can lead to less accuracy in both recognition of a rhythm and comparisons of rhythm characteristics among CGs in peripheral tissues (70), across species and studies, and by extension, to pathophysiologic conditions. Indeed, it has been pointed out that the analysis of circadian rhythms is important for a complete understanding of both physiology and pathology in mammalian and other species (71).

With a focus on the molecular clock and cardiology, we sought to apply inferential statistical procedures (i.e., ANOVA, the single cosinor method, parameter testing) (72) to previously published CG data which were described macroscopically (22) in order to compare and establish rhythm probability and point and interval estimates for amplitudes and acrophases for 14 clock and clock-controlled genes in mouse heart. The importance of CGs to cardiovascular health, as well as the general treatment and prognosis of human disease, are also reviewed.

Materials and Methods

In 3 separate studies, 5-week-old male Balb/c mice were exposed to 2 weeks of 12 h light/12 h dark (LD) cycles, then kept in complete darkness (DD) as a continuation of the dark span. In each study, organs were harvested from a single mouse every 4 h for 24-h (6/study; 18 total) beginning at

the onset of the 3rd DD cycle (i.e., at circadian time [CT] = 00h, which corresponded to the previous onset of light during LD) and CG mRNA expression was examined. Results for 14 CGs[1] obtained by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR) (performed in triplicate) included: three isoforms of *Period* (*mPer1*, *mPer2*, *mPer3*), two isoforms of *Cryptochrome* (*mCry1*, *mCry2*), *mBmal1*, *mCK1*δ, *mCK1*ε, *mClock*, *mDbp*, *mNpas2, mRev-erb*α, *mRev-erb*β, and *mTim*. The relative levels of each RNA were normalized to the corresponding G3-PDH RNA levels used as control. Full study details, including CG molecular assays, are reported elsewhere (22).

Prior to combining the data across the 3 studies for graphing and statistical analyses, relative levels of all mRNAs were standardized by re-expressing each value as percent of the highest value (max = 100%) for each CG in each study. Each time-series was analyzed for time-effect across the 6 timepoints by one-way analysis of variance (ANOVA) and for circadian rhythm characteristics by the single cosinor procedure involving the fit of a 24-h cosine to the data by least-squares linear regression (73), using the Chronolab statistical package (74). An R^2 value (= % reduction of total variability by fitted cosine; R^2 x 100 is also known as PR or %R = % Rhythm) and a *p*-value from the zero-amplitude assumption were determined for the cosine model, with rhythm detection considered statistically significant if $p \leq 0.05$. Rhythm characteristics and their dispersion indices determined from the best-fitting 24-h cosine model include: the "Mesor" (the middle of the cosine representing an adjusted 24-h average if unequal sampling); the "Amplitude" (the distance from the Mesor to the peak or trough of the fitted curve, indicating a predictable range of change); and the "Acrophase" (the peak of the cosine in hours from an external reference event, such as local midnight or time of light onset, indicating the estimated time of highest values). A parameter test was also applied to test for the equality of amplitudes and of acrophases between all CGs (75).

Results

In two panels for each CG, chronograms of the 3 individual studies (left panel) and overall timepoint means±SE along with the best-fitting 24-h cosine (right panel) are shown in Figures 1 & 2 (arranged left to right chronologically by acrophase). In general, 24-h patterns were highly reproduci-

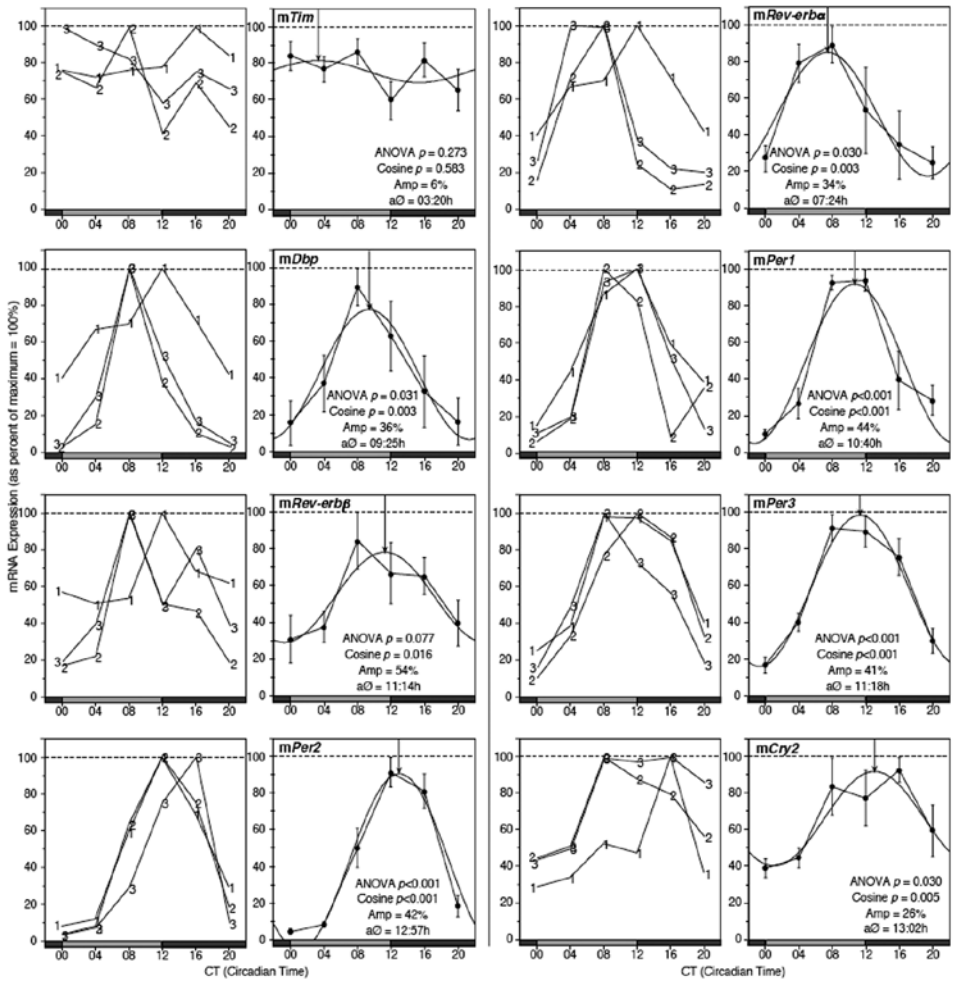

Fig. 1. Circadian patterns for mRNA expression (%) of 8 of 14 key clock genes (CG) in mouse heart (chronograms for mTim, mRev-erbα, mDbp, mPer1, mRev-erbβ, mPer3 and mPer2 arranged in order of chronological peak (acrophase) from CT 00h). Male Balb/c mice exposed to LD12:12 for two weeks followed by complete darkness (DD), then examined every 4 h for 24-h on the 3rd day in DD beginning at previous light onset in LD (= 00h Circadian Time [CT]). Relative CG mRNA levels normalized to the corresponding G3-PDH RNA levels and each sample expressed as % of maximum value (= 100%) in each time series. Y-axis = mRNA amounts, x-axis = CT, gray and black bars along x-axis indicate the duration of prior light and dark spans in LD, respectively. Left panels for each CG: 3 single studies showing extent of reproducibility of 24-h patterns. Right panels: overall timepoint means±SE with best-fitting 24-h cosine. For time-effect, *p*-values from ANOVA and 24-h cosine, and amplitude & acrophase (aØ, arrow) are listed.

[1] Per = period, Cry = cryptochrome, Bmal1 = brain and muscle ARNT-like protein 1, Rev-erb = reverse strand of TR alpha(α) or beta (β), Tim = timeless, Clock = circadian locomotor output cycles kaput), Dbp = D-element (or D-box) binding protein, CK1 = casein kinase 1 epsilon (ε) or delta (δ); Npas2 = neuronal PAS domain protein 2, a redox-sensitive Clock homologue. Organisms identified by prefix: m = mouse, r = rat, ha = hamster, h = human.

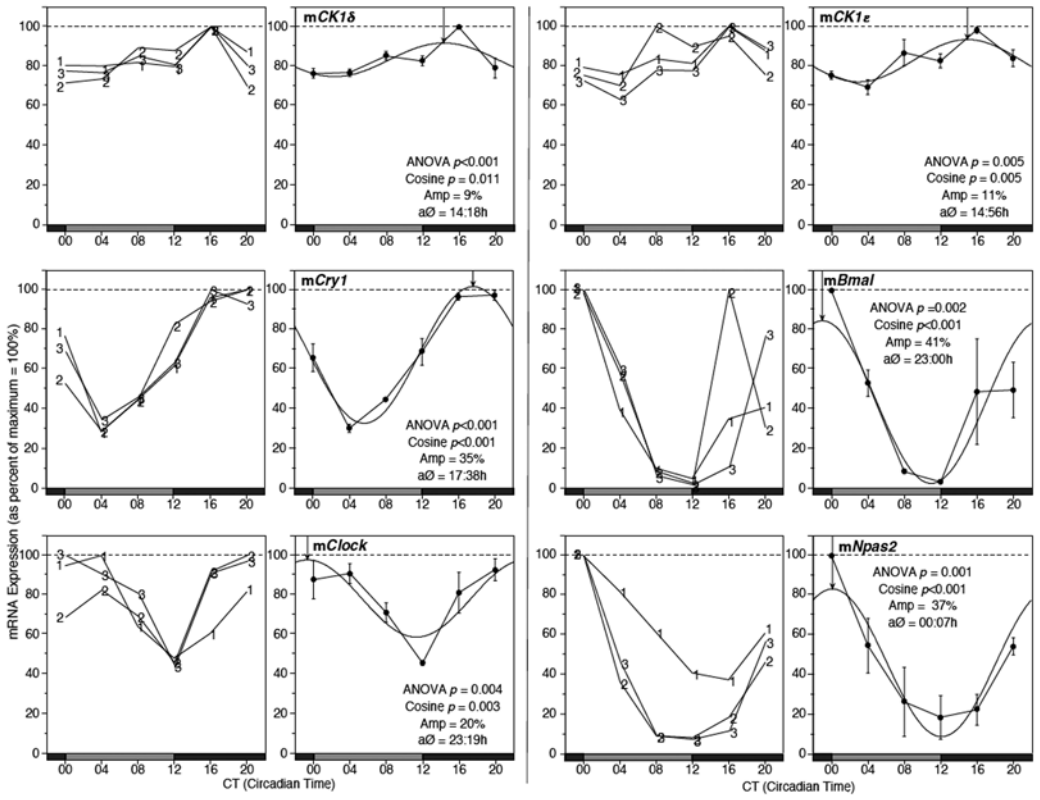

Fig. 2. Circadian patterns for mRNA expression (%) of 6 of 14 key clock genes (CG) in mouse heart (chronograms for mCry2, mCK1δ, mCK1ε, mCry1, mBmal1, mClock and mNpas2 arranged in order of chronological peak (acrophase) from CT 00h). See legend to Figure 1 for details.

ble across the 3 studies on day 3 in DD in terms of amplitudes and the timing of peaks and troughs for a particular CG (left panels). Although circadian variation is suggested in *mTim* in patterns for individual studies (Fig. 1), peaks were widely dispersed across the 3 studies (i.e., at 16h, 08h and 00h), resulting in a suppressed overall circadian pattern.

Results of analyses of each CG for time-effect by ANOVA and rhythm detection by single cosinor are listed in Table 1, with the best-fitting 24-h cosine drawn through the mean values in Figures 1 and 2 (right panels). Out of 14 CGs examined, 12 genes showed a significant time-effect at $p \leq 0.031$ when tested by ANOVA (p-values were 0.273 and 0.077 for *mTim* and *mRev-erb*β, respectively), and 13 genes displayed a significant 24-h rhythm at $p \leq 0.011$ when analyzed by the single cosinor procedure (p-value was 0.583 for *mTim*). Acrophases and 95% limits, representing calculated peak mRNA expression times, are arranged in chronologic order beginning with the earliest from 00h in Table 1 and in an acrophase chart (Fig. 3). Five CGs (*mRev-erb*α*, mDbp, mPer1, mRev-erb*β*, mPer3*) reached their maxima in the second half of the previous light span in LD (i.e., late in the presumed resting span), 5 CGs (*mPer2, mCry2, mCK1*δ*, mCK1*ε*, mCry1*) reached their peak in the first half of the previous dark span in LD (i.e., early in the presumed activity span), while 3 genes (*mBmal1, mClock, mNpas2*) reached their peaks near the end of the previous dark span in LD (i.e., late in the presumed activity span).

While parameter tests confirmed notable (and previously recognized) differences between acrophases of CGs with non-overlapping 95% confidence limits (Table 2), there were some instances where significant differences in timing could be established even though these limits slightly overlapped (i.e., *mRev-erb*α vs. *mRev-erb*β, *mRev-erb*β vs. *mCK1*ε, *mPer3* vs. *mCry2*, and *mCK1*ε vs. *mCry1*). Parameter tests also suggest that the amplitude for *mCry1* was significantly different from those of 4 CGs (lower than *mPer2*, higher than *mCK1*δ, *mCK1*ε, *mClock*), and that the *mCry2* amplitude was significantly different from those of 3 CGs (lower than *mPer2*, higher than *mCK1*δ, *mCK1*ε). Amplitudes for *mCK1*δ and *mCK1*ε were significantly lower than almost all other CGs, but not between each other, while the *mClock* amplitude was significantly lower than *mCry1*, *mBmal1*, *mCK1*δ, *Npas2* and the 3 *mPer* genes (Tables 1 and 2).

Discussion

In the original publication of the data (22) used as the basis for the current report, visual inspection was used to interpret the temporal expression of 14 clock and clock-related genes in 7 peripheral tissues in the mouse. With a focus on cardiology, we herein chose to concentrate on the 14 CGs in the heart by reanalyzing all the heart data using ANOVA and the single cosine technique in order to make a comparison of macroscopic (i.e., subjective, by eye) vs. microscopic (i.e., objective, statistical) interpretation of the presence and characteristics of circadian variations in CGs.

By way of some background, it should be noted that circadian rhythms are almost always characterized by a stable 24-h period under natural synchronized conditions. When

Table 1. Circadian Rhythm Parameters for mRNA Expression of 14 Key Clock Genes in Mouse Heart on Day 3 of DD: Comparison of macroscopic conclusions on rhythm characteristics with ANOVA and cosinor analyses.*

	Macroscopic Rhythm Observations		Results for Time- Effect by Analysis of Variance (ANOVA) and Single Cosinor** ANOVA				Parameters from 24h Cosine Fit**			
Gene	**amplitude**	**maxima (CT)**	**F**	***p***	**%R**	***p***	**Mesor ± SE**	**Amp ± SE**	**aØ**	**(95% Conf Limits)**
Tim	none	-	1.5	0.273	7	0.583	75.6 ± 4.0	6.0 ± 5.7	03:20h	- -
Rev-erbα	robust	04h - 08h	3.7	0.030	55	0.003	51.4 ± 5.7	33.9 ± 8.0	07:24h	(05:24, 09:24h)
Dbp	robust	08h	3.6	0.031	54	0.003	42.2 ± 6.0	35.6 ± 8.5	09:25h	(07:20, 11:24h)
Per1	robust	12h	17.6	<0.001	77	<0.001	48.5 ± 4.4	43.6 ± 6.2	10:40h	(09:28, 11:48h)
Rev-erbβ	robust	08h	2.7	0.077	43	0.016	53.8 ± 5.3	24.7 ± 7.4	11:14h	(08:36, 13:52h)
Per3	robust	08h - 12h	20,1	<0.001	87	<0.001	57.4 ± 3.0	41.4 ± 4.2	11:18h	(10:28, 12:08h)
Per2	robust	12h - 16h	27.6	<0.001	89	<0.001	42.4 ± 3.1	48.1 ± 4.4	12:57h	(12:12, 13:44h)
Cry2	weak	08h - 16h	3.7	0.030	51	0.005	66.5 ± 4.7	25.9 ± 6.6	13:02h	(10:48, 15:12h)
Ck1δ	none	16h	9.9	<0.001	45	0.011	83.5 ± 1.7	8.5 ± 2.4	14:18h	(11:48, 16:48h)
CK1ε	none	16h	6.2	0.005	51	0.005	83.0 ± 1.9	10.7 ± 2.7	14:56h	(12:44, 17:08h)
Cry1	small	16h - 20h	40.7	<0.001	92	<0.001	67.5 ± 1.8	34.5 ± 2.6	17:38h	(17:04, 18:16h)
Bmal1	robust	20h - 00h	7.7	0.002	63	<0.001	43.6 ± 5.8	41.1 ± 8.2	23:00h	(21:20, 00:40h)
Clock	weak	20h - 04h	6.6	0.004	53	0.003	78.4 ± 3.3	19.5 ± 4.7	23:19h	(21:16, 01:24h)
Npas2	robust	20h - 00h	8.2	0.001	68	<0.001	46.1 ± 4.7	37.3 ± 6.7	00:07h	(22:36, 01:36h)

* Mice synchronized to LD12:12 for 2 weeks and released into constant darkness (DD) by a continuation of D in 3 studies. Mice (6/study, 18 total) sampled every 4h for 24h beginning on day 3 in DD at previous L-onset in LD.
Units = values normalized to corresponding control gene G3-PDH RNA level and expressed as % of maximum value, with highest value = 100%. CT of reported maxima & comments from text and Figs 1& 2 in Yamamoto, et al., 2004 (22).
** Single cosinor analysis = fit of a 24h cosine to all data by least-squares linear regression.
Cosine parameters = %R (percent rhythm) = % reduction by cosine of overall variability; *p* from zero-amplitude test;
Mesor = 24h mean; Amp (amplitude) = distance from Mesor to peak or trough of cosine;
Acrophase (aØ, in order chronologically) = peak of best-fitting 24h cosine; 95% limits added if $p \leq 0.05$.
Phase time units (hh:mm) in Circadian Time (CT), where L-onset = 00:00h.

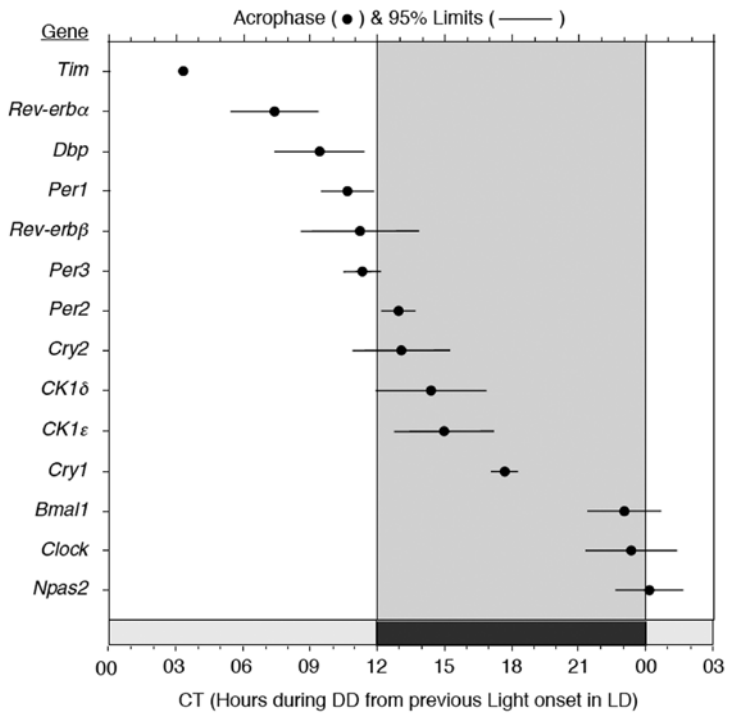

Fig. 3. Acrophase chart indicating timing of peak expression of mRNA for 14 key clock genes in heart of mice (see Figure 1 legend for details). Acrophase (Ø) = peak of fitted 24-h cosine using all data from 3 studies (n = 18); 95% confidence limits added to Ø if $p \leq 0.05$ from non-zero amplitude test. Lighter and darker gray bars along x-axis indicate the duration of light (L) and dark (D) spans, respectively, during LD schedule prior to DD (continuous darkness); CT = circadian time.

Table 2. Results of Parameter Tests for Circadian mRNA Expression of 13 Key Clock Genes (CGs) in Mouse Heart on Day 3 of DD.*

	Acrophase:	07:24h	09:25h	10:40h	11:14h	11:18h	12:57h	13:02h	14:18h	14:56h	17:38h	23:00h	23:19h	00:07h
	±SE:	00:54h	00:55h	00:32h	01:08h	00:24h	00:21h	00:58h	01:05h	00:58h	00:16h	00:45h	00:56h	00:40h
Amp ± SE	*Gene*	*Rev-erbα*	*Dbp*	*Per1*	*Rev-erbβ*	*Per3*	*Per2*	*Cry2*	*Ck1δ*	*CK1ε*	*Cry1*	*Bmal1*	*Clock*	*Npas2*
33.9 ± 8.0	*Rev-erbα*	-	0.153	0.011	**0.036**	0.003	<0.001	0.008	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
35.6 ± 8.5	*Dbp*	0.886	-	0.245	0.234	0.076	0.005	0.026	0.015	0.008	<0.001	<0.001	<0.001	<0.001
43.6 ± 6.2	*Per1*	0.345	0.452	-	0.661	0.340	0.002	0.058	0.018	0.005	<0.001	<0.001	<0.001	<0.001
24.7 ± 7.4	*Rev-erbβ*	0.408	0.345	0.060	-	0.946	0.179	0.257	0.091	**0.045**	<0.001	<0.001	<0.001	<0.001
41.4 ± 4.2	*Per3*	0.412	0.543	0.774	0.062	-	0.005	**0.013**	0.031	0.008	<0.001	<0.001	<0.001	<0.001
48.1 ± 4.4	*Per2*	0.133	0.203	0.554	0.012	0.279	-	0.943	0.263	0.083	<0.001	<0.001	<0.001	<0.001
25.9 ± 6.6	*Cry2*	0.445	0.374	0.059	0.912	0.058	0.009	-	0.400	0.194	0.003	<0.001	<0.001	<0.001
8.5 ± 2.4	*Ck1δ*	0.007	0.007	<0.001	0.052	<0.001	<0.001	0.023	-	0.667	**0.019**	<0.001	<0.001	<0.001
10.7 ± 2.7	*CK1ε*	0.013	0.012	<0.001	0.091	<0.001	<0.001	0.047	0.553	-	0.025	<0.001	<0.001	<0.001
34.5 ± 2.6	*Cry1*	0.944	0.904	0.187	0.229	0.169	0.013	0.238	<0.001	<0.001	-	<0.001	<0.001	<0.001
41.1 ± 8.2	*Bmal1*	0.534	0.644	0.807	0.149	0.970	0.454	0.158	0.001	0.002	0.451	-	0.796	0.289
19.5 ± 4.7	*Clock*	0.134	0.112	0.004	0.556	0.002	<0.001	0.440	0.050	0.119	0.010	0.031	-	0.493
37.3 ± 6.7	*Npas2*	0.748	0.877	0.491	0.219	0.601	0.185	0.233	<0.001	0.001	0.701	0.720	0.038	-

**p*-values from comparison of amplitudes (left diagonal quadrants) and acrophases (right diagonal quadrants) from 24h cosinor analysis.
Acrophases arranged chronologically. *p*-value for acrophase comparison in bold if 95% confidence limits for two arophases overlapped.
Single cosinor analysis = fit of a 24h cosine to all data by least-squares linear regression.
Cosine parameters = Amplitude (Amp) = distance to peak or trough of cosine from 24h mean (Mesor);
Acrophase = peak of best-fitting 24h cosine; Phase time units (hh:mm) in Circadian Time (CT). where L-onset = 00:00h.
Note: an additional gene. *mTim.* did not show a significant circadian rhythm and its parameters cannot be compared.

isolated from external cycles, the period in an organism will differ slightly from precisely 24-h and become about (*circa*) 24-h. Thus, after several days under such constant conditions (CC), such as continuous light (LL) or darkness (DD), the period will usually become established somewhere between 20h and 28h. This period is known as the desynchronized or "free-running" circadian period and usually after some unstable cycles (i.e., transients) between the period established under synchronizing LD conditions and the free-running period in CC, remains comparatively stable with regard to length (76). When studying CGs on the 3rd day in constant conditions of DD, it could therefore be expected that the underlying circadian period in the mice might have started to slowly drift either longer or shorter from precisely 24-h, resulting in a movement of the acrophase away from its previous location during synchronized LD conditions.

An earlier report of *rPer1* and *rPer2* in rat pineal studied for 24-h in LD and on the 2nd day in DD observed nearly identical circadian patterns in both lighting conditions for each CG, indicating the endogenous nature of the rhythmic expression in CGs that persists under CC, but does not change very much during at least the first few days in DD in the rat (31). Indeed, when we applied cosinor analysis and parameter testing to these published data, we could confirm statistically that the amplitudes did not differ significantly, and the acrophases differed by <1h between LD & day 2 in DD. In the current study, CGs in mice were not measured during LD, so no data were available in LD vs. on the 3rd full day in DD in order to test for any change in rhythm characteristics during CC. Therefore, using cosinor analysis we made comparisons between circadian patterns for CGs in mice in DD in this study with 24-h patterns found in the literature in some of the same CGs studied in LD for mice or rats. We found that amplitudes and acrophases on day 3 in DD in the current study were virtually identical for 7 CGs in liver, spleen, and kidney (heart was not studied in LD) for mice sampled in LD (29), e.g., acrophases differed by an hour or less between CGs in LD, while for 8 CGs in rat heart, amplitudes were comparable and acrophases were slightly but not significantly advanced by 1-2 hours in LD (33). These findings suggest that the rhythms observed on day 3 in DD in CG mRNA expression were still comparable to patterns found during LD and thus allow for analysis and discussion of circadian characteristics for CGs in the current study using a precise 24-h cosine to describe the oscillations observed during DD in relation to the previous synchronized light-dark/rest-activity cycle.

With regard to rhythm detection, the original publication (22) reported that mRNA expression of 8 genes (*mPer1,2,3, mBmal1, mRev-erbα, mRev-erbβ, mNpas2 and mDbp*) "showed a robust circadian rhythm" in all peripheral tissues, including the heart, and speculated that these 8 genes "likely constitute the core molecules of a molecular circadian clock." It was also noted that the rhythm of mRNA expression for *mCry1* "was obviously circadian …, but the peak-trough amplitude was relatively smaller than that of the above genes." In addition, it was mentioned that *mCry2* and *mClock* were "rather weak and not clearly circadian", and that "no circadian rhythms were observed in … *mCK1δ, mCK1ε,* and *mTim.*" When these same data were subjected to ANOVA and cosinor statistics, however, a significant time-effect and/or circadian rhythm was found in all CGs, except for *mTim*. There was good agreement between the original estimates of maxima by eyeballing and with the point and interval estimates of the acrophase for each CG. In addition, objective differences in timing of peaks in CG mRNA expression (by virtue of non-overlapping 95% confidence intervals (CIs) of individual CG acrophases) were confirmed by a parameter test, that also found a few additional significant differences in timing where the 95% CIs slightly overlapped between two CGs (Table 2).

With regard to amplitude, the original report of a circadian rhythm in only 8 CGs was based upon a subjective

determination of a "robust" peak to trough variation in mRNA expression over 24 hours. However, this perception that a high-amplitude rhythm should be considered as more biologically important, and thus more consequential, overlooks the possibility that a low-amplitude protein rhythm upstream might drive a high-amplitude physiological rhythm if the protein participates in a specific downstream supportive process (21, 55). Reanalysis of the data found that, apart from *mTim*, a statistically-significant circadian rhythm was found in the remaining 13 CGs in mouse heart by objective chronobiological inferential procedures even though 5 CGs (*mCry1, mCry2, MCK1*δ*, mCK1*ε*, mClock*) had comparatively small amplitudes (Table 1). Parenthetically, the lack of a significant time-effect for *mTim* is not surprising, since mammalian *Tim (Timeless)* has been reported to be a developmental gene without substantial circadian function (7). Thus, most reports have failed to detect a significant time-of-day change or only a weak circadian oscillation for *Tim* in the SCN and its functional role in peripheral mammalian clocks, such as its interaction with CRY proteins in DNA repair pathways, is still open to question (77).

With the cosinor technique we demonstrate that statistical determination of rhythm parameters for 13 CGs allowed for a quantitative description of a circadian amplitude and acrophase for each CG, and propose that both values could be useful in making objective comparisons of CG circadian characteristics between other peripheral organs, species, and/or between studies utilizing various experimental conditions, such as different photoperiods and/or phase shifts (10, 37, 43, 78, 79), feeding schedules (16-18, 79, 80), during development and aging (3-6, 32, 40, 81), as well as between health and disease or induced disorders, such as gene mutations. In addition, the statistical determination of the acrophase by itself can also be of importance, since a phase shift can be equivalent to a down-regulation at a defined timepoint, indicating that gene interaction needs to be interpreted in the context of the phase of a circadian oscillation (55). In a search of the literature, time-series analysis to obtain rhythm detection probability and characteristics has been applied using one form of cosinor or curve-fitting technique or another (82) to CG data in some studies of mouse SCN (2, 70), skeletal muscle, kidney and thymus (25), liver and mammary tumor (83), bone marrow (28), adipose, cardiac and liver tissues (80), liver, kidney, spleen, testis, thymus, and blood (29), calvarial (skull) bone (30), and gastrointestinal tract (18, 84); in rat SCN and pineal (38); and in human skin and oral mucosa (45), bone marrow (47), and peripheral blood leukocytes (46, 49, 85), as well as in blood pressure and heart rate of *Clock*-mutant mice (86).

Circadian mechanisms influence nearly all aspects of physiology, psychology and behavior, which includes sleep-wake cycles and nearly all functions involving cardiovascular, respiratory, digestive, integumentary, nervous, endocrine, immune/lymphatic, excretory (urine), reproductive, muscular, skeletal, behavioral and whole body systems; circadian information has been established in health for 100's of variables (58, 87). Numerous reports of humans or mice with mutations or targeted knock-out disruptions of core circadian genes have shown that the molecular clock influences different biochemical pathways involved in pathophysiology. Consequently, studies have linked altered function of specific CG components of the circadian clock with numerous metabolic and psychopathological disorders, including: cardiovascular control and disease [*mClock* (20, 86), *mPer2*, *mBmal1* (88), *mRev-erb*α (89), *Dbp*, *mBmal1* (90), *mBmal1, mPer2, mCry1,2* (91), and *mBmal1, mClock, mNpas2* (92)]; aging-related pathologies [m*Bmal1* (93), and *mClock* (94, 95)]; cancer development and regulation [breast *hPer1, hPer2, hPer3* (96), *hPer1* (97) and *hPer1, hPer2* (98); endometrial *hPer1* (99); lymphoma *mPer2* (100); colorectal *hPer1* (101) and *hPer1, hClock* (102); Lewis lung and mammary *mPer2* (103); non-small cell lung *hPer1* (97, 104); hepatocellular *hPer1, hPer2, hPer3, hCry2, hTim* (105); and ovarian *hPer1,2,3, hCry1,2, hBmal1, hClock, hCKI*ε (106)]; obesity, diabetes and metabolic syndrome [*mBmal1*, *mClock* (107), *mClock* (108), *hClock* (109), *hBmal1, hPer1, hCry1* (110), and *hClock, hBmal1, hPer1,2,3, hCry1* (50)]; hepatotoxicity [*mPer2* (111)]; sleep apnea [*hPer1* (112)]; delayed sleep-phase syndrome [*hPer3* (85, 113, 114)]; seasonal affective disorder [*hPer2, hBmal1[Arntl], hNpas2* (115)]; reproductive performance [*mClock* (116, 117)]; and bipolar disorders [*hBmal1[Arntl]*, *hPer3* (118), and *hClock* (119)], among others [Table 2 in (8)].

Appreciating relationships between circadian clocks and normal vs. abnormal organ physiology can have important impact relevant not only to cardiovascular health, but also to the general treatment and prognosis of human disease (88). Information on the molecular clock presents new avenues for therapeutic intervention in conditions where disturbance of circadian CG expression is an important cause of morbidity in chronic illnesses and diseases with a strong circadian component, including coronary vascular disease (120), epilepsy (121), cancer (83, 122) and asthma (123, 124). It could also lead to new strategies for pharmacological manipulation of the human clock to improve the treatment of various clock-related sleep and psychiatric disorders, jet lag and other human diseases (125).

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