Circadian rhythms govern cardiac repolarization and arrhythmogenesis

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Sudden cardiac death exhibits diurnal variation in both acquired and hereditary forms of heart disease1–3, but the molecular basis of this variation is unknown. A common mechanism that underlies susceptibility to ventricular arrhythmias is abnormalities in the duration (for example, short or long QT syndromes and heart failure)4–6 or pattern (for example, Brugada’s syndrome)7 of myocardial repolarization. Here we provide molecular evidence that links circadian rhythms to vulnerability in ventricular arrhythmias in mice. Specifically, we show that cardiac ion-channel expression and QT-interval duration (an index of myocardial repolarization) exhibit endogenous circadian rhythmicity under the control of a clock-dependent oscillator, krüppel-like factor 15 (Klf15). Klf15 transcriptionally controls rhythmic expression of Kv channel-interacting protein 2 (KChIP2), a critical subunit required for generating the transient outward potassium current7. Deficiency or excess of Klf15 causes loss of rhythmic QT variation, abnormal repolarization and enhanced susceptibility to ventricular arrhythmias. These findings identify circadian transcription of ion channels as a mechanism for cardiac arrhythmogenesis.

Circadian rhythms govern cardiac repolarization

Sudden cardiac death from ventricular arrhythmias is the principal cause of mortality from heart disease worldwide and remains a major unresolved public health problem. The incidence of sudden cardiac death exhibits diurnal variation in both acquired and hereditary forms of heart disease1–2. In the general population, the occurrence of sudden cardiac death increases sharply within a few hours of rising in the morning, and a second peak is evident in the evening hours1. In specific hereditary disorders, for example, Brugada’s syndrome, fatal ventricular arrhythmias often occur during sleep7. A common mechanism in both acquired and hereditary forms of heart disease that enhances susceptibility to ventricular arrhythmias is abnormal myocardial repolarization8. Clinically, three common types of alterations in myocardial repolarization are evident on the surface electrocardiogram (ECG). First, prolongation of repolarization is seen in acquired disorders (for example, heart failure)9 and congenital disorders (for example, long QT syndrome)10. Second, shortening of repolarization is found in the short QT syndrome11. Third, early repolarization is the hallmark ECG finding in Brugada’s syndrome12. Interestingly, all three modifications of repolarization increase vulnerability to ventricular arrhythmias8. Despite rigorous investigation of the biophysical and structural characteristics of ion channels that control myocardial repolarization, the molecular basis for the diurnal variation in occurrence of ventricular arrhythmias remains unknown.

Biological processes in living organisms that oscillate with a periodicity of 24 h are said to be circadian. This cell-autonomous rhythm is coordinated by an endless negative transcriptional–translational feedback loop, commonly referred to as the biological clock9. Several physiological parameters in the cardiovascular system such as heart rate, blood pressure, vascular tone, QT interval and ventricular effective refractory period exhibit diurnal variation10–13. Recent studies have also identified a direct role for the biological clock in regulating cardiac metabolism, growth and response to injury9. Previous studies have also reported that expression of repolarizing ion channels and ionic currents (I\textsubscript{Na}) exhibit diurnal changes14. However, a potential link between circadian rhythms and arrhythmogenesis remains unknown. We made the serendipitous observation that Klf15 expression exhibits endogenous circadian rhythmicity in the heart (Fig. 1a). Gene expression microarrays in hearts of mice that are deficient in Klf15 led us to identify KChIP2 (also called KCNIP2), the regulatory β-subunit for the repolarizing transient outward potassium current (I\textsubscript{Na}) as a putative target for this factor in the heart. These observations led us to question whether the circadian clock may regulate rhythmic variation in repolarization and alter susceptibility to arrhythmias through Klf15.

First, we explored mechanisms through which the circadian clock regulated rhythmic expression of Klf15 in the heart. Examination of approximately 5 kb of the promoter region of Klf15 revealed four canonical ‘E-box’ regions, that is, consensus binding sites for CLOCK and its heterodimer BMAL1 (also called ARNTL), which are essential transcription factors involved in the circadian clock (Supplementary Fig. 1a, inset). Consistent with this finding, Klf15 luciferase (approximately 5 kb) was activated in a dose-dependent manner by the CLOCK–BMAL1 heterodimer (Supplementary Fig. 1a). To confirm this interaction, we performed chromatin immunoprecipitation (ChIP) and identified rhythmic variation in BMAL1 binding to the Klf15 promoter in the hearts of wild-type mice, but not in the hearts of Bmal1-null mice (Fig. 1b). In accordance with the observations above, the expression of Klf15 was disrupted in Bmal1-null, and Perl- and Cryl-null hearts (Supplementary Fig. 1b). Thus, our data strongly suggest that the circadian clock directly regulates the oscillation of Klf15 in the heart.

To determine whether myocardial repolarization and ion-channel expression exhibit ‘true’ (endogenous) circadian rhythms—that is, oscillate in the absence of external cues such as light—wild-type mice were placed in constant darkness for 36 h and telemetry-based ECG intervals were measured every 2 h for 24 h. Under these conditions, the heart rate and the QT interval corrected to heart rate (QTc) were both rhythmic and exhibited true endogenous circadian rhythmicity (Fig. 1c, d). Next, to examine whether expression of repolarizing ion channels had endogenous circadian rhythms, mice were placed in constant darkness for 36 h, and hearts were collected every 4 h over a 24-h period. The expression of the α-subunit for the transient outward
potassium current ($I_{\text{K}}$), Kv4.2 (encoded by Kcnd2) (Fig. 1e), and the regulatory β-subunit, KChIP2 (Fig. 1f), exhibit endogenous circadian rhythmicity, as did components of the circadian clock in the heart (Supplementary Fig. 2). In contrast, the expression of two other major repolarizing currents in the murine ventricle, Kv1.5 (the α-subunit for the ultra-rapid delayed rectifier potassium current) and Kir2.1 (the α-subunit for the inward rectifier potassium current), did not reveal notable rhythmic variation (Supplementary Fig. 3). In addition, we observed a 24-h rhythm in the oscillation of Bmal1, Klf15 and KChIP2 after serum shock in cultured neonatal rat ventricular myocytes (Supplementary Fig. 4). These data indicate that myocardial repolarization and the expression of some repolarizing ion channels exhibit an endogenous circadian rhythm.

Next, to elucidate the role of Klf15 in regulating rhythmic changes in repolarization, we used complementary in vivo loss- and gain-of-function approaches in mice. For loss-of-function, a previously described systemic Klf15-null mouse was used for; for gain-of-function, a cardiac-specific Klf15 transgenic (Klf15-Tg) mouse driven by an attenuated α-myosin heavy chain (α-MHC) promoter was developed (Supplementary Fig. 5). First, we examined whether rhythmic expression of Kcnd2 or KChIP2 was altered in the Klf15-deficient state. Kcnd2 expression exhibited altered rhythmic variation in Klf15-null mice with reduced expression at zeitgeber time 6 (ZT6), and increased expression at ZT22 compared to wild-type controls (Fig. 2a). KChIP2 expression was devoid of any discernable rhythm in the Klf15-null mice and sustained reduction was observed at all time points (Fig. 2b, c and d).

**Figure 1** Klf15 expression, ECG QTc interval and expression of repolarizing ion channels exhibit endogenous circadian rhythm. a, Klf15 expression exhibits endogenous circadian variation in wild-type (WT) hearts from mice in constant darkness ($n = 4$ per time point). CT, circadian time. b, Effect of Bmal1 ChIP on the Klf15 promoter, showing rhythmic variation in binding of Bmal1 to the Klf15 promoter in wild-type hearts ($n = 3$ per group). c, Duration of ECG QTc interval (ms) in conscious mice exhibits endogenous circadian variation in constant darkness ($n = 4$). d, Representative ECGs from WT (non-Tg) and Klf15-Tg in wild-type hearts measured every 4 h after 36 h in constant darkness at CT 0 and CT 12. e, f, Endogenous circadian variation in transcripts for Kcnd2 and KChIP2 in wild-type hearts measured every 4 h after 36 h in constant darkness ($n = 4$ per time point). Error bars, mean ± s.e.m.
Supplementary Fig. 6a). Next, we examined whether Kcnkd2 or KChIP2 serve as transcriptional targets for Klf15 in the heart. Adenoviral overexpression of Klf15 in neonatal rat ventricular myocytes robustly induced KChIP2 expression but had no effect on Kcnkd2 expression (Supplementary Fig. 6b). Notably, in Klf15-Tg hearts, expression of KChIP2 was twofold greater but with no effect on Kcnkd2 expression (Fig. 2d, e). Examination of the KChIP2 promoter region revealed numerous consensus krüppel-binding sites, that is, C(A/T)CCC (Supplementary Fig. 7a). The activity of KChIP2 luciferase was induced by full-length KLF15 but not by a mutant that lacked the zinc-finger DNA-binding domain (Supplementary Fig. 7b). To identify the specific Klf15 binding site, deletion constructs of the KChIP2 promoter were generated, and transcriptional activity was mapped to the proximal 555 bases (Supplementary Fig. 7a). Mutation of one krüppel-binding site within this region (A1) was sufficient to cause complete loss of activity in the full-length KChIP2 promoter (Supplementary Fig. 7c). Chromatin immunoprecipitation of Flag–KLF15 from Klf15-Tg hearts confirmed that KLF15 was enriched on the endogenous KChIP2 promoter (Supplementary Fig. 7a). The activity of Klf15-Tg hearts was similar to their controls at ZT6 (Supplementary Fig. 6). In the Klf15-deficient state, the ECG QTc interval was prolonged in the dark phase and failed to oscillate (Fig. 3a, c). This occurred despite Klf15-null mice having similar heart rates to their wild-type counterparts (Supplementary Fig. 11). In contrast, the Klf15-Tg mice had persistently short QT intervals with no rhythmic day–night variation (Fig. 3b, d). Again, this occurred despite minimal difference in heart rates when compared to wild-type controls (Supplementary Fig. 11). Next, we examined whether transient outward current (Ito_fast)-dependent changes in repolarization in isolated myocytes were responsible for the ECG changes mentioned above in Klf15-null and Klf15-Tg mice. In Klf15-null mice, there was a marked reduction in Ito_fast density (Fig. 3e) and prolongation of action potential duration (APD) (Fig. 3g). In contrast, Klf15-Tg mice exhibited a substantial increase in Ito_fast density (Fig. 3f) with a dramatic shortening of APD (Fig. 3h). In the Klf15-Tg mice, in addition to short QT intervals, we observed ST-segment changes indicative of early repolarization that are similar to ECG findings in Brugada’s syndrome (Fig. 3b, arrows). Our data suggest that Klf15-dependent transcriptional regulation of rhythmic KChIP2 expression in murine hearts plays a central part in rhythmic variation in ventricular repolarization.

Next, we examined whether excessive prolongation or shortening of repolarization could alter arrhythmia susceptibility and survival. Klf15-null mice show no spontaneous arrhythmias on ECG telemetry, hence we used intracardiac programmed electrical stimulation to examine arrhythmia susceptibility. In contrast to wild-type mice, a marked increase in occurrence of ventricular arrhythmias was seen in Klf15-null mice (Fig. 4a). Notably, Klf15-Tg mice exhibit spontaneous ventricular arrhythmias on ECG telemetry (Fig. 4b) and succumb to ~35% mortality by 4 months of age (three out of eight deaths in Klf15-Tg versus no deaths out of eight in wild-type non-transgenic controls, data not shown). As the Klf15-null mice show no evidence of overt ventricular dysfunction, apoptosis or fibrosis in the basal state, the enhanced susceptibility to arrhythmias is probably primarily driven by...
of the human heart. Nevertheless, these data may provide a mechanistic foundation for future efforts to prevent or treat cardiac arrhythmias by modulating the circadian clock through behavioural or pharmacological means.

**METHODS SUMMARY**

Mice used in the present study, messenger RNA quantification using polymerase chain reaction with reverse transcription (RT–PCR), promoter reporter analysis, western immunoblot analysis, chromatin immunoprecipitation, telemetry ECG and interval analysis, isolated myocyte studies for action potential or \( I_m \) measurements, in vivo electrophysiological studies for arrhythmia susceptibility, cosinor analysis for rhythm assessment, and statistical methods are detailed in the Methods.

**Full Methods** and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 31 March 2011; accepted 12 January 2012.

Published online 22 February 2012.

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Figure 4 | **Klf15** deficiency or excess increases susceptibility to ventricular arrhythmias. **a**, Programmed electrical stimulation in wild-type and Klf15-null mice. Onset of ventricular tachycardia after premature stimuli is shown (arrows) in Klf15-null mice (none of the seven wild-type mice were inducible; \(* P < 0.05\)). **b**, Spontaneous ventricular arrhythmia in Klf15-Tg mice. (none of the four wild-type mice exhibited spontaneous arrhythmias but three of the four Klf15-Tg mice exhibited ventricular arrhythmias; \(* P < 0.05\)). VT, ventricular tachycardia.
METHODS

Mice. All animal studies were carried out with permission, and in accordance with, animal care guidelines from the Institutional Animal Care Use Committee (IACUC) at Case Western Reserve University and at collaborating facilities. Wild-type male mice on C57BL/6J background (Jackson Laboratory) were bred in our facility and used for circadian studies. Mice were housed under strict light-dark conditions (lights on at 6:00 and lights off at 18:00) and had free access to standard chow and water, and were minimally disturbed for 4–6 weeks before the final experiment. Generation of systemic Klf15-null mice was as described previously25. Klf15-null mice have been backcrossed into the C57BL6/J background for over ten generations18 and the BMAL1 mice was as previously described24. For Klf15-Tg mice, Flag–KLF15 was cloned downstream of an attenuated z-mycosin heavy-chain promoter as previously described26. Primer sequences that were used for ChIP of Flag–KLF15 on the Klf15 locus were as follows: forward, 5′-GTGCGGACAACAGTTGCAATTCC-3′; and reverse, 5′-GACAGCACCAAGTCTTCAAA-3′; and probe, 5′-FAM-CCCCGCGCAGTGCAGCTGCTGCAGT-3′BHQ1. Non-target primers were: forward, 5′-GGGACATTAGTGCTTGCTGCTGCTTCTC-3′; and reverse, 5′-GACGACCGAAGCTTTACAA-3′; and probe, 5′-FAM-TGCAAAAAGGGCTTGCACTGGG-3′BHQ1. Primers that were used for ChIP of Flag–KLF15 on the KChIP2 promoter were: forward, 5′-GCTCGAGACCTGCCTGCGCTGCTGCTGCTC-3′; and reverse, 5′-GGGCGGGACAGTTTACAA-3′.

Telemetry ECG and interval analysis. Mice were implanted with telemetry devices (ETA F20, Data Sciences International) and allowed to recover for at least 2 weeks. ECGs were recorded from conscious mice continuously in their native environment using an electrocardiogram device (PhysioTel, Data Sciences International) were stored for future analysis. Owing to rapid changes in the mouse heart rates, a weighted heart-rate approach was used to assess rhythmic changes in QT interval, and measurements were made every 2 h over a 24-h period. First, the average heart rate was calculated for each hour by digital tracking of the ECG RR intervals (time interval between two consecutive R waves) using the DataPath analysis software (Data Sciences International). Then, during the first instance within each hour when the average heart rate was present, the QT interval was measured using electronic calipers from two consecutive beats. The QT interval was corrected for heart rate using a previously validated formula for conscious mice QT(RR(100))/RR(100) (ref. 23). A Cosinor model was applied to assess the 24-h rhythm in QT using a sinusoidal regression function and raw data presented in four hourly blocks for visualization purposes.

Electrophysiological studies in myocytes. Murine ventricular myocytes were isolated using a standard enzymatic dispersion technique following overnight fast as previously described24. Myocytes were re-suspended in media 199, allowed to recover and recordings were conducted within several hours on the same day. The conventional whole-cell mode was used to record action potentials and IM. In brief, myocytes were bathed in a chamber that was continuously perfused with Tyrode’s solution of the following composition (in mmol−1): NaCl, 137; KCl, 5; CaCl2, 2.0; MgSO4, 1.0; glucose, 10; and HEPES, 10 (pH 7.35). Patch pipettes (0.9–1.5 MΩ) were filled with electrode solution composed of (in mmol−1): aspartic acid, 120, KCl, 20; NaCl, 10; MgCl2; 2; and HEPES, 5 (pH 7.3). Action potentials were elicited in current-clamp mode by injection of a square pulse of current of 5 ms duration and 1.5–2 times the threshold amplitude. APD was measured at 90% repolarization. To measure IM were cells placed in Tyrode’s solution (as described earlier) containing 1 mM nisoldipine to block calcium current and calcium-activated chloride current, and tetrodotoxin (100 μmol l−1) to block sodium current. Cells were brought from a holding potential of ~70 mV to ~25 mV for 25 ms. To isolate the fast, transient component of the outward currents, IM fast, the decay phase of outward potassium currents was fit by the exponential functions of the form:

\[ y(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2) + A_3 \]

where \( \tau_1 \) is the time constant of decay of the fast, transient component of outward potassium currents; \( A_1 \) is the amplitude coefficient of IM fast; \( \tau_2 \) is the time constant of decay of the slow, transient component of the outward currents; \( A_2 \) is the amplitude of IM slow; and \( A_3 \) is the amplitude coefficient of the non-inactivating steady-state outward potassium current IM. Consistent with previous studies25, the time constant of decay of the fast, transient component IM fast was 47 ± 5 ms. The measured current amplitudes were normalized to cell capacitance and converted into current densities. All experiments were conducted at 36 °C. Cell capacitance and series resistance were compensated electronically at −80 mV. Command and data acquisition were operated with an Axopatch 200b patch-clamp amplifier controlled by a personal computer using a Digitata 1200 acquisition board driven by pCLAMP 7.0 software (Axon Instruments).

Programmed electrical stimulation. Intracardiac programmed electrical stimulation was performed as previously described24. In brief, mice were anaesthetized using 1.5% isoflurane in 95% O2 after an overnight fast. ECG channels were amplified (0.1 mV cm−1) and filtered between 0.05 and 400 Hz. A computer-based data acquisition system (Emka Technologies) was used to record a 3-lead body surface ECG, and up to four intracardiac bipolar electrograms. Bipolar atrial pacing and right ventricular pacing were performed using 2-ms current pulses delivered by an external stimulator (STG-3000, MultiChannel Systems; Reutlingen). Standard clinical electrophysiological pacing protocols were used to determine all basic electrophysiological parameters. Overdrive pacing, single, double and triple extrastimuli, as well as ventricular burst pacing, were delivered to determine the inducibility of ventricular arrhythmias, which was tested twice.

Statistical analysis. A cosinor model was adopted to determine whether there is a substantial 24-h rhythm in each physiological and molecular variable of interest. By pooling data points of all mice, the model fits data to a fundamental sinusoidal function. To determine the coefficients (amplitude and phase) of the sinusoidal function and to see whether there were significant relationships, a mixed model analysis of variance was performed using standard least-square regression and the restricted maximum likelihood method (IIM 8.0, SAS Institute) as previously described24. Data are presented as mean ± s.e.m., the Student’s t-test was used for assessing the difference between individual groups and P ≤ 0.05 was considered statistically significant.

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