Cardiomyocyte specific Bmal1 deletion in mice triggers diastolic dysfunction, extracellular matrix response and impaired resolution of inflammation

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Running title: Clock dysfunction triggers fibrotic response in the heart

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Abstract

The mammalian circadian clock consists of multiple transcriptional regulators that coordinate biological processes in a time-of-day-dependent manner. Cardiomyocyte-specific deletion of the circadian clock component, Bmal1 (aryl hydrocarbon receptor nuclear translocator-like protein 1), leads to age-dependent dilated cardiomyopathy and decreased lifespan in mice. We investigated whether cardiomyocyte-specific Bmal1 knockout (CBK) mice display early alterations in cardiac diastolic function, extracellular matrix (ECM) remodeling, and inflammation modulators by investigating CBK mice and littermate controls at 8 and 28 weeks old (i.e., prior to overt systolic dysfunction). Left ventricles of CBK mice exhibited (p<0.05): 1) progressive abnormal diastolic septal annular wall motion and reduced pulmonary venous flow only at 28 weeks of age; 2) progressive worsening of fibrosis in the interstitial and endocardial regions from 8 to 28 weeks of age; 3) increased (>1.5 fold) expression of collagen I and III, as well as the matrix metalloproteinases MMP-9, MMP-13, MMP-14 at 28 weeks of age; 4) increased transcript levels of neutrophil chemotaxis and leukocyte migration genes (Ccl2, Ccl8, Cxcl2, Cxcl1, Cxcr2, Il1β) with no change in Il-10 and Il-13 genes expression; and 5) decreased levels of 5-LOX, HO-1 and COX-2, enzymes indicating impaired resolution of inflammation. In conclusion, genetic disruption of the cardiomyocyte circadian clock results in diastolic dysfunction, adverse ECM remodeling, and pro-inflammatory gene expression profiles in the mouse heart indicating signs of early cardiac aging in CBK mice.
New and noteworthy

Cardiomyocyte specific Bmal1 gene deletion in heart progress to;
1) diastolic dysfunction with significant age-dependent hypertrophy.
2) dilative hypertrophy marked with endocardial fibrosis and interstitial fibrosis in age-dependent manner.
3) age-dependent ventricular fibrosis display aggravated extracellular matrix deposition and defective resolution of inflammation response.
Introduction

The circadian clock is a timekeeping system which regulates physiological performance and behavior relative to day-night cycles. Oscillations in cardiovascular functions are firmly established, including time-of-day-dependent fluctuations in blood pressure, heart rate, and cardiac output (9) (10) (31). Night shift work and frequent time zone changes result in a dissociation between this intrinsic timekeeping mechanism and the environment, which is associated with increased risk of adverse cardiovascular effects (such as myocardial infarction and sudden cardiac death) (33) (5) (15). In mammals, the timekeeping system can be divided into two sub-systems, the central (located in the superchiasmatic nucleus) and the peripheral (present in essentially all cells of the body) clocks. Central and peripheral clocks coordinate biological processes in a time-of-day-dependent manner, thus facilitating homoeostasis (34). In the past two decades, it has become clear that the circadian clock modulates numerous physiological processes, in addition to influences on pathological events (e.g., myocardial infarction) (43). The circadian clock mechanism is composed of several core components (PER1, PER2, PER3, CRY1, CRY2, CK1e, and TIM), including CLOCK (circadian locomotor output cycles protein kaput) Bmal1 (synonym - aryl hydrocarbon receptor nuclear translocator-like protein 1), and REV-ERBα. Genetic disruption of clock components in mouse models initiates metabolic abnormalities, including an increased incidence of adiposity and insulin resistance (38). In humans, interrupted sleep duration, altered sleep-wake cycle or insomnia directly associates with disturbed circadian rhythm and sudden sensorineural hearing loss marked with reduced levels of PER1 and CRY2 in peripheral leukocytes (41). In the rodent hearts, the impact of disruption of two main
transcription factors, CLOCK and BMAL1, has been investigated (42). These studies reveal important roles for these clock components in the heart at the levels of contractility, electrophysiology, metabolism, signaling, translation, and transcription (7). Of these models, genetic ablation of BMAL1 in the heart elicits the most striking phenotype from 28 week onwards; age-onset development of dilated cardiomyopathy associated with reduced life span. The mean survival for cardiomyocyte specific Bmal1 deletion (CBK) mice was 33 weeks, while no deaths were observed in littermate controls (i.e., Bmal1<sup>fl</sup>/<sup>fl</sup>) or wild-type (C57BL/6J) within one year of the study (42).

The mechanism(s) by which genetic ablation of Bmal1 in the heart results in cardiomyopathy and an accelerated aging phenotype is (are) currently unknown. We determined that Bmal1 deletion initiates an extracellular matrix (ECM) response which proceeds to an inflammatory response by 28 weeks of age. Compared to littermate controls, germline Bmal1 deletion results in the development of chronic inflammation (6). Here, we report that ECM genes are increased in CBK mice at the age of 28 weeks. As expected, with increased ECM deposition, the transforming growth factor (TGF-β) signaling cascade, including SMAD2 (Mothers against decapentaplegic homolog 2), was activated in CBK hearts by 28 weeks of age. Thus, Bmal1 deletion in mice initiated dilative hypertrophy, diastolic dysfunction, and ECM responses and an impaired resolution of inflammation axis with marked signs of accelerated early cardiac aging by 28 weeks of age.

**Materials and Methods**

**Mice**
All animal procedures were conducted according to the “Guide for the Care and Use of Laboratory Animals” (Eight Edition, 2011) and were approved by the Institutional Animal Care and Use Committees at the University of Alabama at Birmingham, USA. CBK (BMAL1\textsuperscript{floxflox}/α-MHC-CRE\textsuperscript{+/-}) mice on the C57Bl/6J background were developed as previously described. (12, 42) Male CBK and C57BL/6J background littermate control mice (control) were maintained on a standard diet. Both littermate control and CBK mice were subjected to pulse wave and doppler echocardiography at 8 and 28 weeks of age. A total of 33 mice were used in the experiment, 6 for control at 8 weeks of age, 12 for control at 28 weeks of age, 5 for 8 weeks old CBK, and 10 for 28 weeks old CBK.

**Diastolic function measurements using echocardiography**

Diastolic function was assessed in mice using echocardiography; assessments were made in mice under 1.5-2.0% isoflurane in an oxygen mix using Vevo 770 (VisualSonics Inc., Canada) high-resolution imaging system. This system was equipped with a RMV707B transducer containing a 30 MHz high frequency probe and data was analyzed using VisualSonics software. Temperature and heart rates of mice were monitored and kept in ranges of 37.0°C±0.5°C and >450±100 beats per minute, respectively. The mouse was placed in supine position on adjustable rail to allow coordination of the ultrasound transducer. The placement of the transducer was manipulated to obtain multiple views, such as parasternal LV long and short axis, apical four-chamber and suprasternal views. Parasternal long axis views were obtained by aligning the scan head at 30-45 degrees counter clockwise to the head of the mouse. The images obtained included LV long axis B mode, left atrium M-mode, tissue Doppler imaging of LV and pulsed wave doppler of pulmonary venous flow. Parasternal short
axis B mode images were acquired by rotating approximately 90 degree clockwise from the parasternal long axis view. Pulse wave doppler images of the mitral valve, showing an apical four chamber view, were obtained by moving the scan head transverse at the lower left side of the thorax. Aortic valve velocity was obtained via a pulse wave doppler image in suprasternal view.

**Necropsy of control and CBK mice**

Mice were anesthetized with 2% isoflurane in an oxygen mix and injected with 4 IU/g heparin. After 5 minutes, the carotid artery was cut and plasma from the blood was collected. The left ventricle (LV) was injected with 2 ml cardioplegic solution to arrest the heart in a diastolic state, the whole heart and lungs were then removed. The right ventricle and LV were separated. Lungs were removed and all masses were recorded. The tibia was removed and placed in 1.5 M potassium hydroxide to digest for 24 hrs and the length was measured with a vernier caliper. The LV was cut into 3 sections, with the middle being stored in 10% formalin for 24 hours, then preserved in 70% ethanol for histological analysis and the remaining LV sections are snap frozen and stored at -80 C° for further cellular and molecular analysis.

**LV wheat germ agglutinin staining**

To analyze myocyte area, wheat germ agglutinin (WGA) staining was performed. Formalin-fixed, paraffin-embedded LV blocks were sectioned at 5 μm thickness, deparaffinized and rehydrated, as previously described (26). Alexa Fluor 488-conjugated WGA (Invitrogen) solution was added to the tissue in 1:1000 dilution for 1 hr. Samples were then washed with PBS and mounted with pro-longed gold antifade reagent (Invitrogen). Myocyte area and cardiac fibrosis were quantified from 5-6 high-
power fields per section using Image J software (NIH). Data for each group were calculated from 30 cardiomyocyte section and 4-5 mice/group.

LV picrosirius red staining

For picrosirius red (PSR) staining, paraffin-embedded, unstained sections of LV tissue for control and CBK mice were deparaffinized in citrusolv and rehydrated through subsequent washes of ethanol. After a wash with water, phosphomolybdic acid 0.2% aqueous was added to the slides. Another subsequent wash with water was followed by addition of sirius red, 0.1% in saturated picric acid (26357-02) and then application of 0.01 N hydrochloric acid. Following this step, the slides were dehydrated and then mounted using permount. The slides were allowed to dry for image analysis. The collagen staining in polarized light was determined using Image Pro Premier 64-bit software.

RT² profiler inflammatory and ECM PCR array

Frozen samples of LV tissue from CBK and control mice were used for RNA extraction. 4-8 mg of LV tissue was homogenized using a sonic dismembrator (Fisher Scientific Inc. USA, Amplitude: 10-100) and RNA was isolated with TRIzol as per manufacturer’s instruction. RNA concentration was determined using the ND1000 nanodrop and cDNA synthesis was performed with a RT² first strand kit using 0.5 µg RNA per sample. Each sample was prepared on a RT²-PCR plate for inflammatory genes (Inflammatory Cytokine and Receptor by Qiagen PAMM-011E) and extracellular matrix genes (Mouse ECM & Adhesion molecules by Qiagen PAMM-011E), then ran on an ABI 7900HT. Gene levels were normalized to hypoxanthine phosphoribosyltransferase 1 (HPRT-1) as the housekeeping gene control. The results were reported as $2^{-ΔCt}$ values.
RT-PCR for measurements of resolving gene transcripts

For qPCR, reverse transcription was performed with 2.5 μg of total RNA using SuperScript® VILO cDNA Synthesis Kit (Invitrogen, CA, USA). Quantitative PCR for pro-resolving (hmox-1, alox-5, ptgs-1) and pro-inflammatory (alox-12 and alox-15) genes was performed using taqman probes (Applied Biosystems, CA, USA) on master cycler ABI, 7900HT. The mRNA expression was normalized with the reference genes (β-Actin). The results were reported as $2^{-ΔCt}$ ($ΔΔCt$) values. All the experiments were performed in duplicates with n=3-4 mice/group.

LV protein extraction and Immunoblotting

The LV tissue from CBK and control mice was homogenized in reagent A (1xPBS from Invitrogen, without calcium and 1x proteinase inhibitor). Homogenates were centrifuged at 14,000 rpm for 5 minutes at 4°C and supernatant was snap frozen in liquid nitrogen and used as the fraction A soluble protein. The pellet was re-suspended reagent B (Reagent 4 from Sigma and 1xPI). The new homogenous solution was snap frozen and used as fraction B insoluble protein. Total protein content was determined using the Bradford assay (26). A total of 10 μg protein was loaded, electrophoresed on Criterion XT Bis-Tris 4-12% 18-gel, MOPs Buffer and transferred to a nitrocellulose membrane using material from BioRad. The blots were probed with primary antibody (COX-2 (abcam) 1:1000, 5-LOX (abcam) 1:1000, HO-1 (Enzo lifescience) 1:2000, Collagen I(abcam) 1:1000, Collagen III (abcam) 1:1000, TIMP-1(abcam) 1:1000, MMP-9 (abcam) 1:1000, phospho-Smad2/3 (Ser465/467) 1:1000 and total smad2 (millipore 1:1000) overnight at 4°C followed by secondary antibody (Biorad). The blots were stripped using stripping buffer (ThermoFisher, cat no. 46428) and re-probed for β-actin and total-
smad2 as loading control. The proteins were detected using the femtochemiluminescence detection system (Pierce Chemical, Rockford, IL, USA). Densitometry was performed using Image J software.

**Statistical analysis**

Data are expressed as mean and SEM. Statistical analyses were performed using Graphpad Prism 5. Analysis of variance (ANOVA) followed by Newman–Keuls post-hoc test was used for multiple comparisons. All immunoblotting densitometry data was normalized to β-actin/total protein lane. For 2 group’s comparison, the student-t test (unpaired) was applied. p<0.05 was considered as statistically significant.

**Results**

**CBK mice develop age-related cardiac hypertrophy**

To determine if CBK mice develop age-dependent changes in LV mass, we measured the weights of the right and left ventricles at 8 and 28 weeks. At 28 weeks of age, CBK mice had higher RV mass, RV/body weight and larger LV mass/ body weight and LV mass/tibia ratios than littermate control 28 week old controls (Table 1); in contrast, no genotype-dependent effects were observed at 8 weeks of age. LV cardiac hypertrophy was next assessed by measuring myocyte area cross-sections using WGA staining. Myocyte area was not different between control and CBK mice at 8 weeks of age (Figure 1a and b). However, at 28 weeks of age, CBK mice showed a 3 fold increase in the surface area of myocyte, indicative of cardiomyocyte hypertrophy (Figure 1a and b).

**CBK mice develop age-dependent diastolic dysfunction**

CBK mice have been reported to develop an age-dependent dilated cardiomyopathy (initially observed after 28 weeks of age), with a shorter life-span compare to control
mice (42). To determine whether development of diastolic dysfunction occurred at a
cything age in CBK mice, we measured diastolic function in CBK and control mice
using doppler echocardiography at 8 and 28 weeks. At 8 weeks of age, no significant
differences were observed for markers of diastolic function between control and CBK
mice (Table 2). However, at 28 weeks of age, CBK mice showed changes in several
key functional parameters, including increased mitral valve E/A ratio, abnormal diastolic
septal annular wall motion and reduced pulmonary venous flow (Table 2; Figure 1c).
Mitral valve E/A ratio was ~1.5 in control mice at both 8 weeks and 28 weeks, in
comparison, the E/A ratio was progressively increased >2 from 8 weeks to 28 weeks in
CBK mice. The 28 weeks old CBK mice showed increased left atrium diameter and
increase in left atrium filling pressure compared to both age-matched control indicating
multiple signs of diastolic dysfunction. End diastolic volume (EDV) and end-systolic
volume (ESV) were increased and ejection fraction (EF; p<05) was significantly lower
for CBK mice at 28 weeks of age compared with all other groups, implying a diastolic
dysfunction and clear indication of systolic dysfunction in aging prone CBK mice.

**CBK mice exhibit interstitial and endocardial fibrosis at 28 weeks of age**

Since diastolic dysfunction and cardiac hypertrophy primarily contribute to stiffening of
the left ventricle, we next examined the intensity of fibrosis in control and CBK hearts at
8 weeks and 28 weeks of age. Myocardial collagen content was slightly higher at 8
weeks of age in CBK, compared to control, examined using picrosirius red staining
(Figure 2a, PSR images top panel, and 2b % stained area lower panel). However, at 28
weeks of age, CBK mice showed even greater interstitial and endocardial ECM
deposition than age matched control, as shown in PSR stained LV images and
quantification of stained areas (Figure 2a, 2b, and 2c). At 28 weeks of age, CBK mice also displayed a clear hypertrophic response with larger dilation compared to control mice, as shown by representative 1.25x LV middle pieces (Figure 2d). Along with 1.25x magnification and 40x magnification H&E images are shown for 28 week old mice to indicate the hypertrophy of the cardiomyocytes in CBK mice (Figure 2d) at 28 week but not at 8 weeks. Further, LV sections at 28 weeks of age were also imaged using polarized light, where large collagen fibers appear yellow/orange, and thinner fibers appear green (20). Plane polarized light images showed that CBK and control mice collagen deposition varied in thickness, with CBK displaying elevated levels of the thicker, orange tinted type I collagen fiber and control showing more of the thinner, green/yellow type III collagen fibers (Figure 2c bottom panel). The orange collagen fibers in CBK mice at 28 weeks of age appeared to be denser compared with age-matched control controls indicating an accelerated fibrotic response.

CBK mice displayed pathological remodeling of ECM transcripts

Data presented thus far suggest that ventricular hypertrophy in CBK mice, indicated by an increase in size, shape, mass and LV dysfunction, is associated with the changes in collagen density. Thus, to elucidate the mechanism which initiated the reactive or reparative fibrosis in CBK hearts, we performed an 84 gene ECM array at 8 and 28 weeks for control and CBK mice. At 8 weeks, none of the 84 genes were statistically different indicating reparative or adaptive fibrosis (supplementary table 1). However, at 28 weeks, CBK mice showed a robust upregulation in 20 genes, indicating reactive fibrosis (Figure 3a). The upregulation of MMP-9, MMP-13, MMP-14 and MMP-1a is consistent with a rapid alteration of the collagen weave, known to lead to increased...
stiffness, muscle fiber slippage, and an increase in chamber size. Further activation of
genes encoding for collagen types I-V (Col1a1, Col2a1, Col3a1, Col4a1, Col4a2, Col4a3,
Col5a1) in CBK mice suggested the presence of active interstitial fibrosis. Furthermore,
increased Timp-1 and Timp-3, along with MMPs, in CBK mice suggests a potential
stimulation of feedback loop. The upregulation of Icam-1, Thbs3, Vcan, Itgax, Lamc1,
Tnc and Emilin-1 indicates an activated reparative fibrosis (Figure 3b). Thus, our data
suggest a hypertrophic and fibrotic response in CBK mice at 28 weeks, which overlaps
with myocyte hypertrophy and accelerated ECM deposition indicating signs of early
cardiac aging.

**CBK mice evidenced the activation TGF-β pathway**

Upregulation of TGF-β isoforms, and activation of the Smad2/3 and Smad1/5 pathways,
is typically observed during the cardiac fibrotic response (40). Thus, we investigated the
activation status of Smad2/3 in CBK hearts. Phosphorylation of Smad2/3 at serine
465/467 was increased in LV of CBK mice at 28 weeks of age (figure 3c and d),
suggesting TGF-β activation. In addition, we also observed phosphorylation of Smad2/3
at 8 weeks in CBK mice indicating activation of Smad2/3 occurs as an earlier event prior
to reparative fibrosis. Thus, an early event in the progression of LV remodeling and
development of dilated cardiomyopathy in CBK hearts appears to be Smad2/3
activation and initiation of fibrosis.

**Aging prone CBK mice showed accelerated ECM remodeling**

CBK mice displayed higher collagen content, accelerated fibrosis and ECM gene
response in mRNA levels, which was initiated as reparative fibrosis by 8 weeks of age,
and progressed to reactive pathological fibrosis by 28 weeks of age. In order to confirm these changes at the protein level at 28 weeks, we performed immunoblotting. Protein analysis revealed an increase in the expression levels of collagen I & III in CBK hearts (Figure 4a-e). Similarly, matrix metalloproteinase-9 (MMP-9) with TIMP-1 were increased in CBK hearts at 28 weeks of age.

**CBK hearts showed impaired resolution of inflammation with aging**

Given that cardiac dysfunction is often associated with delicate imbalances in pro-inflammatory and resolution of inflammation mechanisms, an array of inflammatory transcripts were determined in control and CBK hearts at 8 and 28 weeks of age. Inflammatory cytokine and receptor array showed that at 8 weeks there were only 3 genes i.e.; *Il2rb, Il6rb, spp1* (all p<0.05) increased in CBK mice and 1 gene decreased i.e. *Mif* (p<0.05) (supplementary table) compared to age matched control (supplementary table 2). These changes revealed no signs of inflammation noticed at 8 weeks. In contrast, by 28 weeks of age, several inflammatory pathways were upregulated in CBK mice compared with control (Figure 5a). The CBK mice showed several genes commonly involved in chemokine binding, neutrophil chemotaxis, T cell migration and leukocyte chemotaxis (*Cxcl1, Cxcr2, Ccr2, Ccl6, Cxcl5*) which were significantly upregulated. Simultaneously, the gene markers for lymphocyte proliferation (*Ccr7, Ccr5, and CXcl13*) were down regulated in CBK mice. Thus, dysregulation of several cytokines/chemokines and adhesion molecules genes indicates that Bmal1 deletion developed unresolved and chronic inflammation in CBK mice at 28 weeks, and aggravated ECM gene response. At 28 weeks of age, CBK mice showed rapid increase in pro-inflammatory cytokines (i.e. *ccl8* and *il-1β* increased, without change in *il-10* and
il-13), which are differentiate cytokines for proresolving genes (Figure 5c) indicating defective resolution axis. Bmal1 deletion in CBK mice triggers a defective inflammation resolution axis and signs of early cardiac aging, and adverse ECM remodeling, thereby impaired LV function. Thus, to understand how Bmal1 deletion affects the inflammation-resolution axis at 28 weeks, we determined mRNA levels of resolving genes. The CBK mice showed a significant decrease in pro-resolving transcripts of Hmox-1, Alox5, and Ptgs-2 (3, 17, 19) with no change in levels of inflammation promoting transcripts Alox12 and Alox15 (21) compared with age-matched controls (Figure 6a-b). Further, our immunoblotting analyses validated that not only the mRNA levels were affected in CBK mice, but also the protein levels of 5-LOX, COX-2, and HO-1 were significantly lower in CBK mice at 28 weeks of age compared with age-matched controls (Figure 6c-d), indicating that altered circadian clock from Bmal1 deletion leads to an impaired inflammation resolution axis compared with control mice.

**Discussion**

Central and peripheral circadian clock influence multiple aspects of cellular physiology, and as a consequence, disruption of clock function invariably results in cardiovascular pathology (11, 32). In this study, we defined the role of Bmal1 in age-related LV remodeling and diastolic dysfunction, through characterization of control and CBK mice at 8 and 28 weeks of age. Importantly, the present study was aimed towards addressing the role of Bmal1 in age onset cardiac remodeling rather than time of day events. Our results highlighted that cardiac-specific deletion of Bmal1 in mice; 1) initiated dilative ventricular hypertrophy and diastolic dysfunction; 2) activated phospho-Smad2 (Ser465/467), triggered ECM responsive genes, and increased collagen deposition; and
3) impaired the inflammation-resolution axis. Thus, our study validates that deletion of Bmal1 showed early signs of cardiac aging by 28 weeks of age, indicated by LV diastolic dysfunction, with increased fibrotic response and impaired resolution of inflammation.

At the molecular level, the clock consists of rhythmic transcription, such as the core clock genes Bmal1 and CLOCK that are essential and is expressed in all mammalian cells, including cardiomyocytes (30). Germline ablation of Bmal1 results in a premature aging phenotype, as described previously, suggesting a central role of Bmal1 in lifespan and survival. A recently published study shows that cardiomyocyte specific Bmal1 deletion leads to development of dilated cardiomyopathy associated with reduced life span. (42) The present study indicates that cardiac-specific deletion of Bmal1 leads to ventricular hypertrophy by the age of 28 weeks and was initiated by reparative form of fibrosis at 8 weeks of age (triggered ECM response but not inflammatory response). In fact, CBK mice displayed lower Mif (macrophage migration inhibitory factor) expression during puberty at 8 weeks of age, but not 28 weeks of age. Mif possess oxidoreductase activity that may regulate macrophage migration and antagonizes myocardial hypertrophy, as the Mif deficient mice are prone to fibrosis and ventricular hypertrophy (22).

In CBK mice, increased LV mass to body weight ratio is indication of cardiac hypertrophy which occurs due to continuous pressure load that may transit to heart failure due to diastolic dysfunction, systolic dysfunction, or combination of both. CBK mice displayed indifferent inflammatory gene expression at 8 weeks of age but marked induction of reactive fibrosis and signs of chronic inflammatory responses at 28 weeks.
of age. These pro-inflammatory cytokines activate inhibitory mediators, such as TGF-β, that counteracts inflammation, but promotes interstitial and perivascular fibrosis. Since ventricular hypertrophy is not associated with significant cardiomyocyte loss, therefore future perspective studies are warranted to identify the mechanism related to TGF-β mediated matrix deposition. Thus, fibrotic response of ventricle is associated with early hypertrophy leading to the chamber dilation, ECM responses and systolic dysfunction in CBK mice. These subsequent changes lead to the age related LV dilative hypertrophy ultimately leading to dilative cardiomyopathy. Present findings are in consistent with an earlier study reported by Young et al (42) that CBK mice begin to develop systolic dysfunction at 20-24 weeks of age. Bmal1 plays a direct role in maintaining cardiomyocyte physiological function and a lack of Bmal1 in the heart results in development of premature and early cardiac aging. This was clearly indicated by development of the dilative cardiomyopathy at 28 weeks of age as indicated by diastolic dysfunction.

The doubling of E/A ratio in CBK mice suggested a restrictive filling pattern and LV stiffness, indicating a severe form of diastolic dysfunction (44). The CBK mice also showed an increase in left atrium size and decrease in S wave velocity confirmed an increased left atrial filling pressure with increase in E/E’ ratio which indicated early development of dilative cardiomyopathy. Lefta et al has shown that the genetic deletion of Bmal1 leads to dilative cardiomyopathy, indicating the importance of Bmal1 in cardiac pathology (25). Our study demonstrated a direct and definitive role Bmal1 in development of dilative cardiomyopathy. Cardiac specific Bmal1 deletion implicates the mechanical input that transduces into a biochemical event and modifies fetal gene
transcription triggering to ECM response. As Bmal1 is a transcriptional regulator and regulates many post translation modification (14), we observed that the deletion of Bmal1 led to minimal phosphorylation of SMAD2 at serine (465/467) at 8 weeks which is then amplified at 28 weeks of age. The multiple tyrosine-phosphorylated kinases and serine-threonine kinases implicated in the signaling of hypertrophy found in the ECM (24) (37). Several pieces of evidence suggest that the disruptions of cell-cell and cell-ECM contact are sufficient in itself to modulate changes which lead to ventricular hypertrophy. Bmal1 deletion leads to early cardiac aging and development of cardiovascular disorders. The role of TGF-β/ SMAD signaling has been well demonstrated in the ECM deposition and fibrotic response (40). Ventricular hypertrophy lead to increase in collagen production and pathological collagen deposition, which is characterized by both perivascular and interstitial fibrosis (4) (39). These studies linked a direct role of Bmal1 in controlling ECM genes, thereby leading to the deposition of interstitial and endocardial fibrosis. Increase in expression of the transcript levels of Col1a1, Col2a1, Col3a1 Col4a1, Col4a2, Col4a3, and Col5a1 along with Lcam-1, Thbs3, Vcan, Itgax, Lamc1, Tnc and Emilin-1 indicated an activated fibrotic response in 28 week old aging prone CBK mice. Thus, our study has defined the role of disturbed Bmal1 signaling in ventricular remodeling in the heart and presents the notion that Bmal1 has a control on multiple factors which regulate ECM remodeling and age-related inflammation.

Persistent, ungoverned, inflammatory microenvironment drives myocardial infarction, and results in diurnal clustering in humans (28). Further, continuous, chronic slow grade inflammation leads to accelerated aging (8). Previous studies have demonstrated
chronic inflammation leads to progressive development of reparative to reactive fibrosis (13). A recent study by Nguyen et al (29) demonstrated the Bmal1 regulates the oscillation of inflammatory Ly6c\textsuperscript{hi} monocyte, which potentiates the chronic inflammatory response both locally and systemically. This potentially leads to chronic inflammatory diseases, such as myocardial infarction leading to heart failure. Predominantly, CBK mice show one of the most prevailing phenotypes of early cardiac aging with chronic inflammation (6, 23). Adrenal specific Bmal1 knock out mice exhibits a pulsating plasma corticosterone secretion, while mice with a Bmal1 deficiency exclusively within pancreatic β cells display hypoinsulinaemia and diabetes (27, 36). Not only cardiomyocyte but also immune cells i.e. particularly leukocytes are also influenced by sleep duration and sleep-wake up cycle which peaks in the circulation during the resting phase and decrease during the active period. The pro-inflammatory cytokines tumor necrosis factor (TNF) and interleukin-1β (IL-1β) peak during the onset of the active phase implicating association of circadian rhythms and the onset of diseases (16). Recent studies have shown that the mice with disrupted circadian clock expressed substantially lower levels of Ccl2 mRNA compared with wild type mice(35). The studies also showed oscillations in the serum levels of IL-6, IL-12, CCL2, CCL5 and CXCL1 when the mice are stimulated with lipopolysaccharide (LPS), however these oscillation are absent in Bmal1\textsuperscript{-ma} macrophages and Nr1d1\textsuperscript{-r1}mice (35). As leukocytes are known to display higher phagocytic ability and cytotoxicity during the active phase the disruption of the circadian clocks leads to acute inflammatory insults (2) (18). Moreover, after myocardial injury diurnal rhythm disruptions alters neutrophils and macrophages kinetics and delays the resolving phase (1). In consistent with previous study, the
current report also displayed that the disruption of cardiomyocyte-specific Bmal1 gene displayed chronic inflammation at the age of 28 weeks. Significant increase in the pro-inflammatory transcripts viz ccl2, il-1β, ccl8 and ccr2 (neutrophil chemotaxis and leukocyte migration genes), while no change in the proresolving/alternative transcript il-10 and il-13 in CBK mice indicates impair defensive mechanism to repair inflammation at 28 weeks of age. This was further supported by decreased levels of 5-LOX, COX-2, and HO-1 (3, 17, 19) impairing the resolution of inflammation axis. Thus, age-related defective resolution of inflammation and dilative ventricular dysfunction leads to reduced survival (42) with marked cardiomyopathy.

In summary, the current study reveals diastolic dysfunction, ventricular hypertrophy and cardiac pathology in CBK mice. Thus, genetic ablation of Bmal1 in the cardiomyocyte leads to dilative cardiomyopathy through Smad2/3 that triggered fibrosis, stimulated inflammation and impaired the resolution of inflammation.

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Figure legends

**Figure 1. CBK mice promoted age on set dilative hypertrophy and diastolic dysfunction.**
a) Images of wheat germ agglutinin (WGA) at 8 and 28 weeks representing increase in cardiomyocyte size indicating age-associated hypertrophy at 28 weeks in CBK mice (scale bars: 10 μm). b) Quantitation of cardiomyocyte area/section/mouse indicated hypertrophic response at 28 weeks of age in CBK mice. *p<0.05 vs age-matched control (CON); n=5 mice/group. c) Representative echocardiographic images with abnormal diastolic septal annular wall motion indicating diastolic dysfunction in CBK mice at 28 weeks compared with age-matched control.

**Figure 2. CBK mice triggered collagen deposition and dilation in LV compared to control.**
a) LV middle regions images (40x magnification) stained with picrosirius red (PSR; at 8 and 28 weeks for WT and CBK mice indicated higher interstitial collagen deposition in CBK mice at 28 weeks. Intense red color indicates collagen deposition. b) Quantification of collagen density in CBK mice compared with control mice at 28 weeks of age 4-5 images/mouse, n=5 mice/group. c) 10x magnification images of PSR stained 28 week WT and CBK mice shows interstitial and endocardial fibrosis (black arrows, top panel) Under plane polarized light endocardial fibrosis is marked by an orange color (white arrows, bottom panel). d) Hematoxylin and eosin stained LV middle cavity (1.25x magnification) indicates hypertrophy and dilation at 28 weeks compared with age-matched control. H&E LV staining indicates dilative hypertrophy at 28 weeks of age (40x images). *p<0.05 vs age-matched control (CON)

**Figure 3. Aging prone CBK mice stimulated early ventricular fibrotic response and activated phosphorylation of Smad2 at 28 weeks of age.** a) Venn diagram presenting changes in ECM and cell adhesion genes expression in LV of CBK mice compared to age matched control littermate control. Black color genes indicate no change in gene expression. Red color genes indicate an increased expression and blue color genes indicates decreased in gene expression compared to control littermate control. p<0.05 for all the increased and decreased genes compared with age-matched control. b) Change in ECM mediated pathways at 28 weeks of age in CBK mice. c) Immunoblot representing phosphorylation of smad2/3 (ser465/467) in control and CBK mice. d) Densitometry analysis of phospho-Smad2/3 control and CBK mice in 8 and 28 weeks. *p<0.05 vs age-matched control. $p<0.01 vs 8 week old CBK mice.

**Figure 4. Aging prone CBK mice showed accelerated extracellular matrix remodeling.** a) Immunoblot representing increase in expression of MMP-9, TIMP-1, collagen I and III in LV of
CBK mice compared with littermates control at 28 weeks of age. b) Densitometric analyses of MMP-9, TIMP-1, collagen I and III, β-actin was used as loading control. *p<0.05 vs age-matched control; n=5 mice/group.

**Figure 5. CBK mice displayed higher inflammation than age-matched control.** a) Venn diagram presenting change in inflammatory cytokines and receptor genes expression in LV of CBK mice at 28 weeks of age compared with age-matched control littermate control. Black color genes indicate no change in expression. Red color genes indicates an increased in expression and blue color genes indicates decreased in expression. *p<0.05 for all the increased or decreased genes compared with age-matched control. b) Change in inflammation mediated pathways at 28 weeks of age in CBK mice. c) Bar graph depicting change in pro-inflammatory and proresolving gene at 28 weeks of age in CBK mice compared with control. *p<0.05 vs age-matched control; n=4 mice/group.

**Figure 6. CBK mice develop a defective inflammation-resolution axis.** mRNA expression of a) Alox-5, Hmox-1, ptgs-2. b) Alox-12, Alox-15. c) Immunoblot representing 5-LOX, COX-2 and HO-1 in control and CBK mice at 28 weeks of age. d) Densitometry analyses of 5-LOX, COX-2 and HO-1. β-actin was used as loading control. *p<0.05 vs age-matched control; n=5 mice/group.

**Figure 7.** Schematic design showing altered circadian rhythm in CBK mice lead to an early age on set hypertrophy and fibrosis accompanied by diastolic dysfunction leading to defective resolution of inflammation.
Figure 1.

(a) 8 weeks  28 weeks

CON

CBK

(b) 3000 2000 1000 0

square μm

Control  CBK

8 weeks  28 weeks

(c) 28 weeks

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**Note:** The figure compares the progression of conditions over 8 and 28 weeks, showing differences in tissue samples labeled as CON and CBK. The histogram illustrates a significant increase in square μm for CBK compared to Control at 28 weeks.
Figure 2.

### LV interstitial fibrosis

|       | Control | CBK |
|-------|---------|-----|
| 8 wks |         |     |
| 28 wks|         |     |

### LV endocardial fibrosis

- **a**: Images showing interstitial fibrosis at 8 and 28 weeks for Control and CBK groups.
- **b**: Graph showing % Red stained area with significant increase in CBK group.
- **c**: Images showing endocardial fibrosis with arrows indicating fibrous tissue.
- **d**: Comparative images of Control and CBK groups at 8 and 28 weeks.
Figure 3.

| Biological pathway                        | Gene altered                                                 | P value   |
|------------------------------------------|--------------------------------------------------------------|-----------|
| Collagen metabolic response              | MMP-9, MMP-13, Col1a1, Col5a1, Col3a1                       | .000011   |
| Collagen biosynthetic process            | Col4a1, Col5a1, Col3a1                                       | .0000131  |
| Extracellular matrix part                | Emilin-1, Col4a1, Col4a2, Col4a3, Col2a1, Col1a1, Col5a1, Col3a1, Timp-1, Timp-3, MMP-13, Tnc, Vcan, Icam1 | .000001   |
| Platelet derived growth factor binding   | Col4a1, Col2a1, Col1a1, Col5a1, Col3a1                       | .0003211  |
| Negative regulation of proteolysis       | Timp-1, Timp-3                                              | .0000211  |
| Wound healing                            | Col4a1, Col5a1, Col3a1                                       | .0000142  |
Figure 4.
Figure 5.

| Biological pathway          | Gene altered                  | P value  |
|-----------------------------|-------------------------------|----------|
| Chemokine receptor binding  | Cxcl1, Cxcr2, Ccr2, Ccl6,     | .000112  |
|                             | Cxcl5, Cxcl13, Cxcl31,       |          |
|                             | Ccr7, Cd12, Cd17             |          |
| Neutrophils chemotaxis      | Ccr7, Ccl2, Cxcl1, Cxcl2,    | .000069  |
|                             | Cxcr2, Ifng                  |          |
| Chemokine activity          | Ccl1, Ccl6, Cxcl5, Cxcl13    | .000212  |
| Cell chemotaxis             | Cxc2, Ccl6, Cxcl13, CC7,     |          |
|                             | Cd12, CC17, Ifng, Pf4        |          |
| Leukocyte migration         | Ccr7, Cxcl13, Cdl2, Cc19,    | .000081  |
|                             | Ccl2, Ccl7, Cxcl1, Pf4,      |          |
|                             | Cxcl12, Cd17, Ccr2, Cxcl2,   |          |
|                             | Cxcl1, Cxcr2, Ifng           |          |
| Lymphocyte chemotaxis       | Ccr7, Cxcl13, Ccl17, Ccr2,   | .000111  |
|                             | Cd12, Cd31                   |          |
| Lymphocyte proliferation    | Ccr7, Ccr5, Cdl2, Ifng       | .000001  |
| T cell migration            | Ccr2, Cxcl13, Ccr2, Cxcl5    | .000037  |
**Figure 6.**

*Inflammation resolving transcripts*

**a**

![Graph: Alox5, Hmox-1, Ptgs-2](image)

**Inflammation promoting transcripts = no change**

**b**

![Graph: Alox12, Alox15](image)

**c**

![Images: 5-LOX, COX-2, HO-1, Actin](image)

**d**

![Graph: 5-LOX, COX-2, HO-1](image)
Metabolic disorder
(e.g. Obesity, diabetes)

Environmental stress
(e.g. Night shifts/ altered sleep-wake cycle)

Altered Circadian
Clock
Bmal\(^{-/-}\)

Asymmetric
ECM Remodeling

Impaired
Inflammation-Resolution Axis

Diastolic dysfunction in aging prone CBK mice
Table 1. Necropsy parameters at 8 weeks and 28 weeks in control and CBK mice.

| Groups                     | Control          | CBK             |
|----------------------------|------------------|-----------------|
|                            | 8 weeks (n=6)    | 28 weeks (n=12) | 8 weeks (n=5) | 28 weeks (n=10) |
| Body Weight (g)            | 26±1             | 32±1            | 25.8±0.8       | 28.9±0.9*       |
| Tibia (mm)                 | 17±0.1           | 18±0.1          | 17±0.1         | 18±0.2          |
| Spleen (mg)                | 73±7             | 90±13           | 75±8.6         | 67±3            |
| Right ventricle (RV; mg)   | 18±1             | 24±1            | 22±1           | 26±2            |
| Left ventricle (LV; mg)    | 83±2             | 97±3            | 96±3*          | 109±8           |
| RV/BW                      | 0.70±0.03        | 0.77±0.04       | 0.86±0.04      | 0.91±0.07       |
| LV/BW                      | 3.1±0.07         | 3.0±0.09        | 3.7±0.05       | 3.82±0.31*      |
| Lung-wet weight (mg)       | 155±13           | 167±12          | 144±7          | 180±9           |
| Lung-dry weight (mg)       | 29±2             | 35±2            | 31±2           | 37±3            |
| LV/Tibia                   | 4.8±0.1          | 5.6±0.2         | 5.4±0.2        | 6.1±0.4         |

Data are mean ±SEM
*p<0.05 vs WT
BW: body weight, LV: left ventricle, RV: right ventricle, control; littermate, CBK: Cardiomyocyte-specific Bmal-1 deleted knockout.
Table 2. Summary of diastolic function measurements using pulse wave and tissue doppler echocardiography in control and CBK mice at 8 and 28 weeks of age.

| Groups                              | Control       | CBK           |
|-------------------------------------|---------------|---------------|
|                                     | 8 weeks (n=6) | 28 weeks (n=7) | 8 weeks (n=5) | 28 weeks (n=5) |
| E/A                                | 1.69±0.07     | 1.55±0.05     | 2.06±0.17     | 2.74±0.79*     |
| Isovolumic relaxation time (ms)     | 14.6±1.6      | 15.0±1.5      | 20.4±9        | 18.1±2.1       |
| Isovolumic contraction time (ms)    | 16.0±1.0      | 18.0±1.6      | 16.0±1.0      | 17.0±2.3       |
| Left atrium diameter (mm)           | 2.4±0.2       | 2.5±0.07      | 2.4±0.13      | 3.0±0.33*      |
| E'/A'                               | 1.44±0.03     | 1.40±0.04     | 1.46±0.02     | 1.5±0.11       |
| E/E'                                | 29.2±2.1      | 31.3±3.1      | 40.1±3.6      | 38.0±5.6       |
| Pulmonary venous flow- systole      | 237±33        | 257±22        | 257±28        | 153±55*        |
| Pulmonary venous flow- diastole     | 475±14        | 489±41        | 510±39        | 264±88*        |
| Pulmonary venous flow- AR           | 143±21        | 122±12        | 117±14        | 100±21         |
| Pulmonary venous flow- AR duration  | 19±1          | 21±1          | 17±1          | 22.7±2         |
| End diastolic volume, µl            | 71±4          | 74±4          | 77±7          | 112±32*        |
| End systolic volume, µl             | 32±4          | 32±6          | 37±4          | 85±31*         |
| Ejection fraction, %                | 56±3          | 58±5          | 52±3          | 28±7*          |

Data are mean ±SEM, WT; Control: littermate control type, CBK; Cardiomyocyte-specific Bmal-1 deleted knockout; E, velocity of early mitral flow; A, velocity of late mitral flow; E’, Early peak velocity of septal annulus; A’ late peak velocity of septal annulus; E/E’ index of left atrial filling pressure; AR, *p<0.05 vs WT