Development of dilated cardiomyopathy in Bmal1-deficient mice

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1Center for Muscle Biology, University of Kentucky, Lexington, Kentucky; 2Department of Physiology, College of Medicine, University of Kentucky, Lexington, Kentucky; and 3Department of Physiology, Wayne State University School of Medicine, Detroit, Michigan

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Lefta M, Campbell KS, Feng H, Jin J, Esser KA. Development of dilated cardiomyopathy in Bmal1-deficient mice. Am J Physiol Heart Circ Physiol 303: H475–H485, 2012. First published June 15, 2012; doi:10.1152/ajpheart.00238.2012.—Circadian rhythms are approximate 24-h oscillations in physiology and behavior. Circadian rhythm disruption has been associated with increased incidence of hypertension, coronary artery disease, dyslipidemia, and other cardiovascular pathologies in both humans and animal models. Mice lacking the core circadian clock gene, brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein (Bmal1), are behaviorally arrhythmic, die prematurely, and display a wide range of organ pathologies. However, data are lacking on the role of Bmal1 on the structural and functional integrity of cardiac muscle. In the present study, we demonstrate that Bmal1−/− mice develop dilated cardiomyopathy with age, characterized by thinning of the myocardial walls, dilation of the left ventricle, and decreased cardiac performance. Shortly after birth the Bmal1−/− mice exhibit a transient increase in myocardial weight, followed by regression and later onset of dilation and failure. Ex vivo working heart preparations revealed systolic ventricular dysfunction at the onset of dilation and failure, preceded by downregulation of both myosin heavy chain isoform mRNAs. We observed structural disorganization at the level of the sarcomere with a shift in titin isoform composition toward the stiffer N2B isoform. However, passive tension generation in single cardiomyocytes was not increased. Collectively, these findings suggest that the loss of the circadian clock gene, Bmal1, gives rise to the development of an age-associated dilated cardiomyopathy, which is associated with shifts in titin isoform composition, altered myosin heavy chain gene expression, and disruption of sarcomere structure.

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we did not detect a change in passive stiffness at the level of the single cardiomyocyte. Systolic performance is decreased before the overt development of DCM in the Bmal1−/− mice, as shown by ex vivo functional measurements of isolated working hearts. Together, these findings suggest that loss of Bmal1 results in an age-associated pathology in the heart that shares some, but not all, characteristics with DCM.

MATERIALS AND METHODS

All animal procedures were conducted in compliance with the guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care and were approved by the Institutional Animal Care and Use Committees at University of Kentucky and Wayne State University.

Animals. The germline Bmal1−/− mice were previously backcrossed over 10 generations to the C57BL6 background (2). Both male and female mice are infertile (1), so the Bmal1−/− and wild-type mice for this study were littermates from heterozygote (Bmal1+/−) breeding. Genotypes were determined as previously described (7). Mice were housed four per cage and kept on a 14-h:10-h light-dark schedule with ad libitum access to food and water.

Echocardiography. Mice were placed on a heated platform set at 37°C and anesthetized with isoflurane gas, and transthoracic measurements were taken using a VisualSonics Vevo 660 with a RMV 707 30-MHZ probe. M-mode images were acquired from the parasternal short-axis view at the papillary muscle level. Data analysis was performed with the use of the Vevo 660 analytic software.

Cardiomyocyte isolation. Cardiomyocytes were isolated as previously described (45a, 68). The composition of all buffers is identical to those of O’Connell et al. (45a), except that trypsin was not included in the digestion buffer. Briefly, mice were euthanized by cervical dislocation, the heart was carefully excised, and the aorta was cannulated. The heart was hung in a Langendorff apparatus and perfused retrogradely with aerated (95% O2-5% CO2, 37°C) perfusion buffer, followed by perfusion with digestion buffer until the heart was pale and swollen. Following digestion, the ventricles were minced, resuspended in digestion buffer, and triturated to create a cell suspension. An equal amount of Stop-1 solution was added and the cardiomyocytes were allowed to settle into a pellet. The pellet was resuspended in Stop-2 solution, and the isolation was considered successful after about 65% of the cells were rod shaped. Isolated cardiomyocytes were used for assessment of cell size or for passive tension measurements.

Cardiomyocyte size determination. Cardiomyocytes in Stop-2 solution were treated with Hoechst dye (1:1,000) for 5 min. A hundred images were taken from each heart at ×100 magnification using a Nikon Eclipse E600 light microscope, averaging 275 cells per heart. Cardiomyocyte area was measured manually using National Institutes of Health (NIH) ImageJ software (10). Cardiomyocytes were binned according to their cell area in bins of 500 μm², and histograms with the percentage of cardiomyocytes in each bin were generated using GraphPad Prism (43).

Histological sections. At 36 wk of age, mice were euthanized by cervical dislocation; the heart was excised and placed in a dish containing warm phosphate-buffered saline until it stopped beating, placed in 4% paraformaldehyde (24 h at 4°C), and then in 70% ethanol. The fixed hearts were embedded in paraffin, and 5-μm sections were taken starting at the papillary muscle level. Sections were stained with Masson’s Trichrome (Sigma-Aldrich, St. Louis, MO). Low-magnification images (×20) were taken using a Nikon Eclipse E600 light microscope. Left ventricular wall thickness and area were obtained manually using NIH ImageJ software (10).

Electron microscopy. Mice were anesthetized with ketamine-xylazine, the chest cavity was opened, and the heart was exposed. Perfusion through the left ventricle was started first with phosphate-buffered saline (pH 7.4), followed by cold 2% paraformaldehyde-4% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and 130 mM NaCl. The perfusion-fixed hearts were then taken to the electron microscopy core facility at the University of Kentucky for processing and sectioning (14). Images were obtained using a Philips BioTwin 12 transmission electron microscope.

Real-time PCR. Total RNA was extracted from 4-wk-old wild-type and Bmal1−/− hearts using TRIZol (Invitrogen). Isolated RNA was quantified using the Nano Drop 2000 spectrophotometer (Thermo Scientific). First-strand cdNA synthesis was done using SuperScript III first-strand synthesis supermix kit (Invitrogen). Real-time PCR was performed using TaqMan probe-based chemistry (Applied Biosystems) and conducted in an ABI 7700 sequence detector. Probes and primers for Gapdh, Myh6, and Myh7 were purchased from Applied Biosystems. After normalization to Gapdh [change in cycle threshold (ΔCt)], gene expression was reported relative to the average expression for that gene in the wild-type group at 4 wk of age (ΔΔCt). Fold change relative to control was calculated as 2−ΔΔCt.

Sample preparation for MHC gels. MHC protein expression was performed on ventricular tissue from 4-, 12-, and 36-wk-old wild-type and Bmal1−/− mice. Control tissue for MHC-α and MHC-β was from neonatal hearts. Frozen ventricular tissue was homogenized using nine volumes of homogenizing buffer/mg tissue, consisting of (in mM) 250 sucrose, 25 NaCl, and 20 Tris (pH 7.4), modified from Talmadge and Roy (57). The homogenate was spun at 20,000 × g for 30 min at 4°C. The pellet, which was enriched in myofibrils, was resuspended in homogenizing buffer and protein concentration was determined using a Bradford assay. Sample buffer (3×), consisting of 1.15 M Tris (pH 6.8), 6% SDS, 75 mM DTT, 0.06% bromophenol blue, and 40% glycerol, was added to a final concentration of 1×. The samples were heated at 100°C for 2 min, and 0.8 μg/lane was immediately loaded on gels (57).

MHC electrophoresis and isoform determination. Gel composition and preparation were identical to Talmadge and Roy (57). The upper and lower chamber buffer composition was the same as in Reiser and Kline (49). The gels were run at 70 V constant voltage for 38 h at 4°C [modified from (57)]. MHC isoforms were visualized with a silver stain plus kit (Bio-Rad, Philadelphia, PA) and scanned using an Epson Perfection V500 photo scanner. Total MHC and the percentage of MHC-α and MHC-β were determined using NIH ImageJ software (10).

Sample preparation for titin gels. Frozen ventricular tissue was placed in sample buffer [described in Warren et al. (63), excluding bromophenol blue, buffer-to-tissue ratio 60:1 (vol/wt)]. Protein concentration of the homogenate was determined using the RC-DC protein assay (Bio-Rad). Samples were heated at 60°C for 10 min; glyceral and bromophenol blue were added to 30% (vol/vol) and 0.1% (vol/vol) final concentration, respectively. Following 10 min centrifugation at 15,000 × g, the supernatant was aliquoted and loaded on gel or stored at −80°C.

Titin electrophoresis and isoform determination. A Bio-Rad Protein II xi XL vertical electrophoresis unit was used. The gel size was 20 cm × 20 cm × 1.5 mm. The composition of the acrylamide plug, the agarose gel, and the upper and lower chamber buffers were the same as in Warren et al. (63). The gels were run at 4°C for a total of 12 h, starting at 80 V and increasing the voltage by 30 V every 100 min. After electrophoresis, the gels were stained with Sypro Ruby (Invitrogen, Eugene, OR) and scanned using a Typhoon scanner operating in fluorescence mode at 450 nm. Scanned images were analyzed using Image Quant software.

Ex vivo working heart preparation and functional measurements. Cardiac function was measured in isolated working heart preparations of 18-wk-old male wild-type and Bmal1+/− mice. As described previously (16), the mice were heparinized and anesthetized before the heart was rapidly isolated. A modified 18-gauge, 6-mm-long, thin-walled needle was used as the aortic cannula. After retrograde perfusion was established, a modified 16-gauge needle was used to cannulate the pulmonary vein for antegrade perfusion through the left atrium. A 30-gauge needle was used to puncture the left ventricle from the apex to make a path for the insertion of a 1.2-Fr pressure-volume.
was measured in single cardiomyocytes isolated from wild-type and stroke volume (\( \frac{L}{H9262 \text{ ml}} \) mg heart tissue) was calculated from the sum of development (\( \frac{H11006}{H9262} \)), and left ventricular volume. Left ventricular pressure, the maximum rate of left ventricular pressure preload of 10 mmHg and afterload of 55 mmHg for the maximum left baseline function of the isolated working hearts was measured at a PowerLab/16 SP digital data archiving system (AD Instruments).

The actual aortic and pulmonary artery flows recorded in real time by (A365, World Precision Instrument) with two microplatinum electrodes attached to the actual aortic and pulmonary artery flows recorded in real time by calibrated drop count using a pair of electrodes feeding to a PowerLab/16 SP digital data archiving system (AD Instruments). Baseline function of the isolated working hearts was measured at a preload of 10 mmHg and afterload of 55 mmHg for the maximum left ventricular pressure, the maximum rate of left ventricular pressure development (\( \pm \frac{H1006}{H9262} \text{ dP/dt} \)), and left ventricular volume. Left ventricular stroke volume (\( \mu\text{l/heart tissue} \)) was calculated from the sum of aortic flow and coronary effluents, normalized to heart rate.

**Single cardiomyocyte mechanical measurements.** Passive tension was measured in single cardiomyocytes isolated from wild-type and \( Bmal1^{+/−} \) hearts, using a method described by Warren et al. (62) with a few modifications. The composition of relaxing and pCa9 solutions was identical to Ferreira et al. (17). Cardiomyocytes were resuspended in relaxing solution and were chemically permeabilized in relaxing solution containing 0.5% (vol/vol) Triton X-100 (Thermo Scientific, Rockford, IL) for 6 min at room temperature. Cardiomyocytes were washed twice in relaxing solution and stored on ice for up to 12 h. Cardiomyocytes were attached between stainless steel insect pins extending from a motor (308B, Aurora Scientific, Ontario, Canada) and a force transducer (406, Aurora Scientific) using silicone adhesive. Once attached, the cardiomyocyte was lifted and placed in a well containing pCa9 solution at 15°C. This apparatus was placed on the stage of a Nikon TE2000-U inverted microscope with the capacity for video capturing. Experiments were performed using SLControl software (8). Each cardiomyocyte was stretched to double its resting length at a rate of 0.1 l/s, and then shortened to 50% l0 before being returned to its resting length. The SLControl software generated traces of passive tension and fiber length over the stretch time. A video of the stretch was captured for subsequent analysis. The SLControl data and the video file were transported to a MatLab program written by K. S. Campbell, which extracted the passive tension values from the SLControl data and plotted them against sarcomere length calculated from individual frames from the video file. We used 20–24 preparations from four mice from each age and genotype group. Experimental results from each group were binned according to their sarcomere lengths in bins of 0.05 \( \mu\text{m} \), and a two-way ANOVA with a post hoc Bonferroni test was run to assess significance. Additionally, passive tension traces from each cardiomyocyte were analyzed with an exponential equation: passive tension = \( \text{exp}[k(\text{SL} - \text{SL0})] \) (19), where SL is the sarcomere length, k is the exponent of stiffness, and SL0 is the sarcomere length at zero load.

**Statistical analysis.** Results from each group (Bmal1^{−/−} vs. wild-type) at each time point are reported as means ± SE. Two-way ANOVA was performed to determine whether a significant interaction existed between factors for each dependent variable under consideration. If a significant interaction was detected, Bonferroni post hoc comparisons were performed to identify the source of significance, with \( P < 0.05 \).

**RESULTS**

**Germline Bmal1^{−/−} mice develop age-associated DCM.** Echocardiogram data showed that at 4 wk of age, the germline Bmal1^{−/−} mice had bigger hearts compared with wild-type littermates, as demonstrated by a 15% increase in the estimated left ventricular weight (LVW) (129 ± 13.79 vs. 110 ± 6.57 mg) (Fig. 1A) and a 43% increase in ratio of LVW to body weight (BW) (8.46 ± 1.14 vs. 5.9 ± 0.4 mg/g, \( P < 0.001 \)) (Fig. 1B). We measured wet LVW and heart weight (HW) in a separate cohort of mice to complement echocardiographic estimates and to show that LVW was increased by 19% in the Bmal1^{−/−} mice, but this increase was not statistically significant (59.47 ± 7.40 vs. 49.98 ± 8.31 mg) (Fig. 1H). However, because of the smaller body size in Bmal1^{−/−} mice, wet LVW-to-BW ratio was significantly increased by 56% (4.74 ± 0.50 vs. 3.04 ± 0.64 mg/g, \( P < 0.05 \)) as was the HW-to-BW ratio (43.65 ± 3.14 vs. 41.05 ± 1.96%) (Fig. 1C), the interventricular septum (IVS) was slightly, but not significantly, thicker (1.62 ± 0.08 vs. 1.54 ± 0.08 mm) (Fig. 1D), and no changes were detected in the left ventricular internal diameter (LVID) (1.67 ± 0.08 vs. 1.68 ± 0.07 mm) (Fig. 1E) or left ventricular posterior wall (LVPW) thickness (Fig. 1F).

To evaluate whether the increase in LVW-to-BW ratio was associated with hypertrophy of cardiomyocytes, we isolated cardiomyocytes and measured cell area. We found that Bmal1^{−/−} hearts have a greater percentage of smaller cardiomyocytes compared with wild-type hearts (Fig. 2A). The percentage of cardiomyocytes smaller than 2.500 \( \mu\text{m}^2 \) is significantly higher in the Bmal1^{−/−} hearts (56.41 ± 3.23 vs. 38 ± 4.22 \( \mu\text{m}^2 \), \( P < 0.01 \)), whereas the percentage of larger cardiomyocytes is significantly decreased (43.59 ± 3.23 vs. 62 ± 4.22 \( \mu\text{m}^2 \), \( P < 0.01 \)) (Fig. 2B). The average cardiomyocyte area is 19% smaller in the Bmal1^{−/−} hearts (2.538 ± 470.88 vs. 3.152 ± 796 \( \mu\text{m}^2 \)) (Fig. 2C).

This increase in LVW-to-BW seen in the 4-wk-old Bmal1^{−/−} mice began to regress by 8 wk of age and declined to wild-type levels by 12 wk of age as assessed by echocardiography (4.67 ± 0.28 vs. 4.48 ± 0.25 mg/g) (Fig. 1B) and wet weight measurements (3.38 ± 0.18 vs. 3.56 ± 0.05 mg/g) (data not shown). Weeks 8–16 were characterized by a regression of LVW-to-BW ratio and myocardial wall thickness, whereas FS continued to be preserved (Fig. 1C). The IVS and LVPW started to get thinner in the Bmal1^{−/−} mice compared with wild-type mice, but these differences did not reach statistical significance by 16 wk of age (IVS, 1.36 ± 0.07 vs. 1.53 ± 0.03 mm; and LVPW, 1.13 ± 0.04 vs. 1.35 ± 0.07 mm) (Fig. 1, D and F). Additionally, the LVID was similar in wild-type and Bmal1^{−/−} hearts (Fig. 1E).

Weeks 20–36 were characterized by a significant decline in FS, indicating systolic dysfunction in the Bmal1^{−/−} mice. By 36 wk of age, FS was 24.3% smaller in the Bmal1^{−/−} mice compared with wild-type mice (24.6 ± 1.16 vs. 32.5 ± 1.06%, \( P < 0.01 \)) (Fig. 1C). During this time period, the IVS and the LVPW continued to thin and by 36 wk of age were on average 26.67 and 24.8% smaller in the Bmal1^{−/−} mice (IVS, 1.10 ± 0.05 vs. 1.50 ± 0.05 mm, \( P < 0.001 \), and LVID, 0.97 ± 0.05 vs. 1.29 ± 0.04 mm, \( P < 0.05 \)) (Fig. 1D, F and G). The LVID got progressively larger in the Bmal1^{−/−} mice during this time, becoming 17.12% larger in the Bmal1^{−/−} mice compared with wild-type by 36 wk of age (3.01 ± 0.10 vs. 2.57 ± 0.11 mm, \( P < 0.05 \)) (Fig. 1E). LVW declined during this time and by 36 wk of age was 22.33% smaller in the Bmal1^{−/−} mice (88.54 ±
3.16 vs. 114.0 ± 6.65 mg) (Fig. 1A). This was during a period of time in which body weight continued to decline (Fig. 1G), thus LVW-to-BW ratio remained unchanged as estimated by echocardiography (3.96 ± 0.20 vs. 3.68 ± 0.24 mg/g) (data not shown). It is also worth noting that body weight (19.90 ± 1.40 vs. 31.11 ± 1.40 g, \( P < 0.001 \)), wet LVW (66.67 ± 2.91 vs. 107.74 ± 9.13 mg, \( P < 0.001 \)), and wet HW (113.86 ± 3.92 vs. 157.06 ± 11.62 mg, \( P < 0.01 \)) were significantly smaller in the Bmal1/−/− mice at 36 wk of age (Fig. 1, G–I).

At 36 wk of age, we evaluated histological cross sections of the heart at the level of the papillary muscles, and this analysis supported the echocardiography data. Representative images are shown in Fig. 3, A and B. We found that the area occupied by myocardium was decreased by 21% in the Bmal1/−/− mice (5.54 ± 0.21 vs. 7.05 ± 0.57 mm², \( P < 0.05 \)) (Fig. 3C). IVS thickness was decreased by 20% (0.68 ± 0.03 vs. 0.85 ± 0.07 mm, \( P < 0.05 \)), and LVPW thickness was decreased by 31% (0.70 ± 0.03 vs. 1.02 ± 0.09 mm, \( P < 0.01 \)) in the Bmal1/−/− mice compared with wild-type controls (Fig. 3D). The left ventricular cavity area was slightly increased (3.13 ± 0.20 vs. 2.75 ± 0.16 mm²), but this difference did not reach statistical significance (Fig. 3C).

Bmal1/−/− myocardium exhibits disorganized sarcomeres. To assess whether the cardiac functional changes were associated with disruptions in sarcomere architecture, we obtained electron micrographs from wild-type and Bmal1/−/− myocardial sections at 14 wk of age. An evaluation of electron micrographs found that some regions had normal sarcomere architecture in the Bmal1/−/− myocardium, whereas other areas exhibited sarcomere disorganization, with diffuse M lines, A bands, and Z disks. We found regions where the distinction between A and I bands was diminished in the Bmal1/−/− myocardium, and this was not seen in any of the images from the wild-type hearts (Fig. 4, A and B).

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Fig. 1. Age-associated dilated cardiomyopathy development in germline brain and muscle aryl hydrocarbon receptor nuclear translocator (ARNT)-like protein-1 knockout (Bmal1/−/−) mice. Graphs show longitudinal echocardiographic changes in left ventricular (LV) mass (A), LV weight (LVW) normalized to body weight (BW) (B), fractional shortening (C), systolic interventricular septum thickness (IVS; D) systolic LV internal diameter (LVID; E), systolic LV posterior wall thickness (LVPW; F), and BW (G). Data are means ± SE; \( n = 7 \). The numbers in the white boxes denote the stage of cardiac pathology observed in that age range. Summarized data for wet LVW (H) and wet heart weight (I) from wild-type (WT) and Bmal1/−/− mice at each representative age are shown. Values are means ± SE; \( n = 4 \) (4 wk and 12 wk), 8 (18 wk), and 7 (36 wk). *\( P < 0.05 \); **\( P < 0.01 \); ***\( P < 0.001 \).
mRNA, but not protein, levels of MHC isoforms were down-regulated in the Bmal1<sup>-/-</sup> myocardium at 4 and 12 wk of age. We measured mRNA expression of MHC-α and MHC-β in the Bmal1<sup>-/-</sup> and wild-type hearts at all three ages (Fig. 5, A and B). MHC-α mRNA levels were significantly decreased by 31 and 32% at 4 and 12 wk, respectively, and were unchanged at 36 wk (Fig. 5A). MHC-β mRNA expression was significantly lower by 57 and 59% at 4 and 12 wk, respectively, but was not significantly decreased at 36 wk (Fig. 5B).

Fig. 2. Bmal1<sup>-/-</sup> cardiomyocytes are smaller than WT cardiomyocytes at 4 wk of age. Average cardiomyocyte area from WT and Bmal1<sup>-/-</sup> mice at 4 wk of age is represented, showing that cardiomyocytes from Bmal1<sup>-/-</sup> hearts are smaller (C). Cardiomyocytes were binned according to their size in 500-μm<sup>2</sup> bins, and the percentage of cardiomyocytes in each bin was calculated. There is a shift toward smaller cardiomyocytes in the Bmal1<sup>-/-</sup> mice (A). The percentage of cardiomyocytes smaller than 2,500 μm<sup>2</sup> was increased in the Bmal1<sup>-/-</sup> hearts, whereas the percentage of larger cardiomyocytes (>2,500 μm<sup>2</sup>) was decreased (B). n = 880 cells from 4 mice (WT) and 1,330 cells from 4 mice (Bmal1<sup>-/-</sup>). *P < 0.05; **P < 0.01; ***P < 0.001.

Fig. 3. Thinning of the myocardial walls and dilation of the ventricular cavity in 36-wk-old Bmal1<sup>-/-</sup> mice. Representative Masson’s trichrome-stained myocardial sections at the level of the papillary muscles, show enlargement of the ventricular cavity and wall thinning in the 36-wk-old Bmal1<sup>-/-</sup> myocardium (B) compared with an age- and sex-matched WT control myocardium (A). Graphs of combined data show a decrease in the area of myocardium (C) and the thickness of both the IVS and LVPW (D). LVC, LV cavity. n = 5 WT and 10 Bmal1<sup>-/-</sup> mice. *P < 0.05; **P < 0.01.
statistically different at 36 wk (Fig. 5B). MHC-β mRNA increased with age in both the wild-type and Bmal1−/− groups. However, we found no difference in MHC isoform composition or total MHC content at the protein level between wild-type and Bmal1−/− hearts (Fig. 5, C and D).

Bmal1−/− mice show increase of N2B and decrease of N2BA titin isoforms. Total titin, reflecting the sum of the N2BA, N2B, and T2 bands, and normalized to MHC and was not different between wild-type and Bmal1−/− hearts at any age (Fig. 6B). Additionally, the percentage of intact titin, calculated as the sum of N2BA and N2B bands over the sum of N2BA, N2B, and T2, was not affected by genotype and ranged between 83 and 90% in the wild-type hearts and 86 and 91% in the Bmal1−/− hearts (Fig. 6C). The T2 band is a degradative product of titin and is a measure of both in vivo degradation as well as degradation that could have occurred during processing of the samples. The levels of total titin normalized to MHC were similar in Bmal1−/− and wild-type hearts during stage 1, increased LVW-to-BW ratio (0.24 ± 0.07 vs. 0.23 ± 0.019); stage 2, regression (0.24 ± 0.027 vs. 0.27 ± 0.017); and stage 3, dilation and failure (0.20 ± 0.014 vs. 0.22 ± 0.011), suggesting that the cardiac pathology is not a result of changes in total titin protein levels.

However, we found that the percentage of the compliant N2BA isoform, calculated as N2BA/(N2BA + N2B), was lower in the Bmal1−/− left ventricles, especially at 4 wk (14.24 ± 1.73 vs. 19.79 ± 0.82%, P < 0.05) and 36 wk of age (12.73 ± 0.76 vs. 18.32 ± 0.90%, P < 0.001) (Fig. 6D). This was at the expense of the stiff N2B titin isoform, which was in turn increased in Bmal1−/− hearts compared with age-matched wild-type controls (4 wk, 85.76 ± 1.7 vs. 80.21 ± 0.82%, P < 0.05; 12 wk, 85.34 ± 0.29 vs. 83.52 ± 1.64%; and 36 wk, 87.27 ± 0.76 vs. 82.22 ± 0.94%, P < 0.01) (Fig. 6E). The N2BA-to-N2B ratio, a well-accepted measure of myocardial stiffness, was decreased in the Bmal1−/− hearts (Fig. 6F) during stage 1 when LVW to BW is increased (0.17 ± 0.02 vs. 0.25 ± 0.01, P < 0.05) and stage 3 when the heart is dilated and failing (0.15 ± 0.01 vs. 0.23 ± 0.01, P < 0.01) but not during stage 2 (0.17 ± 0.004 vs. 0.20 ± 0.02).
when the wild-type and Bmal1<sup>−/−</sup> hearts were functionally similar.

Bmal1<sup>−/−</sup> hearts exhibit compromised systolic function in ex vivo preparations. To complement the echocardiography data, we measured cardiac function in isolated working hearts from wild-type and Bmal1<sup>−/−</sup> mice at 18 wk of age, the beginning of stage 3. As seen in Fig. 7, hearts from Bmal1<sup>−/−</sup> mice exhibited significant depression in left ventricular systolic function as demonstrated by a 9.7% decrease in maximal left ventricular pressure (Fig. 7C), a 22.3% decrease in the rate of contraction (dP/dt) (Fig. 7D), and a subsequent 24.7% decrease in stroke volume (Fig. 7B). At this time point, the heart was larger as shown by the increase in HW-to-BW ratio (Fig. 7A), whereas the rate of relaxation (−dP/dt) was not affected (Fig. 7D).

Passive tension is not different in Bmal1<sup>−/−</sup> cardiomyocytes compared with wild-type controls. We found no difference in passive tension between Bmal1<sup>−/−</sup> and wild-type cardiomyocytes at any age (at sarcomere length of 2.45 μm, 4 wk: 13.63 ± 0.75 vs. 11.54 ± 1.28 kN/m²; 12 wk: 10.16 ± 0.72 vs. 12.54 ± 1.25 kN/m²; 36 wk: 8.78 ± 0.65 vs. 8.78 ± 0.32 kN/m²) (Fig. 8). Additionally, the exponential values, k and SL<sub>0</sub> were not different between Bmal1<sup>−/−</sup> and wild-type cardiomyocytes at any age (Fig. 8).

DISCUSSION

Data presented in this study demonstrate that germline Bmal1<sup>−/−</sup> mice develop age-associated DCM associated with myofilament disorganization that is preceded by changes in titin isoform composition and downregulation of MHC transcript levels. Whereas there is a rich body of evidence linking circadian rhythm dysfunction to the development of cardiovascular pathologies (27, 32, 33, 42), data are lacking on the role of Bmal1 and/or circadian rhythms on the structure and function of the heart with age.

We followed a cohort of Bmal1<sup>−/−</sup> and wild-type mice with echocardiograms starting at 4 wk of age until they reached 36 wk of age. We chose 36 wk as our end point because it has been previously reported that Bmal1<sup>−/−</sup> mice have an average lifespan of 37 ± 12 wk (34). We identified three distinct stages in the progression of the cardiac pathology in the Bmal1<sup>−/−</sup> mice, with stage 1 at 4 wk, characterized by larger hearts in the Bmal1<sup>−/−</sup> mice, whereas FS was not affected. We referred to this first stage as a transitory increase in LVW-to-BW ratio, because it was not sustained past 4 wk of age. This indicates that the germline Bmal1<sup>−/−</sup> mice are different from wild-type mice beginning shortly after birth and have bigger hearts for their body weight.

We defined the changes in the heart between 8 and 16 wk of age as stage 2, and this was characterized by regression of LVW-to-BW ratio with preservation of FS. During stage 3 (20–36 wk), there was significant thinning of the ventricular walls and enlargement of the LVID. These morphological changes were associated with a progressive decline in FS to around 55% of the initial value and a significant depression in cardiac systolic function as shown by decreased stroke volume, maximum left ventricular pressure, and dP/dt in the Bmal1<sup>−/−</sup> hearts at the beginning of stage 3. These findings indicate that loss of Bmal1 contributes to the development of features consistent with DCM. The Bmal1<sup>−/−</sup> mice have been considered a model of accelerated aging (34), and as such the
pathologies observed here could be the result of accelerated systemic aging.

One feature we found early in the $\text{Bmal}^1$−/− mouse is an enlarged heart seen with echocardiography and LVW-to-BW analysis. However, upon analysis of cardiomyocyte size we found that the $\text{Bmal}^1$−/− hearts at 4 wk of age had larger percentages of smaller cardiomyocytes and average cardiomyocyte size was reduced by 19%. This suggests that there were more cells, possibly hyperplasia of cardiomyocytes or other cell types to support the larger heart size. Since cardiomyocyte hyperplasia occurs mainly during embryonic development and up to 1 wk postnatally (46), our data suggest a possible role of $\text{Bmal}^1$ in regulating the timing of cardiac development and growth. The link between loss of $\text{Bmal}^1$ in the heart and heart size in our studies is consistent with the findings by Duragan et al. (13), in which cardiac specific loss of $\text{Bmal}^1$ was associated with increased biventricular weight-to-BW ratio and increased expression of the hypertrophic marker, $\text{mcip}^1$. However, the hypertrophy we observed in the germline $\text{Bmal}^1$−/− mouse occurs much earlier in life, at 4 wk of age, than that observed in the cardiac specific $\text{Bmal}^1$−/− mouse, and the heart hypertrophy we detect is transient. Whether the increased size in the Duragan study is due to hyperplasia and whether the hypertrophy persists are not known. Thus there is still much to learn about the interaction among $\text{Bmal}^1$, cardiomyocyte number, and size in the heart.

Much of the data in this study comes from longitudinal echocardiography studies, and, like others, we found that heart rate in the $\text{Bmal}^1$−/− mice was significantly lower compared with wild-type mice at all ages (means ± SD across all time points, 406 ± 49 vs. 482 ± 41 beats/min). To minimize any potential problems with interpretation, we attempted to match heart rate for echocardiography through an adjustment of isoflurane concentration; however, $\text{Bmal}^1$−/− mice were more sensitive to isoflurane and displayed a quicker drop in heart rate. Previous studies have shown that cardiac parameters measured through M-mode echocardiography were stable over a 20-min time span and a range of heart rates from 450–550 beats/min (50), whereas others have found increased LVW and LVID, decreased FS, and no effect on IVS and LVPW thickness when heart rate decreased by 120 beats/min (64). In the current study, we see a 76 beats/min decrease in heart rate in the $\text{Bmal}^1$−/− mice, whereas our wild-type values for heart rate and FS are consistent with previously published work (22, 64). The findings that IVS and LVPW thickness and LVW are decreased in our $\text{Bmal}^1$−/− mice cannot be explained by the decrease in heart rate and suggest that the changes seen in the $\text{Bmal}^1$−/− hearts are due to the pathology they develop and not an artifact of their decreased heart rate.

One characteristic of DCM is impairment in force generation. The observed decline in the rate of contraction in the $\text{Bmal}^1$−/− hearts ex vivo suggested the involvement of MHC, since a shift to the slower MHC-β isoform is both correlated with decreased $dP/dt$ (41) and commonly associated with DCM (20). It has been shown that a shift toward MHC-β occurs in canine models of DCM and a rat model of pressure overload (20, 28). Additionally, a forced expression of MHC-α protects against cardiomyopathy in the rabbit left ventricle (31). We found that levels of MHC-β mRNA were not increased in the $\text{Bmal}^1$−/− hearts at any of the ages in which significant functional changes were detected with ex vivo analysis or echocardiography. This indicates that the pathology observed in the $\text{Bmal}^1$−/− hearts is not associated with MHC isoform changes and does not directly mimic models of DCM. What we did find is that expression of MHC-α and MHC-β mRNAs were downregulated in the $\text{Bmal}^1$−/− hearts at 4 and 12 wk. This is consistent with previous reports from skeletal muscle of clock-compromised mice, showing downregulation of the mRNAs for many sarcomeric and structural genes (39). We were surprised to observe decreases in both MHC isoform mRNA levels with no differences in protein levels at the 4 and 12 wk ages. This suggests that in the early ages, loss of $\text{Bmal}^1$ leads to altered MHC gene expression but posttranscriptional, potentially protein turnover, mechanisms are in place to maintain normal MHC protein levels. If MHC protein turnover is altered, it is possible that the MHC protein present in the $\text{Bmal}^1$−/− hearts may be modified and thus exhibit altered function which could contribute to the observed decline in force development and the rate of contraction in the $\text{Bmal}^1$−/− hearts. Additionally, a decrease in calcium release; mutations in myosin light chain, actin, and troponin T; and altered phosphorylation status of troponin I are common findings in DCM and affect cross-bridge cycle dynamics, actin-myosin ATPase activity, and excitation-contraction coupling leading to decreased force generation (30, 52, 66). Much remains to be done to understand the relationship between $\text{Bmal}^1$ and cardiomyocyte specific gene expression and maintenance of mechanical function in the heart.

Titin plays a critical role in maintaining sarcomere structure. Our total titin/MHC values were similar to previously pub-
lished work (9, 44, 65) and were not different between wild-type and Bmal1−/− mice at any age, suggesting that changes in total titin are not mediators of the structural or functional cardiac pathology observed in the Bmal1−/− mice. In the myocardium, two titin isoforms are expressed, the larger and more compliant N2BA and the smaller, stiffer N2B (23). The N2BA-to-N2B ratio, a measure of cardiac compliance, in our wild-type mice was consistent with previously reported values for mouse myocardium (45, 47). The N2BA-to-N2B ratio significantly decreased in the Bmal1−/− mice, specifically at 4 wk of age when mice had increased LVW-to-BW ratio and at 36 wk of age when mice showed most symptoms of DCM. These data are consistent with the canine study by Wu and colleagues (65), in which rapid-pacing induced DCM resulted in a decrease in the N2BA-to-N2B ratio and a concomitant increase in titin based passive tension. These data suggest that loss of Bmal1 shifts titin isoform composition toward the stiffer isoform and this shift potentially mediates the pathologies seen in the hearts of the Bmal1-deficient mice.

Titin-based passive force constitutes the majority of the total passive tension in cardiac muscle, and previous studies have shown a correlation between changes in titin isoform composition and myocardial stiffness (24). Although we found a decrease in the N2BA-to-N2B ratio, we did not find the expected increase in passive tension. Our reported passive tension measurements are somewhat lower than what has been reported in mice at the same sarcomere length (9), possibly reflecting the lower N2BA-to-N2B ratio reported in this study. However, it is worth noting that others have found similar N2BA-to-N2B ratios to ours, but those studies did not assess passive tension (45, 47). These data suggest the presence of compensatory mechanisms to offset the isoform induced increase in passive tension. Previous studies have shown that phosphorylation of titin decreases passive tension (19, 35, 36). Thus it is possible that in addition to increasing the N2BA-to-N2B ratio, loss of Bmal1 increases basal levels of titin phosphorylation, resulting in a decrease in passive tension even in the light of the isoform changes observed. Another potential explanation for the

Fig. 8. Passive tension is not different in Bmal1−/− cardiomyocytes compared with WT controls. Passive tension measurements in single cardiomyocytes from WT and Bmal1−/− mice at 4 wk (A), 12 wk (B), and 36 wk (C) are shown. Individual data points are binned according to their sarcomere length in bins of 0.05 μm to generate graphs (left). Additionally, each curve was fit to an exponential equation: passive tension = \exp[k(SL - SL_0)], where SL is the sarcomere length, k is the exponent of stiffness, and SL_0 is the sarcomere length at zero load. k and SL_0 are plotted on the scatter plots (middle and right). Data are means ± SE; n = 20–24 cardiomyocytes from 4 mice/group.
discrepancy between your titin isoform data and the passive tension data might be due to sampling. For your titin isoform determination, we generated a homogenate from a portion of the ventricle taken from the apex of the heart, whereas for the mechanical measurements, we digested the entire heart and performed experiments on 5–7 viable cells selected following digestion. Titin isoform expression varies with the region of heart (4). It could be that by using the apex of the heart, we got an equal representation of all three myocardial layers (epi, mid, and endo), and maybe we have lost this equal representation of the three layers when using only 5–7 cells per mouse for the mechanical studies. Another possibility could be the selective loss of stiffer cells (lower N2BA-to-N2B ratio) during the cardiomyocyte isolation process for the mechanical measurements. With the isolation protocol, roughly 30% of cardiomyocytes are lost during the isolation (45a).

In conclusion, this is the first study to show that Bmn1l-deficient mice develop a cardiac pathology that starts with a transitory increase in myocardial mass and progresses to dilatation and failure until their time of death at ~36 wk of age. The systolic dysfunction observed is associated with early down-regulation of both MHC isoform transcripts, but not with protein, implicating both transcriptional and post-transcriptional changes in myosin gene expression. Bmn1l−/− hearts show loss of the normal sarcomere architecture that is associated with a shift in titin isoform composition toward the stiffer isoform. However, titin-based passive tension is not affected, associated with a shift in titin isoform composition toward the stiffer isoform. In conclusion, this is the first study to show that Bmn1l-deficient mice develop a cardiac pathology that starts with a transitory increase in myocardial mass and progresses to dilatation and failure until their time of death at ~36 wk of age. The systolic dysfunction observed is associated with early down-regulation of both MHC isoform transcripts, but not with protein, implicating both transcriptional and post-transcriptional changes in myosin gene expression. Bmn1l−/− hearts show loss of the normal sarcomere architecture that is associated with a shift in titin isoform composition toward the stiffer isoform. However, titin-based passive tension is not affected, associated with a shift in titin isoform composition toward the stiffer isoform.


