Depletion of lamina-associated polypeptide 1 from cardiomyocytes causes cardiac dysfunction in mice

Ji-Yeon Shin1,2, Caroline Le Dour1,2, Fusako Sera1, Shinichi Iwata1, Shunichi Homma1, Leroy C Joseph1, John P Morrow1, William T Dauer2,4,*, and Howard J Worman1,2,*

1Department of Medicine; College of Physicians & Surgeons; Columbia University; New York, NY USA; 2Department of Pathology and Cell Biology; College of Physicians & Surgeons; Columbia University; New York, NY USA; 3Department of Neurology; University of Michigan Medical School; Ann Arbor, MI USA; 4Department of Cell and Developmental Biology; University of Michigan Medical School; Ann Arbor, MI USA

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Abbreviations: ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-jun N-terminal kinase; LAP1, lamina-associated polypeptide 1; MCK, muscle creatine kinase; Myh6, myosin heavy chain 6

We previously showed that striated muscle-selective depletion of lamina-associated polypeptide 1 (LAP1), an integral inner nuclear membrane protein, leads to profound muscular dystrophy with premature death in mice. As LAP1 is also depleted in hearts of these mice, we examined their cardiac phenotype. Striated muscle-selective LAP1 knockout mice display ventricular systolic dysfunction with abnormal induction of genes encoding cardiomyopathy related proteins. To eliminate possible confounding effects due to skeletal muscle pathology, we generated a new mouse line in which LAP1 is deleted in a cardiomyocyte-selective manner. These mice had no skeletal muscle pathology and appeared overtly normal at 20 weeks of age. However, cardiac echocardiography revealed that they developed left ventricular systolic dysfunction and cardiac gene expression analysis revealed abnormal induction of cardiomyopathy-related genes. Our results demonstrate that LAP1 expression in cardiomyocytes is required for normal left ventricular function, consistent with a report of cardiomyopathy in a human subject with mutation in the gene encoding LAP1.

Introduction

Mutations in genes encoding nuclear lamins and other nuclear envelope proteins cause a broad range of human diseases.1 Mutations in the LMNA gene encoding lamins A and C cause autosomal dominant Emery-Dreifuss muscular dystrophy, which is classically characterized by early joint contractures, progressive wasting, and muscle weakness in a scapulohumeral peroneal distribution and dilated cardiomyopathy with conduction defects.2-4 Cardiomyopathy is the life-threatening feature of this disease.5,5 The same mutations in LMNA can also cause dilated cardiomyopathy with much more variable skeletal muscle involvement.6-8

Mutations in genes encoding proteins interacting with lamins A and C can also cause muscular dystrophy and cardiomyopathy, suggesting that these may function together in striated muscle physiology. Emerin, the widely expressed inner nuclear membrane protein, binds to lamins A and C and mutations in its gene, EMD, cause X-linked Emery-Dreifuss muscular dystrophy as well as related disorders with a prominent dilated cardiomyopathy, cardiac conduction abnormalities and variable skeletal muscle involvement.9,11 Emerin knockout mice, however, develop only very minimal skeletal muscle pathology and first-degree atrioventricular conduction at ages older than 40 weeks.12 Subsequently, an amino-acid substitution in another integral inner nuclear membrane protein that binds to lamin A and C, lamina-associated polypeptide 2-α, was found in two affected brothers with dilated cardiomyopathy.13 Germline deletion of the gene encoding lamina-associated polypeptide 2-α from mice induces cardiomyopathy with systolic dysfunction and sporadic fibrosis, suggesting its requirement in the maintenance of normal cardiac function.14,15

Lamina-associated polypeptide 1 (LAP1) is an integral inner nuclear membrane protein encoded by the human gene TORAIP1.16,17 Three LAP1 isoforms arise by alternative RNA splicing designated LAP1A, LAP1B, and LAP1C with molecular masses of 75, 68, and 55 kDa, respectively.18,19 Biochemical extractions showed that they are associated with the nuclear lamina.20 Subsequent studies showed that LAP1 interacts with torsinA and protein phosphatase1.21-23 We have shown recently that LAP1 interacts with emerin and that they function together in striated muscle maintenance.24,17 LAP1 conditional deletion from...
striated muscle causes a profound muscular dystrophy leading to early death. As mutations in genes encoding other integral inner nuclear membrane proteins that interact with lamins A and C cause cardiomyopathy as well as muscular dystrophy, we examined the potential physiological significance of LAP1 in heart development and function.

Results

Analysis of hearts in mice with striated muscle-selective depletion of LAP1

We have previously generated a striated muscle-selective conditional LAP1 knockout mouse line by crossing Tor1aip1 floxed mice to MCK-Cre transgenic mice (we referred to these MCK-Cre<sup>f/f</sup>;Tor1aip1<sup>+</sup> mice as M-CKO mice) and demonstrated that they develop muscular dystrophy and have a shortened lifespan with a medial survival for male mice of approximately 21 weeks.<sup>16</sup> As the MCK promoter is also expressed in cardiac muscle, we examined the effect of LAP1 depletion in hearts from M-CKO mice at 9–10 week of age. We confirmed that heart tissue of 9–10 week-old M-CKO mice is almost devoid of LAP1 protein (Fig. 1A). To assess cardiac dysfunction, we performed echocardiography on 9- to 10-week-old control and M-CKO mice (Fig. 1B). Echocardiograms from control and M-CKO mice were analyzed to obtain mean values for heart rate, left ventricular fractional shortening, ejection fraction, and left ventricular end-diastolic and end-systolic diameters (Table 1). Left ventricular end-systolic diameter was significantly increased and left ventricular fractional shortening, which is directly proportional to ejection fraction, was significantly decreased in M-CKO mice (Fig. 1C). This indicated defects in systolic function. Consistent with left ventricular stretching or increased wall tension, expression of NppA and NppB mRNAs respectively encoding atrial natriuretic peptide precursor and brain natriuretic peptide precursor was increased in hearts of M-CKO mice compared with control mice (Fig. 1D).

The structure and organization of myofibrils was not overtly altered in left ventricles of 12 week-old M-CKO mice based on histopathological examination of hematoxylin and eosin-stained tissue sections (data not shown). However, staining of heart sections from the M-CKO mice with Masson’s trichrome showed an increase in fibrosis (Fig. 2A and B). Expression of the
with premature death, it is possible that the observed cardiac selective depletion of LAP1 with the induction of myocardial fibrosis. M-CKO mice compared with control mice (Fig. 3A). The cardiomyocyte-selective LAP1-depleted H-CKO mice (Myh6-Cre; ;Tor1aip1<sup>f<sup>f</sup>) were born in expected Mendelian ratios and were indistinguishable from their littermate controls in body mass and growth rate until 20 weeks of age (data not shown). To confirm selective LAP1 depletion in heart, we compared the expression of LAP1 in hearts and other tissues from control, M-CKO, and H-CKO mice. While LAP1 expression was unchanged in protein extracts of skeletal muscles from control and H-CKO mice, it was selectively depleted in heart protein lysates from H-CKO mice. In contrast, LAP1 was depleted from both skeletal and heart muscle in M-CKO (Fig. 3A). The residual expression of LAP1 from heart extracts of H-CKO and M-CKO mice is presumably from non-muscle cells such as fibroblasts or endothelial cells. The expression of LAP1 in protein extracts of liver and lung was not altered in H-CKO mice (data not shown). Selective depletion of LAP1 in cardiomyocytes of H-CKO mice was confirmed by immunofluorescence microscopy of left ventricle tissue sections double labeled with antibodies against of LAP1 and α-actinin. While LAP1 was co-expressed with α-actinin in left ventricle sections from control mice, LAP1 expression was undetectable in α-actinin-expressing cardiomyocytes in H-CKO mice (Fig. 3B). Expression of lamin A and C and lamin B1 was similar in the left ventricles sections from both control and H-CKO animals (Fig. 3C). To ensure that skeletal muscle was not affected in H-CKO mice, we examined hematoxylin and eosin-stained sections of quadriceps from control, H-CKO, and M-CKO mice. In contrast to quadriceps from 12-week-old M-CKO mice showing myopathic features such as degenerative myofibers and central nuclei, sections from 20-week-old H-CKO mice did not display any myopathic defects (Fig. 3D). These results indicated that LAP1 was selectively depleted in cardiomyocytes but not skeletal muscle of H-CKO mice.

### Analysis of hearts of mice with cardiomyocyte-selective depletion of LAP1

To determine if H-CKO mice have cardiac dysfunction, we performed transthoracic M-mode echocardiography when mice were 20 weeks of age (Fig. 4A). The resulting echocardiograms from control and H-CKO mice were analyzed to obtain mean values for heart rate, left ventricular fractional shortening,
ejection fraction and left ventricular end-diastolic and end-systolic diameters (Table 2). Left ventricular end-systolic diameter was significantly increased and left ventricular fractional shortening (directly proportional to ejection fraction) was significantly decreased by approximately 30% in H-CKO mice compared with controls (Fig. 4B). These findings indicated that H-CKO mice exhibit significant defects of systolic function. Consistent with this finding, the expression of NppA and NppB mRNAs were also increased in hearts of H-CKO mice compared with controls (Fig. 4C). We also performed electrocardiography and did not find significant differences in PR interval or QRS duration between H-CKO and control mice at 20 weeks of age (Fig. S1).

The structure and organization of myofibrils was not overtly altered in left ventricle of 20-week-old H-CKO mice when analyzed by hematoxylin and eosin-stained tissue sections (data not shown). Staining of heart sections from 20-week-old H-CKO mice with Masson’s trichrome showed a trend toward increased fibrosis but the difference was not statistically significant (Fig. 5A and B). However, expression of mRNAs of genes...
encoding collagen 1 and fibronectin was significantly increased in hearts from H-CKO mice compared with controls indicating induction of myocardial fibrosis (Fig. 5C).

Impaired mitogen-activated protein kinase signaling in hearts of mice with cardiomyocyte-selective depletion of LAP1

We have previously shown abnormal activation of the extracellular signal-regulated kinase 1/2 (ERK1/2), c-Jun N-terminal kinase (JNK), and p38alpha branches of the mitogen-activated protein kinase signaling pathways in hearts of mice modeling autosomal (Lmna<sup>H222P/H222P</sup> knock-in mice)<sup>27</sup> and X-linked (emerin null mice)<sup>28</sup> Emery-Dreifuss muscular dystrophy. Furthermore, pharmacological or genetic inhibition of these signaling cascades improve cardiac function in Lmna<sup>H222P/H222P</sup> mice.<sup>29-33</sup> We therefore assessed phosphorylated (activated) ERK1/2, JNK and p38alpha in protein extracts of hearts from H-CKO and controls at 20 week of age (Fig. 6A). H-CKO mice exhibited increased cardiac activation of ERK1/2 and JNK but not p38alpha (Fig. 6B). Consistently, expression of mRNAs encoded by Atf2, Atf4, Nfatc4, Elk4<sup>29</sup>, downstream genes in the ERK1/2 and JNK signaling cascades, was significantly increased in H-CKO mice (Fig. 6C).

In hearts from M-CKO mice, we similarly found increased cardiac activation of ERK1/2 and JNK but not p38alpha compared with controls (Fig. S2). These data indicate that the ERK1/2 and JNK signaling pathways are abnormally activated in hearts lacking LAP1 in mice that develop left ventricular dysfunction.

Discussion

Our findings demonstrate the pathological effects of targeted depletion of LAP1 in intact heart. Both striated muscle-selective and cardiomyocyte-selective LAP1 deletions cause left ventricular systolic dysfunction, leading to decreased fractional shortening and/or ejection fraction, enhanced expression of natriuretic peptides, and myocardial fibrosis-related genes. The consistent results from two different animal models lead us to conclude that LAP1 is required for normal cardiac function.

LAP1 interacts with lamins and emerin,<sup>16,20</sup> suggesting that these proteins may form a complex important for maintaining the integrity of the nuclear envelope. In mice, loss of A-type lamins leads to much more significant, early onset cardiac dysfunction, with Lmna null mice developing a severe dilated cardiomyopathy by 6 to 8 weeks of age.<sup>34</sup> Mice with homozygous amino acid substitutions or a single amino acid deletion from A-type lamins also develop profound dilated cardiomyopathy.<sup>35-37</sup>
In contrast, emerin null mice develop minimal cardiac disease. Loss of LAP1 from striated muscle, or more selectively from cardiomyocytes, causes an intermediate phenotype, with systolic dysfunction but without marked left ventricular dilatation. Hence, the differing phenotypes may be due to differences in the relative importance of A-type lamins, emerin, and LAP1 in forming a protein complex that maintains nuclear envelope integrity.

Defects in a complex composed of A-type lamins, emerin, and LAP1 that functions in maintaining nuclear envelope integrity may make cells more susceptible to stress, leading to abnormal activation of stress-induced signaling cascades in cardiomyocytes subjected to contractile stress. We detected an abnormal activation of the stress-induced mitogen-activated protein kinases ERK1/2 and JNK in hearts of 20 week-old H-CKO mice with reduced LAP1 in cardiomyocytes. We have previously shown abnormally increased activities of the ERK1/2, JNK and p38alpha branches of the mitogen-activated protein kinase pathway in 

<Figure 5. Cardiac fibrosis in H-CKO mice with cardiomyocyte-selective depletion of LAP1. (A) Masson's trichrome staining of heart sections from two different 20 week-old control (Tor1aip1<sup>f/f</sup>) and H-CKO mice are shown. Bar = 50 μm. (B) Ventricular sections from 20 week-old Tor1aip1<sup>f/f</sup> mice (Control) and H-CKO mice were stained with Masson's trichrome as shown in previous panel and percentage of fibrosis per each image was averaged. Two different regions from each section from three mice per group (n = 6) were analyzed. Values are means ± standard errors; n.s. = not significant. Means ± standard errors of relative expression of mRNAs encoded by Col1a1, Col1a2 and Fn1 in hearts of control (n = 4) and H-CKO (n = 4) mice at 20 weeks of age. *P < 0.05, **P < 0.01.>

Materials and Methods

Animals

The Institutional Animal Care and Use Committee of Columbia University Medical Center approved all protocols. Mice were kept at room temperature and fed normal chow. The generation and maintenance of floxed alleles of Tor1aip1 (Tor1aip1<sup>f/f</sup>) and MCK-Cre mice from Jackson laboratory (stock number: 006475) were previously described. Myh6-Cre<sup>−/−</sup> mice were bred with Tor1aip1<sup>f/f</sup> mice to obtain Myh6-Cre<sup>−/−</sup>; Tor1aip1<sup>f/f</sup> mice. As described previously, the expression of three isoforms of LAP1 was eliminated in the presence of Cre recombinase. These animals were fertile and produced at the expected Mendelian frequency. They were subsequently backcrossed with Tor1aip1<sup>f/f</sup> mice to obtain.

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Myh6-Cre<sup>−/−</sup>; Tor1aip1<sup>flf</sup> (referred to as H-CKO) mice. Mice were monitored and body mass measured weekly after weaning. To genotype Myh6-Cre mice, PCR was performed from tail biopsies using the following primer sequences: 5′-ATGACAGACAGATCCCTCCTATCTCC-3′ and 5′-CTCATGATGATGATTCGAC-3′.

**Echocardiography**

Transthoracic echocardiography was performed as described previously. Echocardiograms were recorded from mice sedated with low-dose inhaled isoflurane using the standard four limb leads and a B08 amplifier (Emka Technologies) with minimal filtering. Waveforms were recorded using Iox Software v1.8.9.18 and intervals were measured manually with ECG Auto v1.5.12.50, using the average of three representative consecutive beats. The echocardiographer was blinded to mouse genotype.

**Electrocardiography**

Electrocardigrams were recorded from mice sedated with low-dose inhaled isoflurane using the standard four limb leads and a B08 amplifier (Emka Technologies) with minimal filtering. Waveforms were recorded using Iox Software v1.8.9.18 and intervals were measured manually with ECG Auto v1.5.12.50, using the average of three representative consecutive beats. The electrocardiographer was blinded to mouse genotype.

**Quantitative Real Time PCR**

Total RNA was extracted from mouse hearts using the RNasey isolation Kit (Qiagen) as described previously. Quality and concentrations of RNA were measured using a Nanodrop spectrophotometer (Thermo Scientific). The cDNA was synthesized using Superscript First Strand Synthesis System (Life Technology). For each replicate in each experiment, RNA from ventricular muscle of different animals was used. PCR primers for Nppa, Nppb, Col1a1, Col1a2, Fn1, Atf2, Atf4, Nfatc4, Elk4, and Gapdh have been described previously. Quantitative PCR was performed on an ABI 7300 Real-Time PCR System (Applied Biosystems) using HotStart-IT SYBR green qPCR Master Mix (Affymetrix). Relative levels of mRNA expression were calculated using the ΔΔCT method. Individual expression values were normalized by comparison to Gapdh mRNA.

**Histology and immunohistochemistry**

Mouse hearts or skeletal muscles were placed in 10% neutral buffered formalin for 48 h and embedded in paraffin and sectioned at 5 μm. Sections were stained with hematoxylin and eosin for histopathological assessment. Masson’s trichrome staining was performed to identify collagen-rich fibrotic regions as described previously. Stained sections were photographed using a DP72 digital camera attached to a BX53 upright light microscope (Olympus). Fibrotic area was quantified as described previously. Briefly, Images of Masson’s trichrome-stained sections were analyzed by Jmicrovision software (http://www.jmicrovision.com/). Uploaded images and/or oxygen (1.5%) and all procedures were performed at room temperature. Echocardiography was performed using a Vevo 770 imaging system (Visualsonics) equipped with a 30-MHz linear transducer applied to the chest wall. The echocardiographers who performed the procedures and analyzed the data were blinded to the genotype of the mice.
were set up as 2D measurement to obtain Hue histograms in which colors were represented from 0–255 units. The histogram values corresponding to blue color (140–200 units) were summed and the percentage fibrotic area (blue) was calculated out of total values.

For immunohistochemistry, mouse hearts were fixed in 10% neutral buffered formalin for 24 h and moved to 30% sucrose for 24 h. Cryosections were then cut at 5 µm thickness and processed for immunohistochemistry using an M.O.M Kit (Vector Labs). Primary antibodies were anti-α-actinin (Sigma, #A7811), anti-LAP1,2 anti-lamin A/C (Santa Cruz Biotechnologies Inc #SC-20681) and anti-lamin B14 antibodies. Alexa Fluor 488 and 594 conjugated secondary antibodies (Molecular Probes) were used to visualize the primary antibody labeling. Coverslips were mounted with Prolong Gold Anti-fade with 4′,6-diamidino-2-phenylindole (Life Technologies) and images acquired using an A1 scanning confocal microscope on an Eclipse Ti microscope stand (Nikon). All images were taken using a 40×/1.3 Plan-Fluor objective lens.

**Protein extraction and immunoblotting**

Dissected tissues were homogenized in radioimmunoprecipitation assay buffer (RIPA Buffer, Cell Signaling) containing Protease Inhibitor Cocktail (Roche) plus 1 mM phenylmethylsulfonyl fluoride (Sigma). Proteins in samples were denatured by boiling in Laemmli sample buffer16 containing β-mercaptoethanol for 5 min, separated by SDS-PAGE and transferred to nitrocellulose membranes.

For immunoblotting, membranes containing proteins transferred from SDS-polyacrylamide gels were washed with blocking buffer (5% bovine serum albumin and 0.2% polysorbate 20 in phosphate-buffered saline) for 30 min and probed with primary antibodies in blocking buffer overnight at 4 °C. The primary antibodies used for immunoblotting were anti-LAP1,2 GAPDH (Ambion #AM4300), total ERK1/2 (Santa Cruz Biotechnologies Inc #SC-94), phosphorylated ERK1/2 (Cell Signaling #9101), total JNK (Santa Cruz Biotechnologies Inc #SC-474), phosphorylated JNK (Cell Signaling, #9251), total p38α (Cell Signaling, #9212), phosphorylated p38α (Cell Signaling, #4511). Blots were washed with 0.2% Tween-20 in phosphate-buffered saline and then incubated in blocking buffer with horseradish peroxidase-conjugated secondary antibodies (Amersham) for 1 h at room temperature. Recognized proteins were visualized by enhanced chemiluminescence (Thermo Scientific) and detected by exposure on X-ray films (Kodak).

To quantify signals, immunoblots were scanned and densities of the bands quantified using ImageJ64 software. The LAPI bands density was normalized to GAPDH signal density of each sample. For mitogen-activated protein kinases, band intensity of phosphorylated protein was normalized to the band intensity of respective total protein.

**Disclosure of Potential Conflicts of Interest**

No potential conflict of interest was disclosed.

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**Supplemental Materials**

Supplemental materials may be found here: www.landesbioscience.com/journals/mabs/article/29227

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