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Lamin A/C Haploinsufficiency Causes Dilated Cardiomyopathy and Apoptosis-Triggered Cardiac Conduction System Disease

Cordula M Wolf¹, Libin Wang², Ronny Alcalai², Anne Pizard^{2,3}, Patrick G Burgon^{2,4}, Ferhaan Ahmad^{2,5}, Megan Sherwood^{1,6}, Dorothy M Branco¹, Hiroko Wakimoto¹, Glenn I Fishman⁷, Vincent See⁸, Colin L Stewart⁹, David A Conner², Charles I Berul^{1,*}, Christine E Seidman^{2,10,*}, and JG Seidman^{2,*}

¹Department of Cardiology, Children's Hospital Boston, Harvard Medical School, Boston, MA 02115

²Department of Genetics, Harvard Medical School, Boston, MA 02115

³INSERM Unité 652, Institut des Cordeliers, Paris, France

⁴Cardiovascular Endocrinology, Univ. of Ottawa Heart Inst, Ottawa, Ontario, K1Y 4W7 CA

⁵Cardiovascular Inst and Dept of Medicine and Genetics, Univ of Pittsburgh, Pittsburgh, PA 15213

⁶Department of Pediatrics, Children's Hospital Westmead; Sydney, Australia

⁷Leon H. Charney Div of Cardiology, New York Univ School of Medicine, New York, NY 10016

⁸Cardiology, Boston Medical Center and Boston University School of Medicine, Boston, MA 02118

⁹Cancer and Developmental Biology Laboratory, National Cancer Institute, Frederick, MD 21702

¹⁰Howard Hughes Medical Institute, Boston, MA 02115

Abstract

Mutations in the lamin A/C (*LMNA*) gene, which encodes nuclear membrane proteins, cause a variety of human conditions including dilated cardiomyopathy (DCM) with associated cardiac conduction system disease. To investigate mechanisms responsible for electrophysiologic and myocardial phenotypes caused by dominant human *LMNA* mutations, we performed longitudinal evaluations in heterozygous *Lmna*^{+/-} mice. Despite one normal allele, *Lmna*^{+/-} mice had 50% of normal cardiac lamin A/C levels and developed cardiac abnormalities. Conduction system function was normal in neonatal *Lmna*^{+/-} mice but by 4 weeks of age AV nodal myocytes had abnormally shaped nuclei and active apoptosis. Telemetric and *in vivo* electrophysiologic studies in 10 week-old *Lmna*^{+/-} mice showed atrioventricular (AV) conduction defects and both atrial and ventricular arrhythmias, analogous to those observed in humans with heterozygous *LMNA* mutations. Isolated myocytes from 12-month old *Lmna*^{+/-} mice exhibited impaired contractility. *In vivo* cardiac studies of aged *Lmna*^{+/-} mice revealed DCM; in some mice this occurred without

Contact address: Charles I. Berul, M.D., Department of Cardiology – Children's Hospital, 300 Longwood Ave., Boston, MA 02115 USA, Phone: 617 355-6432, Fax: 617 566-5671, charles.berul@cardio.chboston.org.

*These authors contributed equally to this publication.

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overt conduction system disease. However, neither histopathology nor serum CK levels indicated skeletal muscle pathology. These data demonstrate cardiac pathology due to heterozygous *Lmna* mutations reflecting a 50% reduction in lamin protein levels. Lamin haploinsufficiency caused early-onset programmed cell death of AV nodal myocytes and progressive electrophysiologic disease. While lamin haploinsufficiency was better tolerated by non-conducting myocytes, ultimately these too succumbed to diminished lamin levels leading to dilated cardiomyopathy, which presumably arose independently from conduction system disease.

Keywords

Cardiac Conduction System; Arrhythmia; Heart Block; Laminopathies; Dilated cardiomyopathy

Introduction

Lamin A and C are widely expressed intermediate filament proteins within the inner nuclear membrane(1) where they provide mechanical support(2) and interact with other proteins, including tissue-specific transcription factors(3). Mutations in the human lamin A/C (*LMNA*) gene cause 16 different diseases with considerable clinical variability, ranging from cardiac and skeletal myopathies to lipodystrophy, peripheral neuropathy, and premature aging(4),(5). Despite this clinical heterogeneity some *LMNA* mutations cause particular phenotypes. For example, mutations that create a particular abnormal lamin A protein can produce aging syndromes(6), while mutations at the carboxyl terminus of lamin C cause lipodystrophy(7).

Cardiac manifestations are particularly prominent in 3 autosomal dominant laminopathies: Emery-Dreifuss muscular dystrophy(8), limb-girdle muscular dystrophy(9) and dilated cardiomyopathy (DCM) with conduction system disease(10). Although a skeletal myopathy predominates in two of these disorders, some patients develop cardiac electrophysiologic disease, progressive left ventricular dysfunction and heart failure(11). Electrophysiologic defects usually precede DCM(12), and may be the only manifestation of cardiac involvement(13)]. Electrophysiologic abnormalities include sinus node dysfunction, progressive atrioventricular (AV) block, paroxysmal atrial fibrillation and ventricular arrhythmias[9,10,14]. Patients with *LMNA* mutations may experience malignant arrhythmias and sudden death despite pacemaker implantation[15,16]. Postmortem study of hearts with *LMNA* mutations show fibrofatty infiltration of the atrioventricular node[10].

The mechanism by which some lamin mutations cause DCM and conduction system disease whereas other mutations cause muscular dystrophy in addition to cardiac disease is uncertain. Several mutations which should produce null alleles cause Emery-Dreifuss muscular dystrophy. While most missense mutations associated with these cardiac phenotypes are predicted to encode dominant negative alleles, at least one mutation[17] predicted to produce haploinsufficiency, causes familial DCM and conduction system disease[8,9,10,12,13].

Mice carrying human *LMNA* missense mutations H222P[11], N195K[18], L530P[19] and a null allele[20] have been previously described. Juvenile, homozygous lamin-null (*Lmna*^{-/-}) mice are runted, have skeletal muscle atrophy[20], DCM[21], and die by 8 weeks, recapitulating some features of human Emery-Dreifuss muscular dystrophy, DCM and progeria. In contrast, heterozygous mutant mice, which replicate the mutant gene dosage found in humans, are reported as indistinguishable from wildtype mice[21]. Whether this reflects fundamental differences in lamin biology, survival differences, or incomplete phenotyping is unclear.

Given the prominence of cardiac conduction system disease in multiple human LMNA mutations, we examined cardiac function and electrophysiology in mice heterozygous for a targeted deletion of the *Lmna* gene. Despite one normal allele, reduced levels of lamin A/C protein were found in *Lmna*^{+/-} hearts. Using a molecular marker to demarcate myocytes with electrophysiologic properties, conduction system anatomy and function were studied longitudinally. Structure and function were normal in young *Lmna*^{+/-} mice, but older *Lmna*^{+/-} mice had altered atrioventricular nodal architecture and functional electrophysiologic deficits, and arrhythmias. These abnormalities reflected increased apoptosis in conduction system myocytes. Aged *Lmna*^{+/-} mice, like humans with LMNA mutations, also developed DCM, sometimes without overt conduction system disease. We conclude that haploinsufficiency of lamin A/C protein caused cardiac electrophysiologic deficits. Although embryonic development and maturation of these specialized myocytes appeared normal, reduced physiologic levels of this nuclear membrane protein causes programmed cell death of electrically-active cardiomyocytes.

Materials and Methods

Mice

Lmna^{+/-} mice carry a lamin-null allele producing neither full length lamin A/C mRNA or truncated protein[20]. *CCS:lacZ* mice express the β -galactosidase gene in myocytes of the conduction system[22]. The *CCS:lacZ* allele was bred into *Lmna*^{+/-} mice (designated *Lmna*^{+/-}/*CCS:lacZ*). For detailed information see online methods section.

Western blot assays

Protein extracts and Western blots were performed as described[23]. Whole heart lysates were transferred onto Hybond membrane and blotted with primary antibodies against lamin A/C, tubulin, Elk1, ERK 2, pERK1/2, JNK1, p44/42 MAPK, pc-Jun, and GAPDH. Horseradish peroxidase-conjugated secondary antibody was used for chemiluminescence detection. Proteins levels were measured using NIH ImageJ software (<http://rsb.info.nih.gov/>).

Electrocardiography, Electrophysiology and Echocardiography

Electrocardiographic (ECG) recordings, electrophysiological studies, continuous telemetry and echocardiographic analyses were performed as previously described[24,25] (see online methods for detailed protocols).

Gross Morphology, Histopathology and TUNEL assays

Cardiac pathology was defined using previously described methods[25,26]. β -galactosidase activity was detected by staining for 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside (X-Gal) in hearts carrying the *CCS:lacZ* allele as described[27]. Paraffin sections were stained with hematoxylin and eosin, Masson trichrome, One-step Gomori Masson or Van Kossa stain[26] to define areas of fibrosis[25] and/or necrosis in *Lmna*^{+/-}/*CCS:lacZ* and *Lmna*^{+/-}/*CCS:lacZ* hearts.

TUNEL assays

Terminal deoxynucleotidyltransferase (TdT) nicked-end labeling assays were performed as previously described[28]. Nuclei were counterstained with DAPI. Cells were quantified using fluorescence microscopy.

Immunohistochemistry

Apoptosis-specific activation of caspase-3 was studied using a polyclonal rabbit antibody specific for large (17 to 20 kD) fragments and counterstained with hematoxylin and eosin[29].

Myocyte isolation and morphology

Ventricular cardiomyocytes were isolated via a Langendorff hanging heart preparation and enzymatic digestion as described[30]. Resting sarcomere length and sarcomere shortening/re-lengthening were determined from striation positions in myocytes isolated from mice using real-time power spectrum analysis software[25].

Creatine kinase activity measurement

Quantitative measurements of creatine kinase (CK) activity were assessed in unstressed mice using the Roche CK-NAC liquid assay.

Statistical analyses

Significance was compared using SPSS statistical software (version 12.0, SPSS Inc.). Data are presented as mean $\pm$ SD. Differences between mean values were determined using unpaired or paired Student t-test and ANOVA for multiple comparisons followed by the Bonferroni post-hoc test. Dichotomous variables were compared using Fisher exact test for four-field comparison and Pearson Chi-Square for multiple comparisons.

Results

Lamin A and C protein levels in *Lmna*^{+/-} and wildtype hearts

Lamin A and C protein levels are undetectable in nuclear extracts from homozygous *Lmna*^{-/-} null mouse embryonic fibroblasts[20], but not described in *Lmna*^{+/-} heart tissue. Western blot analyses of total protein lysates from cardiac tissue showed significantly decreased levels of lamin A (3-fold) and C protein (2.3-fold) levels in *Lmna*^{+/-} compared to wildtype hearts (Supplement Figure 1).

Electrophysiology

To characterize longitudinal changes of cardiac electrophysiologic properties in *Lmna*^{+/-} mice were studied at 0-2 weeks (neonatal), 4 weeks (juvenile), 10 weeks (adult), and >50 weeks (aged). Only ECG recordings were obtained in neonatal mice due to small size (<10 grams) and frailty. Serial multi-lead ECG recordings were obtained daily, between postnatal day 1 through day 7, and at day 14 on neonatal mice. Neither wildtype (N=15) nor *Lmna*^{+/-} (N=13) mice had ECG abnormalities or differences in standard ECG intervals or morphologies (data not shown).

Sedated ECG recordings, conscious Holter monitoring and intracardiac electrophysiologic recordings were performed in 10-week-old adult wildtype and *Lmna*^{+/-} mice. There were no differences between the averaged baseline SCL, PR, QRS and QTc intervals of wildtype and *Lmna*^{+/-} mice on surface ECG recordings or Holter monitor (Supplement Table 1).

However, 62% of *Lmna*^{+/-} mice (n=18) but no wildtype mice (n=17) had conduction system disease or arrhythmias (p=0.0001). Holter monitoring showed a variety of rhythm abnormalities, including sinus bradycardia in 5/18 (28%) *Lmna*^{+/-} mice (p=0.03), and heart block in 8/18 (44%) *Lmna*^{+/-} mice, but no wildtype mice (n=17, p=0.003); 5 had first degree AV block, 2 high degree AV block with complete intermittent AV block, and 1 had second degree AV block (Figure 1). Electrophysiologic studies demonstrated defective AV nodal conduction in 10-week old *Lmna*^{+/-} mice. Antegrade AV and retrograde conduction time

was significantly slower and AV nodal effective refractory period was prolonged in *Lmna*^{+/-} mice (p=0.02; Table 1). Pacing induced atrial fibrillation (Figure 1D) in 39% of *Lmna*^{+/-} mice and ventricular tachycardia (Figure 1E) in 33% of *Lmna*^{+/-} mice (p<0.006).

To better define the onset and progression of electrophysiologic defects, younger mice underwent intracardiac electrophysiologic studies and aged mice had telemetric and intracardiac electrophysiologic studies. There were no differences in AV nodal conduction or refractory properties between 4-week old *Lmna*^{+/-} and wildtype mice (Table 1). As reported[31], juvenile wildtype mice are vulnerable to pacing-induced ventricular tachycardia, and both *Lmna*^{+/-} mice and wildtype mice had comparable susceptibility to these arrhythmias with pacing (16% *Lmna*^{+/-}, 10% wildtype; p=NS). However, pacing induced atrial fibrillation in 21% of *Lmna*^{+/-} mice but not wildtype mice (Table 1).

Studies of aged *Lmna*^{+/-} mice showed that conduction system abnormalities persisted but did not progress beyond adulthood: telemetric studies undertaken in 9 aged *Lmna*^{+/-} mice showed cardiac conduction system disease in 67% of *Lmna*^{+/-} mice, similar to adult mice (p>0.1 Chi-square). Sinus nodal disease was seen in 44% and varying degrees of heart block in 56% of aged *Lmna*^{+/-} mice. Arrhythmia inducibility in aged *Lmna*^{+/-} mice was comparable to adult mice. Taken together, cardiac conduction system disease and overall arrhythmia vulnerability progressed during early adolescence and persisted with age.

Morphology and histology of the cardiac conduction system

To examine cardiac conduction system anatomy, *Lmna*^{+/-} mice were bred with mice carrying a *CCS:lacZ* allele, which is selectively expressed in the conduction system[22]. Gross morphology of the conduction systems from 10-week old *Lmna*^{+/+}/*CCS:lacZ* (Figure 2A-C) and *Lmna*^{+/-}/*CCS:lacZ* mice (Figure 2D-F) were indistinguishable. Similar lacZ expression was detected in the atria and in the AV node and AV junction, bundle branches and Purkinje fibers of both mutant and wildtype *Lmna*/*CCS:LacZ* mice (Figure 2C,F), evidencing normal development and maturation of the conduction system in *Lmna*^{+/-} mice[27].

Sagittal sections were obtained from 3 *Lmna*^{+/-}, 3 *Lmna*^{+/+}, 3 *Lmna*^{+/-}/*CCS:lacZ* and 3 *Lmna*^{+/+}/*CCS:lacZ* mice to optimize AV node visualization. AV node β-galactosidase staining appeared less intense and more disorganized in *Lmna*^{+/-}/*CCS:lacZ* (Figure 3C,D) than *Lmna*^{+/+}/*CCS:lacZ* hearts (Figure 3A,B). Similar sections from *Lmna*^{+/-}/*CCS:lacZ* (Figure 3G,H) and *Lmna*^{+/+}/*CCS:lacZ* (Figure 3E,F) hearts were stained for β-galactosidase (dark blue) and fibrosis (Gomori, light blue). No fibrosis was observed within the AV node of wildtypes (Figure 3E,F), but increased fibrosis was observed in mutant AV nodal region (Figure 3G,H). Similar findings were observed after Masson's trichrome staining of wildtype (Figure 3I,J) and mutant (Figure 3K,L) sections. Taken together, histochemical analyses showed greater fibrosis in the *Lmna*^{+/-} AV node.

Abnormal nuclei in isolated cardiac conduction cells

Myocytes were isolated from 10-week old *Lmna*^{+/+}/*CCS:lacZ* and *Lmna*^{+/-}/*CCS:lacZ* mice and specialized conduction system cells were identified by β-galactosidase expression. Comparable numbers of β-galactosidase positive cells (7.6±2.2 % of atrial and ventricular myocytes), were isolated from *Lmna*^{+/+}/*CCS:lacZ* and *Lmna*^{+/-}/*CCS:lacZ* mice, indicating appropriate specification and differentiation of conduction system cells occurred despite *Lmna* haploinsufficiency. DAPI stain visualized nuclei and cytoplasmic area, cell lengths and nuclear area of conduction system and non-conduction system cells were measured (Figure 4). Cell sizes of *Lmna*^{+/+}/*CCS:lacZ* and *Lmna*^{+/-}/*CCS:lacZ* myocytes were indistinguishable (Supplement Table 2). However, *Lmna*^{+/-}/*CCS:lacZ* cell nuclei were

significantly longer ($p < 0.001$), and occupied more cytoplasmic area ($p < 0.001$) than did the oval shape of *Lmna*^{+/-}/*CCS:lacZ* nuclei. The *CCS:lacZ* allele did not account for these morphologic changes, since nuclei in myocytes isolated from wildtype and *Lmna*^{+/-} mice with or without the *CCS:lacZ* allele were comparable in size and morphology.

Cell death in *Lmna*^{+/-} AV nodes

Because *Lmna*^{+/-} mice, which had normal cardiac electrophysiology as neonates, were susceptible to inducible atrial arrhythmias at 4 weeks, and had both functional abnormalities and structural defects by 10 weeks of age, we hypothesized that progressive demise of conduction system cells accounted for the evolution of electrophysiologic dysfunction. To test this model, we studied atrioventricular specimens from 4-week old wildtype and *Lmna*^{+/-} mice for necrosis and/or apoptosis. Van Kossa stain, which identifies calcium depositions produced during necrotic cell death, was negative in both wildtype and *Lmna*^{+/-} hearts (data not shown). Specimens were examined for apoptosis by two independent assays, deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL), and immunohistochemical analyses for caspase-3 activity. Atrial sections derived from the atrioventricular junction and above the annulus fibrosis of 4-week old wildtype mice had approximately 6% apoptotic cells ($N = 1038$ DAPI positive nuclei counted from six sections from 4 mice, Figure 5B), and did not react with antibody to activated caspase-3 (Figure 5C). In contrast, the corresponding sections from 4 – week-old *Lmna*^{+/-} mice had significantly more TUNEL positive cells (18%, $N = 1671$ DAPI positive nuclei from six sections obtained from 5 mice; $p < 0.001$; Figure 5E). In addition more *Lmna*^{+/-} cells were caspase-3 positive than wildtype cells (Figure 5F). No significant apoptosis was detected in left ventricular free wall sections from wildtype or mutant (Figure 5H,I) mice.

Lmna^{+/-} mice develop late-onset cardiomyopathy without overt skeletal myopathy

To determine if reduced lamin A/C protein affected myocardial and skeletal myocytes in addition to specialized electrophysiologic myocytes, the morphology and function of hearts and myocytes from young (10 weeks) and old (50-75 weeks, median 62 weeks) *Lmna*^{+/-} mice (Table 2) were studied. Among the initial cohort of 10 *Lmna*^{+/-} mice, 2 died (at 52 and 55 weeks). Both had enlarged hearts and distended livers, consistent with heart failure (data not shown); these mice were not included in subsequent analyses. All wildtype mice ($n = 9$) survived to 90 weeks.

LV wall thickness, chamber sizes and fractional shortening were compared in 10 week-old and 50 week-old wildtype and *Lmna*^{+/-} mice (Table 2). At 10 weeks, mutant and wildtype cardiac morphology and fractional shortening were indistinguishable. However, 50 week-old *Lmna*^{+/-} mice had enlarged ventricular chambers in systole ($p = 0.01$) and diastole ($p = 0.002$), corresponding to significantly decreased fractional shortening ($p = 0.02$). Histopathologic examination of cardiac sections from aged *Lmna*^{+/-} mice showed more fibrosis than wildtype mice (Figure 6). In 3 aged *Lmna*^{+/-} mice, ventricular dysfunction occurred without overt evidence of conduction system disease.

Isolated myocytes revealed similar cell dimensions in 10-week and 50-week old *Lmna*^{+/-} and wildtype mice, but myocytes from aged *Lmna*^{+/-} mice had significantly greater sarcomere length ($p < 0.01$) than aged wildtype mice (Supplement Table 2). Cell and sarcomere shortening (Figure 6G) were also decreased in *Lmna*^{+/-} myocytes compared to wildtype ($p < 0.001$), with ventricular dilation and depressed cardiac contractility, consistent with DCM in aged *Lmna*^{+/-} mice.

To determine if skeletal muscle pathology was present in *Lmna*^{+/-} mice, serum creatine kinase levels were measured. Values were not different between wildtype and *Lmna*^{+/-} mice

($p=0.88$; $n>5$ for each group; data not shown). The soleus and bicep muscles were examined in aged mice (age >50 weeks; 4 wildtype mice, representative figure 6E, and 5 *Lmna*^{+/-} mice, Figure 6F including inset). Characteristic features of skeletal myopathic pathologies such as variable myocyte size, immature muscle fibers with central nuclei (on hematoxylin-eosin stain), or increased connective tissue (on Masson trichrome stain) were notably absent in all specimens. Muscle fiber variability was similar between *Lmna*^{+/-} and wildtype mice (standard deviation of muscle fiber area in arbitrary units: *Lmna*^{+/-} 2.37 ± 0.8 , $N=5$ vs. WT 2.98 ± 1.2 , $N=3$, $p=0.4$).

Mitogen-activated protein kinase (MAPK) signaling in the disease process of *Lmna*^{+/-} mice

Activation of MAPK signaling cascade has recently been identified in hearts from mice homozygous for the LMNA mutation H222P[23], and this molecular pathway has previously been implicated in the development of cardiomyopathy, conduction defects[32,33] and apoptosis[34].

To further identify molecular mechanisms leading to heart failure and cardiac conduction disease in *Lmna*^{+/-} mice, expression of proteins in the MAPK cascade were measured by Western blot analyses from whole hearts lysates of three 20 week-old *Lmna*^{+/-} and two age-matched wildtype mice. There was no activation of MAPKs or downstream targets in heart tissue from *Lmna*^{+/-} mice. Immunoblots with antibodies against selected encoded proteins such as Elk-1: a transcription factor activated by MAPKs, ERK2: extracellular signal-regulated kinase 2 or p42 MAPK, JNK1: MAPK8, a member of the MAPK family, P44: p44/42 MAPK, and pc-Jun: phosphorylated c-Jun, downstream in c-Jun N-terminal kinase (JNK) pathway, showed similar expression in *Lmna*^{+/-} heart tissue as in wildtypes (Supplement Figure 2).

Discussion

We demonstrate that a heterozygous *Lmna*-null mutation reduced lamin A/C protein levels in hearts and caused early-onset cardiac conduction system disease and late onset DCM in mice. These phenotypes recapitulate cardiac manifestations caused by dominant human LMNA mutations. As previously described, cardiac dimensions and contractile properties of young *Lmna*^{+/-} mice were indistinguishable from wildtype. However, despite normal cardiac function, young 4-week old *Lmna*^{+/-} mice already had active conduction system disease with apoptotic cell death of the specialized cells of the AV node (Figure 5) and enhanced induced arrhythmogenicity that worsened with age (Table 1). In aged *Lmna*^{+/-} mice, ventricular function was significantly worse than wildtypes, and in 30% occurred without detectable conduction system disease. Both the absence of overt electrophysiologic disease in some aged *Lmna*^{+/-} mice with DCM and contractile abnormalities in isolated myocytes suggested an intrinsic myocyte deficit. We suggest that late onset DCM is a direct consequence of lamin deficiency in myocytes that can arise independent of electrophysiologic abnormalities.

Lmna^{+/-} mice exhibited the full spectrum of conduction system abnormalities and histopathology, as well as atrial and ventricular tachyarrhythmias, all disease features found in human patients with dominant LMNA mutations[10,35]. As such, *Lmna*^{+/-} mice were informative of the mechanism leading to AV node histopathology (absence of normal nodal myocytes and extensive fibrofatty replacement) found in individuals who succumb from laminopathies. We demonstrated normal anatomy of the cardiac electrophysiologic system in young *Lmna*^{+/-} mice, therein precluding the possibility that mutation in this nuclear membrane protein disrupted developmental patterning or lineage specification of the conduction system. However, changes in AV node structure were detected by 10 weeks. We interpret the finding of reduced lamin A/C protein and progressive demise of initially normal

conduction system myocytes as evidence that physiologic levels of nuclear lamins are an essential requirement for postnatal viability of AV nodal myocytes.

Several lines of evidence suggest intrinsic myocyte deficits rather than macroscopic structural changes as cause for increased arrhythmogenicity in *Lmna*^{+/-} mice: first, we showed that 10 week old *Lmna*^{+/-} mice displayed increased vulnerability to both induced atrial fibrillation as well as induced ventricular tachycardia in the absence to enlarged atria or ventricular chamber sizes and in the absence of ventricular dysfunction (see Figure 2 and Table 2); second, there was no increased fibrosis or rate of apoptosis in atria or ventricles of 10 week old *Lmna*^{+/-} mice. In addition we found that 40% of 10 week old *Lmna*^{+/-} mice with inducible arrhythmias showed no evident cardiac conduction system disease, suggesting that arrhythmogenicity was less likely a consequence of conduction system disease.

Reduced lamin A/C protein levels in heterozygous mutant mouse hearts were surprising, since mutations that create null alleles in structural proteins (e.g., cardiac troponin T, α tropomyosin) often do not alter cardiac structural protein levels[36,37]. However, as part of the inner nuclear membrane, lamin A/C possesses additional properties such as interaction with transcription factors [3], distinct from other structural proteins.

Lamin deficiency causes abnormal nuclear architecture[20,21]: on electronmicroscopy, *Lmna*^{+/-} nuclei had irregularities of shape and peripheral heterochromatin clumping[21]. We confirmed this and found that nuclei from both isolated conduction and non-conduction system myocytes of *Lmna*^{+/-} mice were elongated and dysmorphic (Figure 4).

Presuming that the abnormal nuclear morphology was mechanistically coupled to progressive loss of conduction system cells, we examined whether AV nodal myocytes were positive for markers of apoptotic cell death. Both TUNEL assays and immunohistochemical detection of activated caspase-3 confirmed apoptosis, not necrosis was the mechanism for cell death (Figure 5). Apoptosis has been hypothesized to account for conduction system demise in patients with lamin pathologies[38,39], although analyses have been limited to post-mortem case studies of end-stage disease. Our data on young *Lmna*^{+/-} mice provide evidence for a direct role of apoptosis in the evolution of cardiac electrophysiologic deficits. Further, we conclude that reduced lamin A/C levels induce apoptosis in conduction system cells.

Prior studies demonstrated apoptosis in ventricular myocytes from *Lmna*^{+/-} mice as the probable cause for early-onset DCM and heart failure[21]. We found no apoptotic myocytes in cardiac ventricles of young adult *Lmna*^{+/-} mice, despite their physical proximity to apoptotic AV nodal myocytes (Figure 5). Given that the nuclei from both AV nodal and ventricular myocytes appeared comparably abnormal, the selective cell death may imply a different threshold or trigger for apoptosis activation in distinct *Lmna*^{+/-} myocyte populations and similarly in skeletal myocytes.

Mechanical stress, one proposed apoptotic trigger in *Lmna*-deficient cells[40], was unlikely to be significantly different in myocytes that populate or surround the cardiac electrophysiologic system. However, a different susceptibility to apoptosis might reflect intrinsic molecular properties that distinguish conduction system myocytes from atrial and ventricular myocytes. For example, some molecules with enriched expression in conduction system myocytes (e.g., connexin 43 and transcription factor Hf1b) have altered levels and mis-localization in homozygous lamin mutant (N195K/N195K) mice[18]. The effects of *Lmna* mutations on proteins particularly important for conduction system structure or function might weaken these specialized cells and promote early apoptotic death. Activation of c-jun N-terminal kinase (JNK), a stress-activated protein kinase, has been previously

implicated in the process of cardiomyopathy and cardiac conduction system disease by downregulation of the gap junction protein connexin 43[32,33], and cardiomyocyte apoptosis[34]. However, the present study showed no c-Jun activation in hearts from *Lmna*^{+/-} mice.

A recently described function of lamin A/C provides another mechanism by which mutation could selectively predispose conduction systems myocytes to apoptosis. Because lamin A/C sequesters cFos at the nuclear envelope of fibroblasts and suppresses the DNA binding activity of transcription activator protein-1 (AP-1) by affecting cFos and cJun dimerization[3], reduced levels of lamin protein could have consequences on downstream transcription. If AV nodal myocytes, like other excitable neural cells, are particularly sensitive to AP-1 mediated transcriptional regulation, including caspase activation and stress-induced apoptosis[41], mutations that reduce lamin A/C levels could activate pro-cell death signals selectively in these specialized myocytes.

Using genome-wide profiling in hearts of mice carrying the *Lmna* H222P mutation, activation of mitogen-activated protein kinase (MAPK) signaling has been proposed as disease mechanism in LMNA-null mice[23]. In the present study, expression levels of distinct MAPKs and downstream targets were evaluated by immunoblotting and expression of these signaling molecules was not altered in *Lmna*^{+/-} mice in contrast to the *Lmna* H222P mouse model[23]. This finding emphasizes the difference between lamin haploinsufficiency and complete lamin deficiency. Furthermore, those analyses were only performed in whole heart and not dissected specialized conduction system tissue [23]. We suggest that different signaling pathways are involved in the development of cardiac conduction system disease and cardiomyopathy in the *Lmna*^{+/-} mouse model and humans with laminopathy than in homozygous mice bearing lamin mutations. We conclude that heterozygous mutations in *Lmna* in mice, and probably in humans, reduce cardiac lamin A/C and trigger apoptosis. Although cell death initially predominates in the AV node, over time ventricular myocytes also succumb, resulting in late-onset DCM and heart failure. We speculate that specialized properties of conduction system myocytes make these more susceptible than surrounding myocytes to pro-apoptotic signals triggered by reduced lamin levels. By extrapolation, variability in the manifestations of different LMNA mutations may reflect in part differences in the levels of nuclear lamin protein. This dosage effect was evident in the phenotypes of *Lmna*^{-/-} and *Lmna*^{+/-} mice. A corollary to this conclusion is that strategies that provide even modest increases in lamin levels may reduce laminopathy-associated heart disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviation list

<i>LMNA</i>	Human Lamin A/C gene
<i>Lmna</i>	Mouse Lamin A/C gene
DCM	Dilated cardiomyopathy
ECG	Electrocardiography
AV	Atrioventricular
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Figure 1.

Telemetry (A-C) and electrophysiologic studies (D, E) of 10-week old mice demonstrated progressive atrioventricular conduction defects and arrhythmia vulnerability. Representative telemetry recordings from (A) wildtype mouse showing normal heart rate, PR and QRS intervals; (B) *Lmna*^{+/-} mouse showing sinus bradycardia and first degree atrioventricular block (prolonged RR and PR intervals); (C) *Lmna*^{+/-} mouse with higher degree atrioventricular block (AV II Wenckebach). Rapid atrial pacing in SCL of 60msec (first 3 beats) results in atrial fibrillation lasting 1.8 seconds (D). RV pacing at SCL of 50 msec results in ventricular tachycardia, which terminates spontaneously after 16 seconds (E). In both D and E panels the upper trace represents surface ECG and the lower trace represents the corresponding right atrial intracardiac electrogram (RA IEGM). Abbreviations: SCL, sinus cycle length; S1, extra-stimulus;

Figure 2.

Anatomy of the cardiac conduction system is similar in 10-week old *Lmna*^{+/-} and wildtype mice. X-Gal staining (blue) was used to identify β-galactosidase expression in the whole heart (A), ventricles (B) and AV junctions (C) of wildtype (CCS:lacZ) and the whole heart (D), ventricles (E) and AV junctions (F) of *Lmna*^{+/-} (CCS:lacZ) mice. Bars: A, D: 800 μm; B, E: 1 mm; C, F: 200 μm.

Figure 3.

Histology of the AV node and surrounding tissues in 10-week old *Lmna*^{+/-} mice (C, D, G, H, K, L) and wildtype mice (A, B, E, F, I, J). *Lmna*^{+/-}/*CCS:lacZ* (A, B, E, F) and *Lmna*^{+/-}/*CCS:lacZ* (C, D, G, H) mice were stained with X-Gal to identify β -galactosidase activity and counterstained with hematoxylin-eosin stain (panel A through D) and with One-step Gomori Masson stain (panel E through H). The AV node (star) in *Lmna*^{+/-}/*CCS:lacZ* mice (C, D) was more disorganized than that of *Lmna*^{+/-}/*CCS:lacZ* (A, B) mice. Fibrosis was detected in the atrioventricular region of *Lmna*^{+/-}/*CCS:lacZ* (G, H: fibrosis light green in one-step Gomori Masson stain; orange arrowhead) but not *Lmna*^{+/-}/*CCS:lacZ* (E, F) and in *Lmna*^{+/-} mice (K, L: fibrosis blue in Masson trichrome stain, black arrowhead) but not wildtype mice (I, J). Bars: A, C, E, G, I, K: 200 μ m; B, D, F, H, J, L: 50 μ m.

Figure 4.

Isolated conduction system and non-conduction system myocytes (demarcated by arrowheads in (A) and (C) from 10-week old *Lmna*^{+/+}/*CCS:lacZ* (A, B) and *Lmna*^{+/-}/*CCS:lacZ* (C, D) mice. Conduction system myocytes were identified by their β-galactosidase activity (blue under white light). Myocyte sizes and shapes from *Lmna*^{+/-} mice (C) were indistinguishable from wildtype myocytes (A), but nuclei (DAPI stained blue under fluorescent light) from *Lmna*^{+/-} mice (D) were longer and thinner than nuclei in wildtype myocytes (B). Bars: A-D: 60 μm; Inset B, D: 7 μm.

Figure 5.

Apoptosis assessed by TUNEL and immunohistochemical detection of activated caspase-3 in AV node sections from 4-week old *Lmna*^{+/-} (D, E, F) and wildtype (A, B, C) mice, and ventricular sections in *Lmna*^{+/-} mice (G, H, I). Sections were stained with DAPI to detect nuclei (A, D, G). TUNEL-positive cells (green nuclei B, E, H), and activated caspase-3 (brown nuclei; C, F, I) were approximately 3-fold more in AV node sections from *Lmna*^{+/-} mice than in wildtype mice. Note lack of TUNEL-positive cells or activated caspase immunopositive cells in ventricular sections (H, I) from *Lmna*^{+/-} mice. Bars: A, B, D, E: 25 μ m; C, F, G, H, I: 75 μ m.

Figure 6.

Aged *Lmna*^{+/-} mice (age 50 weeks and greater) develop cardiac dilation and fibrosis without overt skeletal myopathy. Images depict cardiac morphology (A, B), cardiac histopathology (C, D) and skeletal muscle histology (E, F) of aged *Lmna*^{+/-} (B, D, F) and wildtype (A, C, E) mice (all age 50 weeks and greater). Hearts of *Lmna*^{+/-} mice are enlarged (B) and show fibrosis (D) as compared to wildtype (A, C). Skeletal muscle hematoxylin-eosin histology of *Lmna*^{+/-} (F) and wildtype (E) mice was indistinguishable. There was no increased fibrosis on Masson trichrome stain in *Lmna*^{+/-} (F inset) as compared to wildtype (E inset) skeletal muscles.

Bars: A, B: 1mm; C, D: 100μm E, F (including insets): 50 μm. (G) Sarcomere shortening (μm) of myocytes from aged *Lmna*^{+/-} mice was less than sarcomere shortening of aged wildtype myocytes.

Table 1

Electrophysiology data in 4-week and 10-week old mice.

	Age: 4 weeks			Age: 10 weeks		
	WT*	<i>Lmna</i> ^{+/-*}	P	WT*	<i>Lmna</i> ^{+/-*}	P
No. of mice	21	19		21	18	
AI [#]	2/21	5/19 (24%)	NS	0/21	10/18 (56%)	<0.001 [§]
AF	0/21	4/19 (21%)	0.037 [§]	0/21	7/18 (39%)	0.003 [§]
VT	2/21 (10%)	3/19 (16%)	NS	0/21	6/18 (33%)	0.006 [§]
AH (ms)	29.8±2.6	31.7±1.1	NS	33.6±3.6	34.4±5.5	NS
HV (ms)	10.3±1.9	13.4±1.6	NS	11.9±2.0	15.8±4.6	NS
AVI (ms)	40.1±2.8	45.1±2.5	NS	45.5±4.3	50.2±6.1	NS
AV WB (ms)	94±10	97±11	NS	96±11	109±12	0.028 [¶]
VA WB (ms)	130±22	123±31	NS	119±13	143±15	0.005 [¶]
AVERP (ms)	69±10	73±10	NS	63±11	83±22	0.024 [¶]
RVERP (ms)	43±19	45±18	NS	48±16	38±14	NS

* A subset of mice also carried the *CCS:lacZ* allele

[#] Abbreviations: WT, wildtype; AI, arrhythmia inducibility; AF, atrial fibrillation; VT, ventricular tachycardia; AH, AH interval; HV, HV interval; AVI, AV interval; AV WB, atrioventricular Wenckebach cycle length; VA WB, ventriculoatrial Wenckebach cycle length; AVERP, atrioventricular effective refractory period; RVERP, right ventricular effective refractory period; NS, not significant; ms, milliseconds

[§] Fisher's Exact test, WT vs. *Lmna*^{+/-}

[¶] Independent t-test, WT vs. *Lmna*^{+/-}

Table 2
Echocardiography of 10-week and aged (≥ 50 weeks old) wildtype (WT) and *Lmna*^{+/-} mice

	Age: 10 weeks			Age: >50 weeks		
	WT	<i>Lmna</i> ^{+/-}	p	WT	<i>Lmna</i> ^{+/-}	p
No. of mice	5	4		9	8	
LVWT* (mm)	0.91±0.04	0.87±0.04	NS	0.89±0.12	0.89±0.15	NS
LVEDD (mm)	2.60±0.28	2.73±0.11	NS	2.95±0.27	3.75±0.60	0.002 [¶]
LVESD (mm)	0.79±0.21	0.93±0.12	NS	1.02±0.29	1.92±0.88	0.012 [¶]
FS (%)	70.0±6.7	65.8±5.7	NS	65.7±7.8	50.9±14.9	0.019 [¶]

All echocardiographic measurements were performed at HR of more than 500bpm

* Abbreviations: WT, wildtype; LVWT, maximum left ventricular wall thickness; LVEDD, left ventricular end diastolic diameter; LVESD, left ventricular end systolic diameter; FS, Left ventricular fractional shortening (%) = [(LVEDD-LVESD)/LVEDD] × 100; HR, heart rate; NS, not significant;

[¶] Independent t-test, WT vs. *Lmna*^{+/-}