**Lmod2 piggyBac** mutant mice exhibit dilated cardiomyopathy

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**Abstract**

**Background:** Leiomodin proteins, Lmod1, Lmod2 and Lmod3, are key regulators of the thin filament length in muscles. While Lmod1 is specifically expressed in smooth muscles, both Lmod2 and Lmod3 are expressed in striated muscles including both cardiac and skeletal muscles. We and others have previously shown that Lmod3 mainly functions in skeletal muscles and the mutant mice display disorganized sarcomere. Lmod2 protein has been found to act as an actin filament nucleator in both cell-free assays and in cultured rat and chicken cardiomyocytes.

**Results:** To better understand the function of Lmod2 in vivo, we have identified and characterized a piggyBac (PB) insertional mouse mutant. Our analysis revealed that the PB transposon inserts in the first exon of the Lmod2 gene and severely disrupts its expression. We found that Lmod2⁴⁺/⁺ mice exhibit typical dilated cardiomyopathy (DCM) with ventricular arrhythmias and postnatal lethality. Electron microscope reveals that the Lmod2⁴⁺/⁻ hearts carry disordered sarcomere, disarrayed thin filaments, and distorted intercalated discs (ICDs). Those ICDs display not only decreased convolutions, but also reduced electron-dense staining, indicating less ICDs component proteins in Lmod2⁴⁺/⁻ hearts. Consistent with the phenotype, the expression of the ICD component genes, β-catenin and Connexin43, are down-regulated.

**Conclusions:** Taken together, our data reveal that Lmod2 is required in heart thin filaments for integrity of sarcomere and ICD and deficient mice exhibit DCM with ventricular arrhythmias and postnatal lethality. The Lmod2⁴⁺/⁻ mutant offers a valuable resource for interrogation of pathogenesis and development of therapeutics for DCM.

**Keywords:** Dilated cardiomyopathy, Leiomodin2 mutant, Sarcomere, Intercalated discs

**Background**

The Leiomodin proteins, Lmod1, Lmod2 and Lmod3, are a subgroup of the Tropomodulin (Tmod) protein family and are regulators of the thin filament length in muscles [1–3]. While Lmod1 is specifically expressed in smooth muscles, both Lmod2 and Lmod3 are expressed in striated muscles including both cardiac and skeletal muscles [4–6]. We and others have previously shown that Lmod3 mainly functions in skeletal muscles [6–8] and Lmod3 mutants exhibit muscle atrophy in fast fibers [6]. The mutant mice display disorganized sarcomere and the presence of nemaline bodies in skeletal muscles, a hallmark of the disease nemaline myopathy (NM), consistent with the finding that LMOD3 is mutated in the NM patients [7].

Lmod2 protein has been found to act as an actin filament nucleator in both cell-free assays and in cultured rat and chicken cardiomyocytes [2, 5]. Overexpression of Lmod2 results in the elongated thin filaments and knockdown exhibited disrupted sarcomere assembly in cultured cardiomyocytes [2, 5]. Furthermore, it has been shown that Lmod2 is an antagonist of Tmod1 in cardiomyocytes [2, 5]. Knockout mice of Tmod1 are embryonic lethal due to cardiac defects, and overexpression of Tmod1 in the heart causes myofibril disorganization.

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and dilated cardiomyopathy (DCM) [9–13]. However, the physiological function of Lmod2 remains unknown. We hypothesized that the phenotype of loss of Lmod2 in mice might mimic that of the overexpression of its antagonist Tmod1, and the mutant mice are high likely to carry DCM.

Dilated cardiomyopathy is a common form of cardiomyopathies and the third most common inheritable heart disease [14]. DCM is diagnosed as dilated left ventricular accompanied by systolic dysfunction, less than 50 % ejection fraction (EF) [15]. These symptoms may be accompanied by other complications, including arrhythmias, coagulation, and congestive heart failure (CHF), which often lead to lethality [16, 17]. It has been estimated that DCM causes at least half of heart failures and less than 50 % of the DCM patients survive beyond year 5 after the diagnosis [16, 18, 19].

To better understand the function of Lmod2 in vivo, we have identified a piggyBac (PB) insertional mutant that disrupts Lmod2 expression and carried out phenotypic characterization of this mutant. We show here that Lmod2 is crucial for postnatal survival and essential for cardiac function. Lmod2 deficient mice display DCM with disrupted sarcomeres and intercalated discs (ICDs) including the expression of ICD genes, which offer a new mouse model for this deadly disease.

Results

Generation of Lmod2-deficient mice

Our group has shown that the PB transposon is highly active in mice and human cells and could be used to rapidly generate a large collection of insertional mouse mutants in a cost-effective manner [20]. One of the PB mutants that we generated has an insertion in the Lmod2 gene (Lmod2PB/+ in FVB/N background) [21]. This PB transposon is inserted in the non-coding region of the first exon of the Lmod2 transcriptional unit (Fig. 1a) and significantly down regulated the expression of the gene as revealed by quantitative RT-PCR (less than 5 and 10 % in homozygous male and female in comparison to wild-type control Fig. 1b).

Tmod1 is an antagonist of Lmod2 at the pointed end of the thin filaments in cardiac muscle [5]. It has been suggested that the expression of Tmod1 could be affected by Lmod2 [5]. We therefore analyzed the transcriptional level of Tmod1 by quantitative PCR and found that the mRNA level of Tmod1 remains the same in 25 days old Lmod2PB/+ hearts compared to controls (Fig. 2a).

Lmod3, another member of the Leiomodin family, also expresses in heart together with Lmod2. It might be up regulated to functionally compensate the down-regulation of Lmod2. However, Western analysis revealed that the Lmod3 protein level in the hearts of Lmod2PB/PB is unchanged compared to its controls (Fig. 2b). Together, these data indicate that the phenotypes observed below are likely caused by reduction of Lmod2 gene activity alone and not a compound effect disrupting other Tmod family members.

Lmod2PB/PB mice exhibit postnatal lethality

Lmod2PB/PB mice are born with the expected ratio as well as their Lmod2PB/+ and wild-type littermates. This indicates that Lmod2 is not essential for mouse embryonic development.

While Lmod2PB/PB mice are born alive with normal appearance and body weight, the mutant animals exhibit postnatal death around 3rd week of age and are all dead by 9th week (Fig. 3a). Furthermore, male mutant animals are also underweight after three weeks (Fig. 3b). Heterozygous Lmod2PB/+ animals have normal life spans and display no discernable phenotype including fertility. This result indicates that Lmod2 is crucial for postnatal survival.

Lmod2PB/PB hearts display dilated cardiomyopathy defects

The Lmod2PB/PB mice have no overt signs of distress until 1 to 2 days before death. These signs include curling up, less movement, and dyspnea. Given that Lmod2 is expressed in heart [4], these phenotypes suggest that the mutant animals may suffer from cardiac dysfunction.

We therefore conducted histological and physiological analyses of the Lmod2PB/PB mutant hearts from 25 days old mice before the animals display any signs of distress.
Interestingly, all the analyzed \( \text{Lmod2}^{\text{PB/PB}} \) mutants display enlarged ventricular lumens and thin ventricular walls, phenocopying the symptoms in human DCM patients (\( n=17 \), Fig. 4a). The histopathological defects were also confirmed by echocardiography analysis. The mutant hearts had dilated ventricular lumens (Fig. 4b) and thinner heart walls during both systolic and diastolic periods (Fig. 4c). Consistent with these, morphometric...
muscles than the wild-type control (Fig. 5a, b, i). EM analysis of Lmod2 PB/PB hearts in comparison to wild-type control n = 5. b Higher LV end diastolic and systolic diameter. LVID left ventricular diameter. c Thinner LV anterior and posterior wall. LV PW left ventricular posterior wall. d Morphometric ratios (heart/body weight) of both male and female Lmod2 PB/PB mice are significantly increased. e Reduced ejection fraction and fractional shortening values. f Quantitative RT-PCR analysis of atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP) revealed increased expression of the heart hypertrophy and heart failure biomarkers n = 3. LV left ventricle; d diastolic; s: systolic. g, h Electronic Cardiogram revealed lengthening QTc value of Lmod2 PB/PB mice in comparison to controls

Discussion

Using PB insertional mutants, we have studied two members of the Lmod family, Lmod2 and Lmod3, which express in the cardiac and skeletal muscles. We previously reported that Lmod3 deficiency lead to severe skeletal muscle weakness with atrophy specific to fast fibers [6]. EM analysis revealed disorganized sarcomeres in skeletal muscles of Lmod3 mutant mice.

Here we report the characterization of Lmod2-deficient mice caused by PB insertion in the first exon. Unlike Lmod3-deficient mice that are born small with skeletal muscle atrophy [6], Lmod2-deficient animals are born with normal appearance and body weight. Only male Lmod2-deficient mice exhibit underweight in the 4th week. On the other hand, Lmod2-deficient mice exhibit postnatal lethality and all mutant animals die from 3 to 9 weeks after birth. This postnatal lethality together with previous report of Lmod2 in cultured cardiomyocytes [2, 5] suggests a role of the gene in cardiac function.

We have therefore carried out a pathological analysis of the Lmod2 mutant heart. Our study shows that the Lmod2 mutant hearts have enlarged ventricular with systolic dysfunction reflected with EF value less than 50 %, features that are characteristic to DCM patients. Investigation with transmission electron microscopy reveals that the Lmod2 PB/PB cardiac muscles exhibit disordered sarcomeres and ICDs. The morphologies of Z-discs, M-lines, and thin filaments in sarcomeres are all affected. Interconnected disc is composed of highly organized fascia adherens, gap junctions and desmosomes, and glues cardiomyocytes together. Disruption of ICD would also lead to cardiomyopathy [24]. Recently, it has been shown that mutation in non-ICD component protein could also result in ICD structure abnormality and cardiomyopathy [25]. In the previous reported case, an increase in convolutions of ICD was reported in the cardiomyopathy patient [25]. Here we show that Lmod2 PB/PB cardiomyocytes have a decrease in ICD convolution and cardiomyopathy. Disruption of ICD is also confirmed by reduction in electron density and expression of two of major ICD components.
genes, β-catenin and Connexin43 (Cx43), components of fascia adherens and gap junctions. Therefore, Lmod2 PB/PB mouse model represents a new ICD phenotype related to DCM. During preparation of our manuscript, we noticed that a report of Lmod2 knockout mouse characterization [26]. While DCM was observed in both models, there were differences in ultrastructure of cardiac muscles. In contrast to Lmod2 PB/PB phenotypes, Lmod2 knockout model reported widen Z-discs and increased convolutions of ICDs [26]. The difference could be due to the C57BL/6J background for the Lmod2 knockout and FVB/N for Lmod2 PB/PB.

The morphological defects in cardiac muscles proceed the symptom of heart failure in animals. Similarly, the expressions of the heart hypertrophy and heart failure biomarkers, atrial natriuretic factor (ANF) and brain natriuretic peptide (BNP), are elevated before the detection of heart failure. Together, these data show that Lmod2 deficiency leads to structure abnormality of cardiac muscles, which results in DCM, and that Lmod2 PB/PB mice offer a new model for studying DCM mechanisms and developing therapeutics.

**Conclusion**

Taken together, our data reveal that Lmod2 is required in heart thin filaments for integrity of sarcomere and ICD and deficient mice exhibit DCM with ventricular arrhythmias and postnatal lethality. The Lmod2 PB/PB mutant offers a valuable resource for interrogation of pathogenesis and development of therapeutics for DCM.
Methods

Mouse strains

The founder Lmod2PB/+ mouse was produced by a large-scale genome-wide insertional mutagenesis with the PB transposon in the FVB/N mice [20, 27].

Genotyping

Genomic DNA was isolated by a 56 °C overnight digestion of mouse toes in 200 μL lysis buffer (100 mM NaCl, 10 mM Tris pH 8.0, 25 mM EDTA, and 0.5 % SDS) plus fresh 0.1 mg/ml proteinase K. PCR was performed with primers within exon 1 of Lmod2 (forward: 5′-AGCTGTCGCTTCAATTTTTTCC-3′; reverse: 5′-TGTCCTCCAGCTCCCCTCTCAAG-3′, 247 bp product) and primers within the PB transposon (forward: 5′-CTGAGATGTCCTAAATGCACAGCG-3′ and reverse: 5′-TGTCCTCCAGCTCCCCTCTCAAG-3′, 819 bp product). PCR conditions used were as the following: an initial denaturation step at 93 °C for 3 min, then followed by a 40 cycle of 93 °C for 30 s/57 °C for 30 s/65 °C for 2 min, and one more elongation at 65 °C for 10 min as the final step.

Reverse transcription and quantitative RT-PCR

Total RNA was isolated from 3-week-old mouse heart with Trizol (Invitrogen). The cDNA was synthesized then using the PrimeScript™ RT reagent Kit (Takara). Quantitative RT-PCR was performed with AceQ qPCR SYBR Green Master Mix (Vazyme) according to the manufacturer’s instruction. All quantitative RT-PCR primers are listed in Table 1.

Western blotting

Hearts were dissected and lysed in RIPA buffer with Complete Protease inhibitors (Roche) and PMSF (Phenylmercury acetate) (Sigma) on ice. The BCA Assay (Themo) was applied to quantify total proteins extracted. The western blotting procedure was carried out according to standard protocol with a brief introduction here. Protein bands were separated by SDS/PAGE and transferred to nitrocellulose membranes (Immobilon-P). The transferred membrane was blotted first 5 % skim milk for 1 h. Blots were then incubated with a primary antibody and a secondary antibody consecutively to detect protein of interest. Primary antibodies used are the following: Lmod3 (1:1000; HPA036034, Sigma), GAPDH (1:3000; AC001, ABclonal). Secondary antibody used is the Anti-rabbit IgG antibody conjugated to PerCP-Cy5.5 (1:3000; sc-45101, Santa Cruz). Signal was finally visualized by enhanced chemiluminescence (34,080, Themo).

Echocardiography and ECG

Mice were anesthetized by inhalation of 1–2 % isoflurane delivered in a gas mixture with oxygen, medical-grade compressed air, and nitrogen. The anesthetized mice went through echocardiography with the Vevo 770 microultrasound system (VisualSonics Inc. Toronto, Canada). Both parasternal long-axis and short-axis views of the left ventricles were analyzed with a 707B transducer according to the manufacturer’s instruction. Data were recorded in M mode.

Conscious and unrestrained mice were used for ECG analysis with the ECGenie electrocardiography system (Mouse Specifics, Inc., Boston, MA) according to the manufacturer’s instruction. Before experimental tests, mice should be trained for 3–5 days to behave normally in this machine. Only data from continuous recordings of 20–30 ECG signals were included in the final analyses. A physiologic waveform analysis platform “eMOUSE” (Mouse Specifics, Inc.) was applied for data analysis.

Transmission electron microscopy

Samples for transmission electron microscopy were prepared using the standard procedure: hearts were excised, washed in PBS, fixed in 2.5 % glutaraldehyde in 0.1 M cacodylate buffer for more than 2 h, washed in 0.1 M cacodylate buffer for 3 × 15 min, post-fixed with 1 % potassium ferrocyanide reduced OsO4 on ice for 2–3 h, dehydrated through graded ethanol and acetone, and finally embedded in EMbed 812 resin. Ultrathin sections (60 nm) were analyzed on a Tecnai G2 Spirit BioTWIN electron microscope, and images were collected with a 4 × 4 digital camera (Optronics). Length of sarcomere was measured using ImageJ.

Table 1 Quantitative RT-PCR primers

| Gene       | Forward (5′ → 3′) | Reverse (5′ → 3′) |
|------------|------------------|------------------|
| Lmod2      | ACCTTATCCCGAATTTGCTGA | ACCTTGAGCATGTCTCAAGAG |
| GAPDH      | TGTTCTCTACCCCCAAATGTGTA | GGAATGCTGCTGAACTGGCC |
| Tmod1      | TGAGCTAGTATGACTACGCCTGG | GGCCTTAAATTTCTCTCGGC |
| ANF        | CATCACCTCGCTTCTCTCCTG | CATCACCTCGCTTCTCTCCTG |
| BNP        | CGGCCATGATGATCCTCGGAG | GCAGCATGATGATCCTCGGAAG |
| Plakoglobin| TGGCAACAGACACATACACCTTGG | GTGGTAGCTTCTTCTGATGTG |
| N-cadherin | AGGCCAGTCTTACCGAGGG | TGGCGTCTTACACATGGAA |
| Vinculin   | GAGGCTGAACTGCTTCAATC TTACCA | CCAAGATGACAGGACTGGCTA |
| β-catenin  | ATGGAGCCGCGACAGAAAAATGCA | CTGCTCACCTCAGGAAGGAGGA |
| Cx43       | ACAGCCGTTGATCGACCTTG | GAGAGATGGGGGAAGGAGGCTTT |

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Histology
Hearts were excised, washed in PBS, fixed in 4 % paraformaldehyde solution (Sangon Biotech) overnight, dehydrated, and embedded in paraffin. Longitudinal or transverse sections (10 μm) were stained with a Masson Trichrome Stain Kit (Maixin Biotech) or according to the classical hematoxylin and eosin (H&E) staining protocol (Sigma), and finally mounted with DPX Mountant (Sigma). Light microscopy images were captured using a Nikon Eclipse microscope with a Nikon DS-RiI camera and/or a Zeiss Axio Zoom V16 stereomicroscope with an AxioCam MRc camera.

Statistics
Data were presented as mean ± SED in figures. Two-tailed unpaired Student’s t-test was used unless otherwise stated. The significance is indicated as the label of one or more * with the following categories: (1) * P < 0.05; (2) ** P < 0.01; 3) *** P < 0.005. Prism 6 (GraphPad Software) was used for plotting.

Abbreviations
ANF: atrial natriuretic factor; BNP: brain natriuretic peptide; CHF: congestive heart failure; DCM: dilated cardiomyopathy; ECG: electrocardiography; EF: ejection fraction; EM: electron microscope; FS: fraction shortening; ICD: intercalated discs; Lmod: leiomodin; Lmod1: leiomodin 1; Lmod2: leiomodin 2; Lmod3: leiomodin 3; LV: left ventricle; LVH: left ventricular anterior wall; LVID: left ventricular diameter; LVPW: left ventricular posterior wall; PB: piggybac; QTc: corrected QT interval; Tmod1: tropomodulin 1.

Authors’ contributions
SL, TX and LVS designed the project; SL, KM, HT, CC, and SS did the experiments and collected data; SL, KM, LT, SD, TL, XW, FL, ZZ, TX and LVS supervised the data; SL, TX and LVS prepared the manuscript; TX and LVS supervised all experiments and collected data; SL, KM, HT, CC, and SS did the experiments.

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Competing interests
The authors declare that they have no competing interests.

Consent for publication
Not applicable.

Ethics approval and consent to participate
All animal procedures in this article have been approved by the Animal Care and Use Committee of the Institute of Developmental Biology and Molecular Medicine, Fudan University, Shanghai, China.

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