Activation of sarcolipin expression and altered calcium cycling in LMNA cardiomyopathy

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A B S T R A C T

Cardiomyopathy caused by A-type lamin genes (LMNA) mutations (LMNA cardiomyopathy) is associated with dysfunction of the heart, often leading to heart failure. LMNA cardiomyopathy is highly penetrant with bad prognosis with no specific therapy available. Searching for alternative ways to halt the progression of LMNA cardiomyopathy, we studied the role of calcium homeostasis in the evolution of this disease. We showed that sarcolipin, an inhibitor of the sarco/endoplasmic reticulum (SR) Ca2+ ATPase (SERCA) was abnormally elevated in the ventricular cardiomyocytes of mutated mice compared with wild type mice, leading to an alteration of calcium handling. This occurs early in the progression of the disease, when the left ventricular function was not altered. We further demonstrated that down regulation of sarcolipin using adeno-associated virus (AAV) mediated RNA interference delays cardiac dysfunction in mouse model of LMNA cardiomyopathy. These results showed a novel role for sarcolipin on calcium homeostasis in heart and open perspectives for future therapeutic interventions to LMNA cardiomyopathy.

1. Introduction

Mutations in the gene encoding A-type nuclear lamins (LMNA) cause dilated cardiomyopathy associated with conduction defects (LMNA cardiomyopathy) [1–3]. Lamins are intermediate filament proteins that polymerize to form the nuclear lamina, a fibrous meshwork underlining the inner nuclear membrane of most eukaryotic cells [4,5]. Despite optimization of conventional therapeutic strategies, terminal heart failure and post transplantation complications represent by far the most frequent cause of death. There are currently no effective treatments for LMNA cardiomyopathy. Thus, it is necessary to clarify its pathological mechanisms and to search for potential compounds to protect against LMNA cardiomyopathy.

To study the pathophysiology of LMNA cardiomyopathy and to test possible therapeutics, murine models of the disease [6] have enabled insights into pathological mechanisms [7–11]. All these mechanisms have been targeted with small-molecules in mice and showed some beneficial impact. However, these approaches were not curative. Searching for alternative ways to slow down the cardiac disease progression, we here examined the involvement of calcium homeostasis in the LmnaH222P/H222P mouse model of the disease. Work from others converges to the conclusion that unhinged calcium (Ca2+) handling in the cardiomyocytes plays a central role in initiation and progression of dilated cardiomyopathy [12]. The regulation of Ca2+ cycling is finely tuned by various proteins and regulatory processes, and is unhinged in cardiomyopathy. Therefore, the components of the Ca2+ cycling apparatus have been widely studied, aiming to restore the Ca2+ cycling process and ultimately improve the cardiac function. We here showed that sarcolipin (SLN), an inhibitor of the sarco/endoplasmic reticulum (SR) Ca2+ ATPase (SERCA), was abnormally elevated in LMNA cardiomyopathy, leading to an alteration of calcium cycling. These results establish a novel role for sarcolipin on calcium homeostasis in cardiac muscle and provide a rationale for future therapeutic interventions in LMNA cardiomyopathy.

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coding sarcolipin (SLN), in This analysis highlighted activation of the expression of a mutation, the Lmna LMNA cardiomyopathy, we studied a mouse model that display a phosphorylation of PLB on residues Ser16 and Thr17 in the hearts from (Fig. 3A, Fig. S2B). This was ensuing an increased relative phosphor-
was increased in these cells relative to HL-1 (Fig. 3D). These results to assess the balance between SLN and PLB expression. We generated
proteins was altered in clinging, we investigated whether the expression of calcium handling type mice [7]. The up-regulation of SLN was also observed at 6 months old male Lmna Lmna H222P/H222P mouse model [13]. Lmna p.H222P mutation was found associated with dilated cardiomyopathy in human [14]. We previously studied expression of mRNAs isolated from hearts of young Lmna H222P/H222P and wild type mice at 10 weeks of age [7]. This analysis highlighted activation of the expression of Sln gene, encoding sarcoplasm (SLN), in Lmna H222P/H222P mice compared with wild type mice [7]. The up-regulation of SLN was also observed at 6 months of age in cardiac ventricles from Lmna H222P/H222P mice (Fig. 1), when the left ventricular function was altered. SLN activation was also observed at a younger age, when no left ventricular dysfunction was reported (Fig. 2).
Given that several proteins tightly regulate defective [Ca2+]i cycling, we investigated whether the expression of calcium handling proteins was altered in LMNA cardiomyopathy. We found that expression of SERCA2a (Fig. 2, Fig. S2A) and NCX (Fig. 2, Fig. S2A) were not affected in hearts of 6-month old Lmna H222P/H222P mice. We also reported that expression of these proteins was not different from wild type mice at 3 months of age, when the left ventricular function was normal (Fig. 2). The expression of phospholamban (PLB), a regulator of SERCA2a, was decreased in the hearts from the Lmna H222P/H222P mice (Fig. 3A, Fig. S2B). This was ensuing an increased relative phosphorylation of PLB on residues Ser16 and Thr17 in the hearts from Lmna H222P/H222P mice (Fig. 3B, Fig. S2B). We then used a cellular model to assess the balance between SLN and PLB expression. We generated HL-1 cells stably expressing FLAG-tagged SLN (Fig. 3C). PLB expression was increased in these cells relative to HL-1 (Fig. 3D). These results demonstrated a correlation between enhanced SLN expression and decreased PLB expression in cardiac cells.

2.2. Altered calcium homeostasis in Lmna H222P/H222P mice caused by SLN up-regulation

Given that SLN is a regulator of cardiac calcium handling through its inhibitory role on SERCA2a, we next hypothesized that over-expression of SLN in Lmna H222P/H222P mice could affect calcium homeostasis before detectable cardiac dysfunction. [Ca2+]i transients were elicited by electrical stimulation in ventricular cardiomyocytes from 3-month old Lmna H222P/H222P mice. Given that A-type lamins are important in nuclear pores organization [15], through which Ca2+ is diffused into the nucleus, and that nuclear Ca2+ is involved in gene transcription activation, we therefore set-up the scanning line through the nucleus to simultaneously record cytosolic [Ca2+]i, and nuclear [Ca2+]n transients (Fig. 4A). The peak [Ca2+]i transient (F/F0) was not significantly different between ventricular cardiomyocytes from Lmna H222P/H222P mice and wild type mice (Fig. 4B), which is consistent with the normal heart contractile function reported in Lmna H222P/H222P mice at early stage (3 months) (Fig. S1). We showed that kinetics of the [Ca2+]i transients, were slowed in ventricular cardiomyocytes from Lmna H222P/H222P mice compared with cardiomyocytes from wild type mice. The [Ca2+]i transient decay time was significantly prolonged in myocytes from Lmna H222P/H222P mice compared with those from wild type mice, suggesting slower Ca2+ uptake by the SERCA2a pump. However, the nuclear [Ca2+]n transient was not significantly different between the two types of cardiomyocytes (Fig. 4B). This suggests that sarcoplasmic reticulum (SR) calcium uptake was slowed in Lmna H222P/H222P mice, although the amplitude of the [Ca2+]n transient was maintained at this age. Because [Ca2+]i transients are triggered by the action potentials (AP), we next investigated whether AP characteristics were altered in cardiomyocytes from Lmna H222P/H222P mice. We showed that the zero current potential (Fig. 5A and B), amplitude (Fig. 5B), maximum dV/dt (Fig. 5C) and duration (Fig. 5D) were not significantly different between cardiomyocytes from Lmna H222P/H222P mice and wild type mice. The densities of outward potassium currents (transient outward Ito, ultrarapid IKur and sustained Iss) involved in AP repolarization phase were also similar between cardiomyocytes from Lmna H222P/H222P mice and wild type mice. The fact that the total membrane surface, measured as membrane capacitance, was smaller in cardiomyocytes isolated from Lmna H222P/H222P mice (Fig. 6A, B, 6C) despite the fact that the total membrane surface, measured as membrane capacitance, was smaller in cardiomyocytes isolated from Lmna H222P/H222P mice (Fig. 6D). Altogether, these data suggest that the alterations in [Ca2+]i handling were independent on an alteration of the electrophysiological characteristics of the cardiomyocyte from Lmna H222P/H222P mice.

The amount of Ca2+ released in each twitch depends on the amount of Ca2+ stored in the sarcoplasmic reticulum (SR), which can be estimated by rapid caffeine application (Fig. 4C). Fig. 4D showed no
Fig. 3. Expression of protein involved in calcium handling in LMNA cardiomyopathy.

(A) Representative immunoblots showing total PLB, phosphorylated PLB(Ser16) and phosphorylated PLB(Thr17) expressions in hearts from 6 months-old male Lmna
H222P/H222P and wild type (WT) mice. GAPDH was shown as loading control.

(B) Schematic representation of the construct.

(C) Representative immunoblots showing total PLB and phosphorylated PLB(Ser16) expressions in HL-1 cells stably expressing FLAG epitope-tagged SLN (FLAG-SLN).

(D) Micrographs showing PLB labeling in HL-1 cells (empty) and HL-1 cells stably expressing FLAG epitope-tagged SLN (FLAG-SLN). Nuclei are counter-stained with 4',6-diamidino-2-phenylindole (dapi).
significant difference in SR Ca$^{2+}$ load (Peak [Ca$^{2+}$], transient (F/F$_0$)), which is consistent with the normal [Ca$^{2+}$], transient amplitude. Moreover, the decay time constant during caffeine perfusion between cardiomyocytes isolated from Lmna$^{H222P/H222P}$ mice and wild type mice was also similar, showing no changes in Ca$^{2+}$ extrusion though the NCX in myocytes from the mutated mice, and thus indicating that the slowing on the decay phase of the electrically evoked [Ca$^{2+}$], transient (Fig. 4B) is due to SERCA slowing.

2.3. SLN up-regulation induces cardiac dysfunction

We next tested the hypothesis that SLN overexpression influences left ventricular function in vivo. We injected adeno-associated virus (AAV) vector expressing GFP-tagged shRNA against SLN (shSLN) in 1 month-old Lmna$^{H222P/H222P}$ mice. Following four months of treatment, the mice were analyzed by echocardiography and sacrificed for biochemical and histological studies. There was less alteration of myocardial fibrosis in hearts from Lmna$^{H222P/H222P}$ mice infected with GFP-tagged shSLN relative to mice injected with PBS (Fig. 7A and B). Compared with non-transduced Lmna$^{H222P/H222P}$ mice, for which the fractional shortening decreased overtime, fractional shortening in shSLN-transduced Lmna$^{H222P/H222P}$ mice did not change at 4 months of age (Fig. 7C, Table 1). This effect on the left ventricular function was transient as shSLN-transduced Lmna$^{H222P/H222P}$ mice showed an altered cardiac function no significantly different to non-transduced Lmna$^{H222P/H222P}$ mice at 5 months of age (Fig. 7C, Table 1). The inhibition of SLN expression in Lmna$^{H222P/H222P}$ mice (Fig. 7D) was followed by an increased cardiac expression of PLB (Fig. 7E, Fig. S3). This was followed by a decreased phosphorylation of PLB on residues Ser16 and Thr17 reported to total PLB expression in the hearts from shSLN-transduced Lmna$^{H222P/H222P}$ mice compared with non-transduced mice. (Fig. 7E, Fig. S3). These results showed that partially inhibiting SLN in vivo is efficient to delay the left-ventricular function in a mouse model of LMNA cardiomyopathy.
3. Discussion

We showed in this study that expression of cardiac SLN, an inhibitor of the sarco/endoplasmic reticulum (SR) Ca\(^{2+}\) ATPase (SERCA), was up regulated in ventricles from *Lmna*\(^{H222P/H222P}\) mice and [Ca\(^{2+}\)], transients were slowed down early before the development of cardiomyopathy symptoms. We therefore hypothesized that chronic SERCA2a inhibition contributes to cardiac muscle pathogenesis in LMNA cardiomyopathy. We tested our hypothesis using shRNA approach in *Lmna*\(^{H222P/H222P}\) mice, and found that reduction in SLN expression was sufficient to delay left ventricular dysfunction. These results suggest a novel mechanism in the regulation of pathophysiology in LMNA cardiomyopathy.

We have reported for the first time an alteration in the Ca\(^{2+}\) handling in cardiomyocytes from *Lmna*\(^{H222P/H222P}\) mice. The prolongation of the [Ca\(^{2+}\)] \(t\) transient duration can be explained either by impairment in Ca\(^{2+}\) re-uptake by the SR through SERCA2a or by a decrease in Ca\(^{2+}\) extrusion though NCX1 [16,17]. Our data suggest that only the re-capture by the SR mechanisms was altered in cardiomyocytes from *Lmna*\(^{H222P/H222P}\) mice. A decreased SERCA2a function is commonly observed in models of heart failure, with both reduced and preserved ejection fraction (HfPEF) [18,19]. While in overt heart failure SR Ca\(^{2+}\) content and [Ca\(^{2+}\)] \(t\) transient amplitudes are depressed, accounting for reduced contractile function, in HfPEF those parameters are normal as in *Lmna*\(^{H222P/H222P}\) mice. The preservation in SR Ca\(^{2+}\) load in *Lmna*\(^{H222P/H222P}\) mice suggests that SERCA2a can keep pumping enough Ca\(^{2+}\) to maintain cell contraction, notwithstanding an alteration of its function. Thus the decreased Ca\(^{2+}\) re-uptake in *Lmna*\(^{H222P/H222P}\) mice is still not enough to decrease SR Ca\(^{2+}\) at normal heart rate, so contractile function can be sustained for a while, but the left ventricular function will ultimately decline overtime (Fig. S1).

SERCA2a function in cardiac muscle is regulated by SR membrane proteins, PLB and SLN, both inhibiting SERCA function [20,21]. Here, we showed that the abnormal activation of SLN in cardiac cells and in the heart of a murine model of LMNA cardiomyopathy was followed by a decreased PLB expression. Our results are divergent from work from others [22], as we showed that SLN overexpression induced changes in the expression of PLB. Because a number of conditions may affect PLB expression including sample preparation, disease state, and model, these findings may require additional experimentation. Our findings suggest that the stoichiometry between PLB and SLN is important for the regulation SERCA2a in cardiac pathophysiology, according to work from others [23]. It has been previously described that overexpression of SLN is slowing calcium reuptake by the SR, demonstrating that SLN inhibits SERCA2a, which leads to decreased rates of calcium uptake and decreased contractility [21,24]. Nevertheless, our data show that the decrease in PLB expression was not sufficient to maintain the physiological Ca\(^{2+}\) re-uptake in cardiomyocytes from *Lmna*\(^{H222P/H222P}\) mice, suggesting that the slowed SERCA2a function was related to the activation of SLN expression.

Ca\(^{2+}\) handling alteration may be involved in cardiac dysfunction that appears progressively in *Lmna*\(^{H222P/H222P}\) mice. [Ca\(^{2+}\)], plays an important role in the progression of cardiac disease by activating the so-called excitation-transcription coupling [25]. This mechanism is dependent on calcineurin activation by Ca\(^{2+}\) in the cytosol and/or Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) in the nucleus [26,27]. The impeded [Ca\(^{2+}\)], transient duration could activate calcineurin signalling, leading to LMNA cardiomyopathy. Supporting this hypothesis, it has been shown that SLN overexpression increases calcineurin activity [28] while SLN deletion tends to inhibit calcineurin activity [29,30].

Regulation of SERCA2a by targeting SLN expression in LMNA cardiomyopathy could be an interesting approach to normalize Ca\(^{2+}\) homeostasis. Consistent with our data, recent work in Duchenne muscular dystrophy (DMD), a severe form of muscular dystrophy with cardiomyopathy [31], reported a cardiac SLN overexpression [32].
In this study, Voit et al. described a decreased SERCA2a function activity in the heart of a DMD mouse model, the mdx mice [32]. Furthermore, these authors and others showed that the reduction in SLN expression in mdx mice was sufficient to improve the SERCA function in heart [32] and in skeletal muscle [33]. These data, together with the present study, suggest that SLN could be a target to improve cardiac function in inherited cardiomyopathies. However, the impact of reduction in SLN expression on SERCA2a function remains to be tested in LMNA cardiomyopathy. The partial effect on cardiac function by reduction in SLN expression could reflect the fact that other molecular and cellular defects reported in LMNA cardiomyopathy [7–11,34,35] were not targeted by our approach and could still hamper the cardiac function. Therefore, our work encourages further approaches to mechanistically assess the role played by SLN on calcium handling.

Our findings suggest that drugs that could correct unhinged calcium handling would ameliorate LMNA cardiomyopathy. In this regard, we previously showed that the use of pyridazinone derivative Ca\(^{2+}\) sensitizing agent SCH00013 was beneficial on cardiac function in Lmna\(^{H222P/H222P}\) mice, inducing increased life expectancy and decreased fibrosis [36]. We therefore brought further evidence that the role of calcium handling is of importance in the development of LMNA cardiomyopathy. Moreover, SLN overexpression has been detected in humans with different cardiac pathologies contributing to the contractile dysfunction [37,38]. Therefore, we could speculate that the regulation of SLN may not be specific to LMNA cardiomyopathy but occurs in several cardiomyopathies.

In conclusion, we showed for the first time that the calcium handling is disturbed in LMNA cardiomyopathy before overt signs of cardiac dysfunction, which can be, at least partially due to SLN overexpression. This work opens novel perspectives for further therapeutic interventions. How mutated A-type lamins regulate SLN expression remains an unanswered question. A-type lamins is known to have regulatory roles on gene transcription [39–41], through interaction with gene promoters [42]. LMNA mutants could therefore lead to alteration of A-type lamins–chromatin associations and transcriptional defects [43], which could have downstream effects on cardiac function [44]. Whether mutated A-type lamins alter SLN promoter is unclear and would requires further investigation.
4. Methods

4.1. Animals

All animal experiments were approved by the French Ministry of Health at the Center for Research in Myology for the Care and Use of Experimental Animals (approval number #5556). Experiments were performed according to guidelines from directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes. Lmna<sup>H222P/H222P</sup> mice were fed standard chow and housed in a disease-free barrier facility with 12h/12h light/dark cycles. Mice were sacrificed with cervical dislocation.

4.2. Isolation of mouse cardiomyocytes

Wild type and Lmna<sup>H222P/H222P</sup> mice were anesthetized with intraperitoneal injection of sodium pentobarbital (50 mg/kg). Ventricular cardiomyocytes were isolated as described earlier [45].

4.3. Confocal microscopy and electrophysiological measurements

Isolated ventricular cardiomyocytes were loaded with the fluorescence Ca<sup>2+</sup> dye Fluo-3 AM and viewed with a Leica microscopy in the line scan mode as earlier described [45]. [Ca<sup>2+</sup>]<sub>i</sub> transients were elicited by field stimulation through two Pt electrodes at 2 Hz. Ca<sup>2+</sup> sparks were recorded in quiescent myocytes and SR Ca<sup>2+</sup> load estimated by rapid caffeine (10 mM) application right after electrical stimulation at 2 Hz. Images were analyzed by home-made routines in IDL (Harrys Aerospatiale). The patch-clamp method in the configuration of whole cell was used to record action potentials in the current clamp configuration and potassium currents in the voltage-clamp configuration. Protocols and solutions were previously described [45].

4.4. Protein extraction and western blot analysis

Total proteins were isolated from heart tissue or cultured cell in extraction buffer (Cell Signaling). The heart samples were extracted with Lysis-D tube using Fast-Prep (3 pulses of 45 s), the cells samples
for immunoblotting were Alexa Fluor 488-conjugated goat anti-chicken IgG (Life Technologies).

were sonicated (5 pulses of 10 s at 30% amplitude) to allow dissociation of protein from chromatin and solubilization. Sample protein content was determined by the Bicinchoninic Acid Assay protein assay (Thermo Fisher Scientific). Extracts were analyzed by SDS-PAGE using Tris-glycine gels (Life Technologies) and transferred onto nitrocellulose 0.2 μm membrane (novex, Life Technologies). Membranes were blocked 1 h with 5% bovine serum albumin (BSA) in phosphate buffer saline containing 1% Tween 20 (PBS-T). Subsequently to blocking, the membranes were incubated with primary antibodies overnight at 4 °C. After PBS-T washes membranes were incubated with anti-anti-chicken, anti-mouse or anti-rabbit antibodies for 1 h at room temperature and washed again with PBS-T. The signal was then revealed using Immobilon Western Chemiluminescent HorseRadish Peroxidase (HRP) Substrate (Millipore) on a G-Box system with GeneSnap software (Ozyme).

4.5. Cell culture and reagents

HL-1 (Merck Millipore) were maintained at 37 °C with 5% CO₂. HL-1 cells were culture in Claycomb Medium (Sigma-Aldrich) supplemented with 10% PBS (Invitrogen), 1% penicillin/streptomycin (Invitrogen), norepinephrine 0.1 mM (Sigma-Aldrich) and L-glutamine 2 mM with the required gelatin/bronectin coating. HL-1-SLN cells were created using lentiviral vector for 24h followed by a 3-day incubation of normal medium and a 3–5 days puromycin selection.

4.6. Antibodies

Primary antibodies used were: SLN for Western blot (Merck Millipore, #ABT13), SERCA2a (Abcam, #ab2861), PLB (Cell Signaling, #14562), phospho-PLB(Ser16) (Santa Cruz, SC-12963-R), phospho-PLB (Thr17) (Santa Cruz, SC-17024-R), FLAG (Sigma, F3165), GAPDH (Santa Cruz, sc-47724). Secondary antibodies for immunoblotting were HRP-conjugated donkey anti-chicken (Invitrogen), rabbit anti-mouse or goat anti-rabbit IgG (Jackson Immunoresearch). Secondary antibodies for immunofluorescence were Alexa Fluor 488 conjugated goat anti-rabbit IgG, Alexa Fluor 568-conjugated goat anti-mouse IgG, Alexa Fluor 488-conjugated goat anti-chicken IgG (Life Technologies).

4.7. Immunofluorescence microscopy

Cells were grown in coated (gelatin/bronectin) coverslips. Cells were fixed (10 min, 4% paraformaldehyde in PBS at room temperature), permeabilized (8 min, 0.5% Triton X-100 in PBS), blocked (1h, in PBS with 0.3% triton X-100 and 5% BSA) and incubated with primary antibodies (1h30 at RT, in PBS with 1% triton X-100 and 1% BSA). The section were then washed in PBS (3 times 5 min) and incubated with secondary antibodies (1 h at RT, in PBS with 1% triton X-100 and 1% BSA) and washed (3 times 5 min).

4.8. Histology

Hearts were fixed in 4% formaldehyde for 48h, embedded in paraffin, sectioned at 5 mm and stained with Sirius red and hematoxylin/eosin. Representative stained sections were photographed using a Microphot SA (Nikon) light microscope attached to a Spot RT Slide camera (Diagnostic Instruments). Images were processed using Adobe Photoshop CS (Adobe Systems) [35].

4.9. Echocardiography

Mice were anesthetized with 0.75% isoflurane in O₂ and placed in a heating pad. Transthoracic echocardiography was performed using a Vivid 7 Dimension/Vivid7 PRO ultrasound with an 11 MHz transducer applied to the chest wall [34,35].

4.10. Construction and injection of AAV encoding sh-SLN

AAV9-GFP-U6-mSLN-shRNA (VectorBiolabs) recombinant AAV viruses were produced through co-transfecting HEK293 cells with (i) the AAV plasmid encoding sh-SLN, (ii) the adenovirus helper plasmid and (iii) the AAV packaging plasmid encoding the rep2 and cap. Two days after transfections, cells were harvested, and AAV vectors were purified through CsCl-gradient ultra-centrifugation. Vector titers (GC/ml - genome copies/ml) were determined by real-time PCR. One month-old mice were injected with AAV9-GFP-U6-mSLN-shRNA into the retro-orbital vein (5 × 10¹³ viral genomes/kg in 100 μl).

4.11. Statistics

Graphics were performed using GraphPad Prism 8 software. For
both in vivo pharmacological studies and each parameter, statistical analyses were performed to evaluate the pathology effect by comparing LmnaH222P/H222P mice versus wild type mice, then for the study from 3 to 5 months to evaluate the effect of treatment on LmnaH222P/H222P mice by comparing the AAV9-shSLN treatment group to the PBS treatment group. For each objective an analysis of variance (ANOVA) with interaction was performed on factors “Time” (repeated) and “Group” or “Treatment” depending on the case. The variances heterogeneity was taken into account if necessary. Appropriate post-hoc analysis was then performed. The significance level is taken to 5%, except for the interaction test for which the significance level is taken to 10%. For in vitro pharmacology studies and each parameter, statistical analyses were performed to evaluate the pathology effect by comparing LmnaH222P/H222P mice versus wild type mice. A student t-test for mRNA expression and Cm parameter, a mixed effect model for calcium cycling, action potentials and sparks characteristics were carried out. The variances heterogeneity was taken into account if necessary. A log-transformation of sarcolipin decreases myocyte contractility and calcium transient, Cardiovasc. Res. 65 (2005) 177–180.

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