Mouse model carrying H222P-Lmna mutation develops muscular dystrophy and dilated cardiomyopathy similar to human striated muscle laminopathies

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Laminopathies are a group of disorders caused by mutations in the LMNA gene encoding A-type lamins, components of the nuclear lamina. Three of these disorders affect specifically the skeletal and/or cardiac muscles, and their pathogenic mechanisms are still unknown. We chose the LMNA H222P missense mutation identified in a family with autosomal dominant Emery–Dreifuss muscular dystrophy, one of the striated muscle-specific laminopathies, to create a faithful mouse model of this type of laminopathy. The mutant mice exhibit overtly normal embryonic development and sexual maturity. At adulthood, male homozygous mice display reduced locomotion activity with abnormal stiff walking posture and all of them die by 9 months of age. As for cardiac phenotype, they develop chamber dilation and hypokinesia with conduction defects. These abnormal skeletal and cardiac features were also observed in the female homozygous mice but with a later-onset than in males. Histopathological analysis of the mice revealed muscle degeneration with fibrosis associated with dislocation of heterochromatin and activation of Smad signalling in heart and skeletal muscles. These results demonstrate that LmnaH222P/H222P mice represent a good model for studying laminopathies affecting striated muscles as they develop a dystrophic condition of both skeletal and cardiac muscles similar to the human diseases.

INTRODUCTION

The nuclear lamina is a fibrous meshwork of type V intermediate filaments, the lamins, localized at the internal face of the inner nuclear membrane. This structure is fundamental in sustaining the structural integrity and mechanical stability of the nuclear envelope. Lamins are also localized within the nuclear matrix (1). In mammals, up to seven different lamins exist: A-type lamins (A, AΔ10, C and C2 encoded by LMNA gene through alternative splicing) and B-type lamins (B1 and B2/B3, which are transcribed from the LMNB1 and LMNB2 genes, respectively). Whereas B-type lamins are present in both embryonic and differentiated cells, A-type lamins are expressed only in later stages of development and in differentiated cells. Lamins have binding affinity with chromatin and integral proteins of the inner nuclear membrane, such as A

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emrin. In addition to their involvement in nuclear stability, it has been suggested that lamins play important roles in DNA replication, chromatin organization, regulation of gene expression, spatial organization of the nuclear pore and the correct anchorage of the nuclear envelope proteins (2).

We identified the first LMNA mutations in the autosomal dominant form of Emery–Dreifuss muscular dystrophy (AD-EDMD) highlighting the unpredicted role of these proteins in the pathophysiology of neuromuscular disorders (3). Since then, within 5 years, mutations in this gene have been identified to be responsible for up to nine disorders, i.e. dilated cardiomyopathy with conduction disease (DCM-CD) (4), limb girdle muscular dystrophy with atrophic ventricular conduction disturbance (LGMD1B) (5), Dunnigan-type of familial partial lipodystrophy (6), autosomal recessive Charcot–Marie–Tooth disease type 2 (7), mandibuloacral dysplasia (8), Hutchinson–Gilford progeria syndrome (HGPS) (9,10), atypical Werner’s syndrome (11) and restrictive dermopathy (12). This group of human hereditary diseases are now recognized as laminopathies and are characterized by the extreme variability of the phenotypes associated with the wide spectrum of LMNA gene mutations. Some phenotypic overlaps between the various types of laminopathies were also demonstrated (13,14). The mechanisms by which mutations in the LMNA gene encoding ubiquitously expressed proteins cause these various tissue-specific disorders remain to be elucidated.

Among laminopathies three disorders, EDMD, LGMD1B and DCM-CD, affect specifically the skeletal and/or cardiac muscles (3–5). The diagnosis of this type of laminopathy is based on clinical symptoms, characterized by conduction defect, arrhythmias, left ventricular (LV) dysfunction and dilation with heart failure. As >40% of these patients die suddenly, implantation of a cardioverter-defibrillator is necessary to prevent sudden cardiac death (15,16). The identification of the precise molecular mechanisms of LMNA mutations leading to laminopathies affecting striated muscles is critical for developing new therapeutic strategies to prevent cardiac dysfunction and sudden death.

At the present time, two genetically engineered mouse strains with modified Lmna gene have been reported. Lamin A/C null mice (Lmna–/–) were created to analyse the functional importance of lamin A/C in the end stages of embryonic development and regulation of the terminal differentiation (17). The replacement of exon 8–11 of Lmna gene by Pkmyo cassette leads to the loss of lamin A/C expression that is not embryonic lethal but leads to the development of muscular dystrophy, DCM and death by 8 weeks of age (17,18). The other genetically engineered mice were created with the aim of producing an EDM model with the introduction of the LMNA L530P mutation that causes AD-EDMD in human. Very surprisingly, LmnaL530P/L530P mice presented clinical defects consistent with human HGPS, including a reduction in growth rate, pathologies of bone, muscle and skin and death by 4 weeks of age (19). These two genetically engineered mouse strains developed some features of the human disorders but failed to reproduce faithfully any type of human laminopathy from the gene mutation to the clinical features.

In the present paper, we chose a missense mutation (H222P) identified in a family with a typical AD-EDMD (20) to create a Lmna knock-in mouse, with the aim of producing a faithful animal model of striated muscle laminopathies. We introduced the c.665A > C, p.H222P mutation in the Lmna murine gene by homologous recombination. Although mice carrying this point mutation do not show any phenotypes in neonatal and sexual maturity stages, at adulthood homozygous mutant (LmnaH222P/H222P) mice develop muscular dystrophy and DCM-CD similar to the clinical features of human laminopathies affecting the striated muscles, with all LmnaH222P/H222P mice dying by 13 months of age.

RESULTS

mRNA and protein expression of lamins A/C and emerin

LmnaH222P mice were created by homologous recombination using standard techniques and the targeting constructs described in Methods section and Figure 1A. After heterozygous intercrossing, we obtained LmnaH222P/H222P and wild-type (WT) mice. We first checked the mRNA expression levels of lamins A/C and emerin in the LV and skeletal muscles (gastrocnemius and quadriceps) by reverse transcriptase–PCR techniques. We observed neither differences for lamin A/C and emerin mRNA expression levels nor splicing abnormality of lamin A and C mRNAs between WT, LmnaH222P/+ and LmnaH222P/H222P mice (data not shown). We then checked the amount of lamins A/C and emerin proteins and detected similar amounts of lamins A/C and emerin in both LV and gastrocnemius muscle of LmnaH222P/+ and LmnaH222P/H222P mice as compared to WT mice (Fig. 1D).

LmnaH222P/H222P mice have a reduced life expectancy and die during adulthood, whereas LmnaH222P/+ mice have a life expectancy comparable to WT mice

Analysis of 133 newborn mice from 15 independent heterozygous crosses indicated that the numbers of mice with the three genotypes WT, H222P/+ and H222P/H222P were 39/56/38, approximating the expected ratio of 1:2:1 of Mendelian inheritance of the genotype. Both the mutated LmnaH222P/+ and LmnaH222P/H222P mice were indistinguishable from their WT siblings at birth and seemed healthy during the sexual maturity stage, indicating that the H222P mutation is not lethal during embryonic and perinatal stages and has no impact on the general health condition at young-age.

At adulthood, LmnaH222P/H222P male mice weighed less than WT and LmnaH222P/+ male mice and their growth rate was reduced after 3 months of age, despite normal feeding habits (Fig. 2B). At this time, LmnaH222P/H222P male mice began to show progressively abnormal phenotypes characterized by a hunched position, stiff walking posture and rapid shallow breathing, and all LmnaH222P/H222P male mice died between 4 and 9 months of age (Fig. 2D). As for female mice, LmnaH222P/H222P mice were slightly lighter than WT and LmnaH222P/+ mice (Fig. 2C), and also exhibited abnormal phenotypes similar to LmnaH222P/H222P male mice but only after 6 months of age. The first LmnaH222P/H222P female mouse died at 7 months of age, and all LmnaH222P/H222P female mice were dead by 13 months of age (Fig. 2D). LmnaH222P/+ mice were indistinguishable from WT mice with regard to phenotype and life expectancy.
Lmna H222P/ H222P mice develop DCM with atrio-ventricular conduction abnormality

For the assessment of cardiac abnormality, we performed echocardiography in the mice at 2, 4 and 6 (male/female) and 8 months (female) of age (Tables 1 and 2) and systolic blood pressure measurement by indirect tail-cuff sphygmomanometer.
Cardiac function was evaluated by transthoracic echocardiographic analyses of the left ventricle: LVM/BW, LV mass-to-body weight ratio; LVEDD/BW, LV end-diastolic diameter-to-body weight ratio; LVESD/BW, LV end-systolic diameter-to-body weight ratio; LVFS, LV fractional shortening.

**Table 1.** Echocardiographic data for male WT and Lmna<sup>H222P/H222P</sup> mice aged 2, 4 and 6 months

<table>
<thead>
<tr>
<th></th>
<th>2 months of age</th>
<th>4 months of age</th>
<th>6 months of age</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n = 5)</td>
<td>Lmna&lt;sup&gt;H222P/H222P&lt;/sup&gt; (n = 7)</td>
<td>Lmna&lt;sup&gt;H222P/H222P&lt;/sup&gt; (n = 12)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>27.2 ± 1.07</td>
<td>23.9 ± 0.83*</td>
<td>30.8 ± 1.30</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.500 ± 0.010</td>
<td>0.514 ± 0.014</td>
<td>0.514 ± 0.014</td>
</tr>
<tr>
<td>PWd (mm)</td>
<td>0.580 ± 0.037</td>
<td>0.557 ± 0.020</td>
<td>0.543 ± 0.020</td>
</tr>
<tr>
<td>LVM (mg)</td>
<td>54.3 ± 6.14</td>
<td>60.8 ± 3.11</td>
<td>66.1 ± 4.70</td>
</tr>
<tr>
<td>LVM/BW (mg/g)</td>
<td>2.02 ± 0.07</td>
<td>2.56 ± 0.13</td>
<td>2.15 ± 0.13</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>3.40 ± 0.17</td>
<td>3.69 ± 0.13</td>
<td>3.89 ± 0.08</td>
</tr>
<tr>
<td>LVESD/BW (mm/g)</td>
<td>0.126 ± 0.059</td>
<td>0.155 ± 0.067*</td>
<td>0.127 ± 0.044</td>
</tr>
<tr>
<td>LVESD/BW (mm/g)</td>
<td>0.127 ± 0.059</td>
<td>0.155 ± 0.067*</td>
<td>0.127 ± 0.044</td>
</tr>
<tr>
<td>LVFS (%)</td>
<td>43.1 ± 2.2</td>
<td>34.6 ± 2.7*</td>
<td>37.4 ± 2.7</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>520 ± 14</td>
<td>490 ± 14</td>
<td>468 ± 31</td>
</tr>
</tbody>
</table>

Cardiac function was evaluated by transthoracic echocardiographic analyses of the left ventricle: LVM/BW, LV mass-to-body weight ratio; LVEDD/BW, LV end-diastolic diameter-to-body weight ratio; LVESD/BW, LV end-systolic diameter-to-body weight ratio; LVFS, LV fractional shortening.

*P < 0.05 versus WT.

**P < 0.01 versus WT.

***P < 0.001 versus WT.

method in male mice at 2 months of age. Although systolic blood pressure was not modified in Lmna<sup>H222P/H222P</sup> male mice (120.9 ± 1.4 versus 127.5 ± 8.6 mmHg in WT mice; n = 5 for each groups, P = 0.45), Lmna<sup>H222P/H222P</sup> male mice developed a progressive LV dilation associated with a progressive decrease of contractile function, evidenced by decreased LV fractional shortening (LVFS), which was significant at 2 months of age (Fig. 3B and Table 1). The development of LV dysfunction was not accompanied with cardiac LV hypertrophy (Table 1). Similar features were observed in female Lmna<sup>H222P/H222P</sup> mice but with a later-onset (Table 2). Indeed, the LV dysfunction in female homozygous mutant mice appeared at 4 months of age and seemed to progress less rapidly than in males (Tables 1 and 2). Both male and female Lmna<sup>H222P/+</sup> mice had no cardiac dysfunction by 1 year of age (data not shown).

Telemetric ambulatory electrocardiogram (ECG) measurements were performed in homozygous mutant and WT male mice at 3, 4 and 5 months of age (Table 3). Abnormalities of the conduction system were observed: Lmna<sup>H222P/H222P</sup> male mice at 3 months of age showed a significant increase of the PR interval when compared with WT male mice, indicating atrio-ventricular conduction defects. This increase of PR interval remained stable at 4 and 5 months of age. The QRS complex duration, as a parameter for intraventricular conduction, was significantly increased between Lmna<sup>H222P/H222P</sup> and WT male mice at 5 months of age. In addition, frequent sinusatrial blocks and ventricular extra-systoles were observed in male Lmna<sup>H222P/H222P</sup> mice at 5 months of age during recording. Such abnormalities were never observed in the age-matched WT mice (data not shown).

Gross examination and histopathological analysis of the hearts were performed at the end stage of the disease on several Lmna<sup>H222P/H222P</sup> mice at 4, 5, 6 and 7 months of age for males and at 7 and 10 months of age for females. In each case, the same abnormalities were observed in these mice and typical abnormalities are presented in Figure 3. Macroscopically, the mice had dramatically enlarged hearts with dilation of the atria (Fig. 3D). Microscopically, ventricular chamber dilatation was obvious (Fig. 3F). Histopathologically, a prominent alteration observed in the heart of Lmna<sup>H222P/H222P</sup> mice was a marked increase of fibrosis. In addition, single or small clusters of cardiac muscle cells with degeneration and necrosis were extensively present in the left and right ventricles in Lmna<sup>H222P/H222P</sup> mice (Fig. 3H). There was no evidence of cellular hypertrophy, disarray or inflammatory cells.

Histopathological analysis of the heart of 1 year Lmna<sup>H222P/+</sup> mice revealed no abnormalities (data not shown).

**Lmna<sup>H222P/H222P</sup> mice have abnormal locomotion activities**

The influence of the H222P mutation also became apparent in the locomotion activity of the mice, as evidenced in the open field test and rotarod assay. The spontaneous locomotion activity at 6 months of age, tested in open field, was significantly impaired in male Lmna<sup>H222P/H222P</sup> mice compared to WT but was normal in females (Fig. 4A). The Lmna<sup>H222P/H222P</sup> female mice exhibited reduced spontaneous locomotion activity at 8 months of age, moving only 6.4 ± 2.0% of the time when compared with 54.8 ± 3.4% of the time spent in motion for WT female mice (P < 0.001, n = 3). In a rotarod assay at 6 months of age, both male and female Lmna<sup>H222P/H222P</sup> mice fell earlier from the rotarod, whereas WT mice managed to stay on the rotarod up to 2 min (Fig. 4B). Similar results were also observed for females at 8 months of age (4.4 ± 1.3% in Lmna<sup>H222P/H222P</sup> versus 100% in WT, P < 0.001, n = 5). The Lmna<sup>H222P/+</sup> mice had normal locomotion activities compared to WT by 1 year of age (data not shown).

**Lmna<sup>H222P/H222P</sup> mice exhibit dystrophic pattern of skeletal muscles**

Histological and histochemical analyses were performed on skeletal muscles at the end stage of the disease on several Lmna<sup>H222P/H222P</sup> mice at 4, 5, 6 and 7 months of age for males and at 7 and 10 months of age for females. We analyzed
Cardiac function was evaluated by transthoracic echocardiographic analyses of the left ventricle: LVM/BW, LV mass-to-body weight ratio; LVEDD/BW, LV end-diastolic diameter-to-body weight ratio; LVFS, LV fractional shortening.

**Table 2. Echocardiographic data for female WT and LmnaH222P/H222P mice aged 2, 4, 6 and 8 months**

<table>
<thead>
<tr>
<th>Age (months)</th>
<th>2 months</th>
<th>4 months</th>
<th>6 months</th>
<th>8 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT (n = 5)</td>
<td>LmnaH222P/H222P (n = 4)</td>
<td>WT (n = 12)</td>
<td>LmnaH222P/H222P (n = 12)</td>
</tr>
<tr>
<td>BW (g)</td>
<td>20.4 ± 0.68</td>
<td>22.0 ± 0.82</td>
<td>21.4 ± 0.65</td>
<td>23.9 ± 0.58</td>
</tr>
<tr>
<td>IVSd (mm)</td>
<td>0.500 ± 0.032</td>
<td>0.525 ± 0.025</td>
<td>0.496 ± 0.010</td>
<td>0.492 ± 0.008</td>
</tr>
<tr>
<td>PWd (mm)</td>
<td>0.520 ± 0.020</td>
<td>0.475 ± 0.025</td>
<td>0.492 ± 0.015</td>
<td>0.467 ± 0.019</td>
</tr>
<tr>
<td>LVM (mg)</td>
<td>46.3 ± 3.53</td>
<td>53.3 ± 1.42</td>
<td>48.7 ± 1.81</td>
<td>61.8 ± 2.86**</td>
</tr>
<tr>
<td>LVM/BW (mg/g)</td>
<td>2.27 ± 0.14</td>
<td>2.43 ± 0.05</td>
<td>2.28 ± 0.08</td>
<td>2.60 ± 0.12**</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>3.26 ± 0.16</td>
<td>3.60 ± 0.14</td>
<td>3.44 ± 0.08</td>
<td>4.02 ± 0.12**</td>
</tr>
<tr>
<td>LVESD (mm)</td>
<td>1.72 ± 0.07</td>
<td>2.18 ± 0.18</td>
<td>2.17 ± 0.09</td>
<td>2.84 ± 0.12**</td>
</tr>
<tr>
<td>LVESD/BW (mm/g)</td>
<td>0.160 ± 0.004</td>
<td>0.164 ± 0.004</td>
<td>0.162 ± 0.005</td>
<td>0.169 ± 0.005</td>
</tr>
<tr>
<td>LVEF (%)</td>
<td>46.9 ± 2.6</td>
<td>39.9 ± 2.6</td>
<td>37.2 ± 1.6</td>
<td>28.3 ± 2.3**</td>
</tr>
<tr>
<td>Heart rate (bpm)</td>
<td>574 ± 21</td>
<td>541 ± 24</td>
<td>511 ± 27</td>
<td>509 ± 19</td>
</tr>
</tbody>
</table>

Cardiac function was evaluated by transthoracic echocardiographic analyses of the left ventricle: LVM/BW, LV mass-to-body weight ratio; LVEDD/BW, LV end-diastolic diameter-to-body weight ratio; LVFS, LV fractional shortening.

*P < 0.05 versus WT.
**P < 0.01 versus WT.
***P < 0.001 versus WT.
features of lipodystrophy (13), we hypothesized that fatty acid accumulation may contribute to the development of these phenotypes in LmnaH222P/H222P mice. Oil Red O staining to determine lipid accumulation was performed at the early stage of the disease on several LmnaH222P/H222P mice, i.e. at 2 and 3 months of age for males and females and at the end stage of the disease, i.e. at 4, 5, 6 and 7 months of age for males and at 7 and 10 months of age for females. However, no lipid accumulation was observed in any LmnaH222P/H222P mice (data not shown). Also, there was no difference in LV and skeletal muscle SREBP1 and PPARγ mRNA levels between WT, LmnaH222P/H222P, and LmnaH222P/H222P male mice. Thus, LmnaH222P/H222P mice did not develop any abnormal features of lipid metabolism in affected tissues.

Normal localizations of lamins A/C and emerin proteins is observed in the LmnaH222P/H222P mice

Localization of lamin A/C and emerin proteins in striated muscle sections was evaluated at 6 months of age. Immunohistochemical analyses of lamins A/C and emerin localizations in the heart (Fig. 6D–F) and gastrocnemius muscle (Fig. 6J–L) of LmnaH222P/H222P mice showed similar localization of these proteins compared to heart and muscle of WT mice (Fig. 6A–C and G–I, respectively). Normal localization of these proteins was also observed in other skeletal muscles (diaphragm, soleus, quadriceps, triceps) (data not shown).

LmnaH222P/H222P mice exhibit alteration of heterochromatin distribution in cardiac and skeletal muscles

We analyzed the ultrastructure in cardiac and quadriceps muscles by electron microscopy. The general architecture of the myocytes was normal, the myofibrils were not disrupted and the distribution of mitochondria was normal. Approximately 10% of the nuclei showed pathologic changes in both heart and quadriceps (Fig. 7A and B). The heterochromatin appeared to be very dark, highly condensed and with an irregular distribution. We did not observe caryoplasma extrusion. Sometimes the nuclear membrane showed deep convolutions, with the appearance of intranuclear tubules. These abnormalities were also observed in satellite cells, fibroblasts and endothelial cells of capillaries (Fig. 7C and D).
Lmna<sup>H222P/H222P</sup> mice exhibit increased nuclear translocation of Smad proteins in cardiac and skeletal muscles

A significant body of literature indicates that TGF-β<sub>1</sub> is a powerful initiator for fibrosis, extracellular matrix deposition and alteration of gene expression in heart (22). The intracellular effectors of TGF-β signalling, the Smad proteins, when activated via TGF-β receptors, translocate into the nucleus where they regulate gene transcription. Briefly, receptor-activated Smad proteins, such as Smad2 and Smad3, are phosphorylated and form a heteromeric complex with Smad4. These phosphorylated Smad2/3–Smad4 dimers then translocate to the nucleus, mediating the target gene responses (22). Previous studies demonstrated elevated levels of Smad proteins and increased nuclear accumulation of phosphorylated Smad2 (P-Smad2) in failing heart, strongly suggesting that activation of the Smad proteins is associated with fibrosis in failing heart (23,24).

We hypothesized that pathologic alterations of the nuclear envelope promoted by the Lmna mutation in Lmna<sup>H222P/H222P</sup> mice might alter the intranuclear import of Smad proteins and hence might contribute to the striated muscle phenotype, especially the extensive fibrosis observed in both skeletal and cardiac muscles. Immunofluorescence staining of heart and diaphragm muscles from 5, 6 and 7 months of age males and 10 months of age female mice revealed that

![Figure 5. Histological and histochemical analysis of skeletal muscles from male WT (A, C, E, G, I and K) and Lmna<sup>H222P/H222P</sup> (B, D, F, H, J and L) mice at 6 months of age. Fresh frozen diaphragm (A and B) and soleus muscles (C–L) from WT and Lmna<sup>H222P/H222P</sup> mice were stained with H&E (A–D), modified Gomori trichrome (E and F) or myofibrillar ATPase, pH 9.3 (G and H), cytochrome c oxidase (I and J) and succinate dehydrogenase (K and L) activity. (A–F) Variability in fibre size with hypertrophic muscle cells and numerous degenerative fibres (black arrowheads) were observed in both diaphragm (b) and soleus (d) muscles in Lmna<sup>H222P/H222P</sup> mice when compared with WT. In addition, the presence of interstitial fibrosis (asterisk), splitting muscle fibres (white arrowheads) and internal nuclei (black arrows) are indicated. (G–L) White arrows in Lmna<sup>H222P/H222P</sup> muscle indicate abnormal reaction for myofibrillar ATPase at pH 9.4 (H). This abnormality is associated with focal loss of mitochondrial cytochrome c oxidase activity (I) and succinate dehydrogenase activity (L); scale bar = 50 μm.]
phosphorylated Smad2/3 proteins (P-Smad2/3) were equally distributed in both cytoplasm and nucleus in heart and skeletal muscle of WT (Fig. 8A–D and I–L, respectively) and LmnaH222P/þ mice (data not shown). In contrast, P-Smad2/3 detection in LmnaH222P/H222P mouse tissues revealed an accumulation of P-Smad2/3 in nuclei, with brightly stained regions co-localizing with nuclei of both myofibres and fibrosis areas (Fig. 8E–H and M–P, respectively). These staining patterns of P-Smad2/3 were not observed on phosphatase-treated tissue sections (data not shown). This increase of translocation of Smad proteins in the nuclei was confirmed by western blot analysis. The protein amounts of total Smad2 (phosphorylated and non-phosphorylated) and Smad4 from LV tissues of female mice of 9–12 months of age were determined after cell fractionation. In LmnaH222P/H222P LV tissues, the amounts of Smad2 and Smad4 proteins were increased in the nuclear fraction when compared with WT and LmnaH222P/þ mouse hearts (Fig. 9).

**DISCUSSION**

In the present study, we developed Lmna knock-in mice carrying the H222P mutation that was identified in the human LMNA gene in a family with typical AD-EDMD (20). We show that at adulthood homozygous mutant mice, LmnaH222P/H222P, develop muscular dystrophy and DCM associated with conduction defects, reminiscent of EDMD in human. Cardiac and skeletal muscle dysfunctions have a later-onset in female than in male LmnaH222P/H222P mice. This is the first Lmna mouse model mimicking human laminopathy from the gene mutation to the clinical features.

As we wanted to reproduce human pathologic conditions, we precisely reproduced a human LMNA gene mutation in the

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**Table 4. Blood chemistry data of WT and LmnaH222P/H222P mice aged 6–8 months**

<table>
<thead>
<tr>
<th>Sex</th>
<th>Genotype</th>
<th>n</th>
<th>Glucose (mmol/l)</th>
<th>TC (mmol/l)</th>
<th>HDL-C (mmol/l)</th>
<th>Triglycerides (mmol/l)</th>
<th>Insulin (µg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>WT</td>
<td>6</td>
<td>3.92 ± 0.36</td>
<td>3.03 ± 0.32</td>
<td>2.19 ± 0.27</td>
<td>0.62 ± 0.05</td>
<td>1.04 ± 0.3</td>
</tr>
<tr>
<td>M</td>
<td>LmnaH222P/H222P</td>
<td>6</td>
<td>2.00 ± 0.31**</td>
<td>3.20 ± 0.36</td>
<td>2.10 ± 0.18</td>
<td>0.98 ± 0.18</td>
<td>0.59 ± 0.1</td>
</tr>
<tr>
<td>F</td>
<td>WT</td>
<td>6</td>
<td>3.02 ± 0.50</td>
<td>3.41 ± 0.30</td>
<td>2.40 ± 0.22</td>
<td>0.77 ± 0.06</td>
<td>0.48 ± 0.13</td>
</tr>
<tr>
<td>F</td>
<td>LmnaH222P/H222P</td>
<td>6</td>
<td>2.16 ± 0.43</td>
<td>2.61 ± 0.24</td>
<td>1.79 ± 0.21</td>
<td>1.34 ± 0.16**</td>
<td>0.71 ± 0.13</td>
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Blood biochemistry was measured after 6 months of age. The mice were matched for body weight; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol.

**Figure 6.** Immunodetection of lamin A/C and emerin in heart and skeletal muscles from male mice at 6 months of age. Immunostaining with lamin A/C Ab (A, D, G and J), emerin Ab (B, E, H and K) and overlay (C, F, I and L) show the localization of nuclear envelope proteins in heart and gastrocnemius muscle of WT (A, B, C, G, H and I) and LmnaH222P/H222P mice (D, E, F, J, K and L). In WT mice, lamin A/C (A and G) and emerin (B and H) proteins were co-localized at nuclear periphery in heart (C) and gastrocnemius muscle (I). In heart and gastrocnemius muscle of LmnaH222P/H222P mice, similar lamin A/C (D and J) and emerin (E and K) co-localization (F and L) compared to WT were observed, respectively; scale bar = 20 µm.

**Figure 7.** Nuclear morphology analysis of heart and quadriceps muscle from male LmnaH222P/H222P mice at 6 months of age. (A) Central deposit of densely stained heterochromatin in the myocyte of quadriceps muscle. (B) Deep indentations of the nuclear membrane in cardiomyocyte. (C and D) Condensation of heterochromatin close to the membrane in fibroblast of the heart (C) and in endothelial cell of a capillary (D) of the heart; scale bar = 1 µm.
mouse genome: the same single nucleotide base change (CAT > CCT) in exon 4 of the murine \textit{Lmna} gene and of the human \textit{LMNA} gene directs the same amino acid replacement from histidine to proline at codon 222 in the murine lamins A/C as in the human proteins (c.665A > C, p.H222P) (20).

The previous mouse model developed with the same aim, i.e. \textit{Lmna}^{L530P} knock-in mice as an AD-EDMD mouse model, led surprisingly to the development of clinical features highly reminiscent of another type of human laminopathy, the HGPS (19). HGPS is a premature ageing syndrome with a median age at death of 13.4 years, clinically characterized by postnatal growth retardation, midface hypoplasia, micrognathia, cardiovascular disease, absence of subcutaneous fat, alopecia and generalized osteodysplasia (9,10). The majority of HGPS cases are caused by the single \textit{LMNA} point mutation G608G leading to the aberrant activation of a cryptic donor splice site in exon 11 (9,10,25). Unexpectedly, the introduction of the AD-EDMD L530P mutation in the mouse genome produced a reduction in growth rate and pathologies of bone, muscle and skin, and \textit{Lmna}^{L530P/L530P} mice died by 4 weeks of age (19). Actually, similar to human HGPS, these progeria-like features were associated with greatly diminished levels of mutant lamin A/C mRNA and proteins due to aberrant splicing (19).

The phenotype differences observed between \textit{Lmna}^{H222P/H222P} and \textit{Lmna}^{L530P/L530P} mice are most probably due to the difference in the expression of mutated protein. Indeed, \textit{Lmna}^{H222P/H222P} mice demonstrated a normal expression level of both mutated lamin A/C mRNA and proteins. The observation for the first time of normal expression level of mutated lamin A/C in a \textit{Lmna}
mouse model carrying an AD-EDMD mutation suggests that stability of the mutated lamins A/C may be important for developing a laminopathy specifically affecting striated muscles in mice.

The other genetically engineered mice, i.e. Lmna null mice (Lmna<sup>−/−</sup>), although not reproducing a human LMNA mutation, develop some features of human laminopathies (7,17,18,26). Regarding striated muscle laminopathy, Lmna<sup>−/−</sup> mice present rapid onset of muscular dystrophy associated with progressive DCM and death by 8 weeks of age (17,18). There are several points of similarity between Lmna<sup>−/−</sup> and Lmna<sup>H222P/H222P</sup> mice such as abnormal growth curve, abnormal gait, muscle weakness, dystrophic muscles, DCM-CD and abnormal chromatin organization. However, there are also critical differences between the two strains as detailed in Table 5. The main difference is the later-onset and the less rapid progression of both muscular dystrophy and DCM-CD in Lmna<sup>H222P/H222P</sup>, mimicking the human pathology more closely. Thus, the progression of the disease can be studied in more detail, particularly in the heart. Indeed, PR interval is increased in Lmna<sup>H222P/H222P</sup> male mice in the early stage of DCM at 3 months of age, indicating atrio-ventricular conduction defects, whereas QRS duration increases at 5 months of age, indicating intraventricular conduction disturbance, probably due to the marked increase of fibrosis and cardiac chamber dilation at this stage of the disease. In contrast, the Lmna<sup>−/−</sup> mice had normal cardiac ECG and echocardiography parameters at 2 weeks, exhibited DCM with prolongations of both PR interval and QRS duration at 4–6 weeks of age and died at 8 weeks (18). Thus, the disease of the Lmna<sup>H222P/H222P</sup> mice quite closely mimics human cardiac disease, in which atrio-ventricular conduction defects are most frequently the first cardiac symptoms in patients, DCM arising usually later during progression of the cardiac disease.

Figure 9. Smad proteins localization in LV of female mice at 9–12 months of age. Representative data for two WT, Lmna<sup>H222P/H222P</sup> and Lmna<sup>H222P/H222P</sup> mice are shown. Experiments were repeated at least three times with three mice from each group. Cytoplasmic and nuclear fractions of LV tissue were solved on SDS–PAGE and hybridized with Ab against total Smad2 (phosphorylated + non-phosphorylated forms) and Smad4 proteins. When compared with WT and Lmna<sup>H222P/H222P</sup> mice, Lmna<sup>H222P/H222P</sup> mice show increased amounts of Smad2 and Smad4 proteins in the nuclear fractions and decreased amount of total Smad2 proteins in the cytoplasmic fraction. Detection of α-tubulin (cytoplasmic protein) and emerin (nuclear protein) confirms that the cytoplasmic and nuclear fractions were correctly separated.

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Disease expression</th>
<th>mRNA expression level</th>
<th>Lamin A/C expression and localization</th>
<th>Chromatin reorganization</th>
<th>Conduction activity</th>
<th>Locomotion activity</th>
<th>Cardiac features</th>
<th>Death</th>
<th>Gender difference</th>
<th>Feature of partial lipodystrophy</th>
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<tr>
<td>Lmna&lt;sup&gt;−/−&lt;/sup&gt;</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Normal</td>
<td>Abnormal</td>
<td>Abnormal</td>
<td>Abnormal gait</td>
<td>Progressed dilated cardiomyopathy</td>
<td>Progressive dilated cardiomyopathy</td>
<td>Progressive dilated cardiomyopathy</td>
</tr>
<tr>
<td>Lmna&lt;sup&gt;H222P/H222P&lt;/sup&gt;</td>
<td>Only homozygous mice are affected</td>
<td>Highly decreased</td>
<td>Absent</td>
<td>Absent</td>
<td>Abnormal</td>
<td>Abnormal gait with a stiff walking</td>
<td>Abnormal gait</td>
<td>Progressive dilated cardiomyopathy</td>
<td>Progressive dilated cardiomyopathy</td>
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<td>Lmna&lt;sup&gt;H222P/H222P&lt;/sup&gt;</td>
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<td>Abnormal gait</td>
<td>Progressive dilated cardiomyopathy</td>
<td>Progressive dilated cardiomyopathy</td>
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Table 5. Comparison of striated muscles phenotype in Lmna<sup>H222P/H222P</sup> and Lmna<sup>−/−</sup> mice and human.
These cardiac dysfunctions are associated with cardiomyocyte degeneration and a marked increase of interstitial fibrosis in the Lmna<sup>H222P/H222P</sup> mouse hearts. These features were not observed in Lmna<sup>−/−</sup> mouse hearts (18) but were reported in patients in whom cardiac histopathological analysis was performed (4,29,32). Significant dystrophic features and fibrosis were also present in the diaphragm of Lmna<sup>H222P/H222P</sup> mice associated with rapid shallow breathing; this muscle being largely unaffected in the Lmna<sup>−/−</sup> mice (18). Histological analysis of diaphragm in patients has never been reported. Diaphragm biopsies are not performed in patients, and respiratory investigations are not routinely carried out most probably due to the fact that respiratory insufficiency is not a hallmark of striated muscle laminopathy. Among 57 cases, seven were identified with severe impairment of respiratory function (33,34). This result suggests that involvement of the diaphragm or accessory respiratory muscles may be underestimated in human striated muscle laminopathy, further investigations are needed to confirm these first results.

A striking feature is the death between 4 and 13 months of age of the Lmna<sup>H222P/H222P</sup> mice. Death was most probably due to a combination of arrhythmia, end-stage heart failure due to DCM with decreased LVFS and respiratory failure as a consequence of weak performance of dystrophic diaphragm. All the Lmna<sup>−/−</sup> mice died even earlier at 2 months of age (17) probably due to the more rapidly progressive cardiac disease, although arrhythmia and diaphragm defects were not reported in the Lmna<sup>−/−</sup> mice (17,18).

Interestingly, the weaker performance of heart and skeletal muscles and early death in Lmna<sup>H222P/H222P</sup> mice had a later-onset in females than in males Lmna<sup>H222P/H222P</sup> mice. This gender difference was not reported in Lmna<sup>−/−</sup> mice, although the early death by 2 months of age may prevent the detection of discrepancies between males and females. Several gene-targeted mouse strains demonstrated that male mice displayed more pronounced abnormalities in cardiac function than females (35–39). It was suggested that steroid-hormones, such as testosterone and estrogens, may explain these gender differences in cardiac dysfunction (40,41). On the other hand, gender differences in mouse strains with skeletal muscle disorders seem to be unknown. We provide here the first evidence of sex-related survival benefits in an animal model of cardiac and skeletal myopathy. Although the mechanism by which this may occur is still unknown, one could hypothesize that ovarian hormones may play a protective role in the onset of disease in females or testicular hormones may accelerate the development of phenotypes in males in this mouse model. Future studies using ovariectomy or castration of the Lmna<sup>H222P/H222P</sup> mice may further elucidate these mechanisms. Additional studies are also needed to determine whether such gender difference may exist in human affected by striated muscle laminopathies, as it has not been reported yet for EDMD, LGMD1B or DCM-CD.

Surprisingly, Lmna<sup>H222P/+</sup> mice showed no abnormalities up to 1 year of age despite the fact that the majority of patients reported so far with striated muscle-specific laminopathy carry heterozygous LMNA mutation. There are numerous precedents for mouse models of human disease for which the mice presenting clinical phenotypes carry homozygous mutated alleles, whereas the patients are heterozygous for the mutated allele (42), just as we observed in the present study, with only Lmna<sup>H222P/H222P</sup> mice developing a dystrophic condition in skeletal muscles and heart. Similarly the other two genetically engineered mice for the Lmna gene, Lmna<sup>−/−</sup> and Lmna<sup>L530P</sup> knock-in mice, exhibit clinical features only when they carry homozygous Lmna mutations (17,19). This may reflect different downstream pathways of the gene mutation between human and mouse. However, homozygous H222Y LMNA mutation in human leads to AR-EDMD (43). Although the substituted amino acids are different (proline or tyrosine), the modification of arginine 222 in lamins A/C seems to be particularly sensitive to the development of diseases in both human and mouse. Also, we cannot completely exclude the possibility that Lmna<sup>H222P/+</sup> mice may develop abnormal phenotypes with advanced age. Further studies will be required to characterize any clinical features of human laminopathies in Lmna<sup>H222P/+</sup> mice.

All differences observed between Lmna<sup>−/−</sup> and Lmna<sup>H222P/H222P</sup> mice are probably due to the distinction between complete loss of lamins A/C function (Lmna<sup>−/−</sup>) and alteration of the function by the existence of particular mutant alleles (Lmna<sup>H222P/H222P</sup>). Development of other knock-in Lmna mouse models with introduction of an EDMD mutation localized in the N-terminal domain of lamins A/C is in progress. Further comparison between phenotypes of each gene-engineered mouse model and human striated muscle-specific laminopathies may throw light on the pathogenesis of this type of laminopathy.

One of the specific and important features of Lmna<sup>H222P/H222P</sup> mice is the extensive fibrosis observed in both cardiac and skeletal muscles, a feature that was also present in patients with striated muscle laminopathies. Interestingly, the observation of abnormal intranuclear accumulation of P-Smad proteins in myocyte and non-myocyte cells in cardiac and skeletal muscles of Lmna<sup>H222P/H222P</sup> mice may partly explain this extensive fibrosis. Indeed, similar to what has been observed in hearts of other animal models of heart failure, activation of Smad proteins in cardiac fibroblasts and myofibroblasts was reported to play important roles in cardiac fibrosis (22–24). This intranuclear accumulation of Smad proteins most probably occurs secondary to an increase of circulating TGF-β generated by the failing heart and dystrophic muscles, even if we cannot completely exclude the hypothesis of abnormal nuclear shuttling of Smad proteins through nuclear pore complexes due to mutated lamins A/C. Thus, the puzzle of pathophysiological mechanisms is far from solved. The precise downstream effects of Smad protein activation in striated muscles of Lmna<sup>H222P/H222P</sup> mice need to be explored further. Global gene expression profiling such as gene chip analysis would be a useful tool for investigating the consequence of P-Smad2/3 and Smad4 import into myocyte nuclei.

Thus, the two main pathophysiological hypotheses proposed for laminopathies, i.e. mechanical stress and gene expression alteration (2,44) are both most probably involved in
Lmna<sup>H222P/H222P</sup> mice, as was recently suggested in analyses of Lmna<sup>−/−</sup> mice in which impaired nuclear mechanics in absence of lamins A/C lead to alterations in gene expression (18,45). But this may be accomplished through a different intracellular pathway. Further investigations are needed to elucidate the pathogenic mechanisms of the Lmna H222P mutation.

In summary, the homozygous knock-in mice carrying the Lmna H222P mutation that we have generated faithfully reproduce the most frequent types of human laminopathies, those affecting striated muscles: a missense mutation identified in human, development of the dystrophic condition in both skeletal and cardiac muscles at adulthood, normal expression and localization of lamins A/C and emerin and abnormal heterochromatin organization. An intriguing difference between men and mice is that the clinical phenotype develops in heterozygous and homozygous, respectively. In addition, although we have not solved all the molecular basis underlying the pathology, demonstration of abnormal behaviour of intracellular TGF-β effectors, the Smad proteins, within the affected tissues, i.e. the striated muscles, shed further light on the pathomechanisms of striated muscle laminopathies.

These knock-in Lmna<sup>H222P</sup> mice represent an important animal model for studies of the pathogenesis of laminopathies specifically affecting striated muscles and provide important tools for the development of new treatment strategies, in particular for DCM.

MATERIALS AND METHODS

Production of targeted Lmna H222P mice

Lmna H222P KI mice were generated by homologous recombination in CK35 ES cells (46). Lmna genomic clones were isolated from a 129/svJ mouse BAC genomic library (Genomysystem™) that contains genomic fragments encompassing the whole murine Lmna gene. The targeting vector for the H222P missense mutation was constructed using a 6 kb XhoI fragment encompassing exons 2–11 of the Lmna gene (Fig. 1A). A BsrGI–Clal PCR fragment containing the H222P mutation in exon 4 (CAT > CCT) and a new Sall restriction site in intron 3 was subcloned in this 6 kb XhoI fragment. A Cre/Neo-loxP auto-deleting selection cassette was introduced in intron 3 via the new Sall site (47). Addition of a thymidine kinase selection cassette after exon 11 allowed negative selection (Fig. 1A). G418<sup>R</sup>–GANC<sup>R</sup> resistant ES clones were selected and screened for homologous recombination by Southern blotting as indicated in Figure 1B. For the H222P mutant allele, the Cre/Neo-loxP cassette introduced a new HindIII site, resulting in HindIII restriction fragments of 5.8 kb, whereas the WT allele produced a 8.5 kb fragment (Fig. 1A and B). In addition to new fragments by HindIII restriction, longer BsrGI fragments (10.1 kb) when compared with WT (6.4 kb) were also produced in the mutant allele. Recombinant ES cells were injected in Balb/c blastocysts and implanted into pseudopregnant females. Founders were bred to WT C57BL/6 females to check for germline transmission. Germline transmission of H222P mutation and self-excision of the Cre/Neo cassette in the germ line of chimerae were confirmed by PCR analyses on genomic DNA from heterozygous animals (data not shown) (47). Subsequent breeding produced animals containing the H222P mutation but lacking the Cre/Neo cassette and the genotypes were detected by PCR analyses and sequencing on tail genomic DNA with the following oligonucleotides: 3F, 5'-cagccatactctctctgg-3' and 4R, 5'-agcaggagggagagaga-3' (Fig. 1C). The single loxP site remaining in the Lmna H222P allele after deletion of the Cre/Neo resulted in an amplified PCR product 73 bp longer than the 580 bp fragment amplified from the WT Lmna allele. These mice and their litter matched WT were used in all subsequent studies. All experiments were performed in accordance with the guide for the care and use of laboratory animals published by the NIH (publication No. 85-23, revised 1985).

mRNA and protein expression

After macroscopic examination of the tissues, mouse heart and skeletal muscles were dissected and rapidly frozen in liquid-nitrogen. Total RNA was extracted from LV or skeletal muscles using RNA plus reagent (Qbiogene, Illkirch, France) as described by the manufacturer. RNA concentrations and purity were determined spectrophotometrically and RNA quality further assessed by electrophoretic separation in agarose gels. mRNA expression levels for Lmna and Emd encoding emerin protein were measured by quantitative RT–PCR using the LightCycler Real Time PCR System with the LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostic GmbH, Mannheim, Germany). mRNA expression levels for SREBP1 and PPARγ encoding SREBP1 and PPARγ proteins were also determined. Expression level of mouse β-actin was used as an internal control.

For western blot analyses, frozen tissues were homogenized in a total protein extraction buffer (2% SDS, 250 mM sucrose, 75 mM urea, 1 mM dithiothreitol and 50 mM Tris–HCl, pH 7.5) with protease inhibitor (25 μg/ml aprotinin and 10 μg/ml leupeptin) by FastPrep System (Qbiogene). After determination of protein concentration using the BC assay kit (Uptima, Montlucon, France), total protein was separated on SDS–PAGE gels and hybridized with primary rabbit anti-lamins A/C polyclonal antibody (Ab) (1:200, Santa Cruz Biotechnology Inc., CA, USA), primary rabbit anti-emerin polyclonal Ab and with secondary rabbit anti-mouse or goat antiserum. The same primary Abs were used in all subsequent studies. All experiments were performed in accordance with the guide for the care and use of laboratory animals published by the NIH (publication No. 85-23, revised 1985).
anti-rabbit IgG HRP-conjugated Ab. Recognized proteins were visualized by enhanced chemiluminescence (Pierce).

**Echocardiography**

Transthoracic echocardiography was performed in the mice using an Acuson 128XP/10 cardiac ultrasound machine with Acuson 10 MHz linear probe (Mountain View, CA, USA) at room temperature. Mice were slightly anesthetized using an Acuson 128XP/10 cardiac ultrasound machine with Transthoracic echocardiography was performed in the mice

**Blood pressure measurement**

Systolic blood pressure measurements were performed by the indirect tail-cuff sphygmomanometer method. Non-anaesthetized mice, kept at 30°C, were trained for restrainers and tail-cuff inflation. Systolic blood pressure was recorded by using a PowerLab/S system connected to CHART software (A.D. Instruments, Milford, MA, USA) (50).

**Electrocardiogram**

Long-term telemetric ambulatory ECG were recorded in mice with implantable transmitters (ETA-F20, Data Sciences International, MN, USA). Transmitters were inserted subcutaneously in the abdominal region under anesthesia with ketamine [75 mg per kg body weight (BW) intraperitoneally], xylazine (15 mg per kg BW intraperitoneally) and midazolam (0.75 mg per kg BW intraperitoneally). The mice were placed in cages on top of a receiver for transmission of ECG signals connected to a computer for display and analysis by Dataquest™ A.R.T™ software (Data Sciences International). Telemetric ECG tracings were obtained in conscious mice during quiet awake time at daytime and the PR interval and QRS duration were measured.

**Locomotion tests**

We examined exploratory locomotion of mice in an open field test. In each experiment, a mouse was placed on a new wide area and the time the mouse spent moving around over 5 min was measured. For the rotating rod task, mice were tested with 16 r.p.m. to test forced locomotion activity. Each mouse was tested three times for 2 min with 30 min recovery between each test (51).

**Histopathological and histochemical analyses**

Fresh specimens from individual skeletal and cardiac muscles were snap frozen in liquid-nitrogen-cooled isopentane (VWR International, Fontenay-sous-Bois, France), and stored at −80°C until further processing. Frozen sections (8 μm) of transversal cardiac and skeletal muscles were stained with H&E, modified Gomori trichrome and Oil Red O stains, and labelled for localization of myofibrillar ATPase, cytochrome c oxidase and succinate dehydrogenase activity by standard methods (52). Sections were analyzed by light microscopy. For immunohistochemical analysis, tissue sections were fixed for 10 min in 100% ethanol and incubated for 1 h with blocking solution (10% goat serum, 0.03% Triton-X in PBS) at room temperature. For detection of lamin A/C and emerin, primary rabbit anti-emerin polyclonal Ab (1:200, kindly provided by G.E. Morris) were diluted in an incubation buffer (1% goat serum, 0.03% Triton-X in PBS) and sections were incubated overnight at room temperature. Sections were washed three times with 0.03% Triton-X in PBS and incubated with a secondary sheep anti-rabbit IgG FITC-conjugated Ab (1:500, Amrad Biotech, Victoria, Australia) for 1 h at room temperature. After washing with 0.03% Triton-X in PBS, immunoreaction against lamins A/C proteins was then performed on the sections using Vector® M.O.M.™ Immuno-detection Kit (Vector Laboratories Inc., CA, USA) according to the manufacturer's instruction. The primary and secondary antibodies used were mouse anti-lamins A/C monoclonal Ab 4A7 (1:200, kindly provided by G.E. Morris) and Alexa fluor 568 goat anti-mouse IgG1 (1:500, Molecular Probes, Leiden, The Netherlands), respectively. For immunohistochemical analysis of P-Smad2/3 and desmin, the primary antibodies used were goat anti-P-Smad2/3 polyclonal Ab (1:100, Santa Cruz Biotechnology Inc.) and mouse anti-desmin monoclonal Ab (1:200, Dako A/S) and the secondary antibodies used were rabbit anti-goat IgG FITC-conjugated Ab (1:500, Dako A/S) and Alexa fluor 568 goat anti-mouse IgG1, respectively. Diphosphorylation of cryostat tissue sections with alkaline phosphatase (10 U per ml at 37°C for 1 h, Promega Biosciences Inc., CA, USA) was performed to obtain a negative control of P-Smad2/3 labelling. All tissue sections were counter-stained with 4',6-diamidino-2-phenylindole dihydrochloride (DAPI), and images were collected and analyzed with confocal Leica TCS4D DMRB microscope or Carl Zeiss Axiophot2 microscope and appropriate software.

**Electron microscopy**

The muscle specimens (LV and quadriceps) were immediately fixed for 2 h in 2.5% cold glutaraldehyde with 0.1 M cacodylate buffer, pH 7.3. After washing in cacodylate buffer, the specimens were post-fixed in 1% osmium tetroxide in the same buffer, dehydrated with graded series of ethanol and embedded in Epon. Then 0.7 μm thick sections were stained with toluidine blue alkaline. The ultrathin sections were stained with uranyl acetate, citrated and observed with a Philips CM 120 electron microscope at 80 kV (53).

**Serum biochemical analyses**

Blood samples were obtained after 18 h fasting from male and female WT and Lmna<sup>H222P/H222P</sup> mice between 6 and 8 months of age. The following biochemical parameters were measured as previously described (54,55): glucose, total
cholesterol, high density lipoprotein cholesterol, low density lipoprotein, triglycerides, uric acids, aspartate aminotransferase and alanine aminotransferase. All tests were performed at the Institut Clinique de la Souris (Strasbourg, France).

Statistical analysis

All data were acquired and analyzed by observers who were blinded to the animals’ genotypes. Continuous variables were analyzed using ANOVA and Student’s t-test for paired values. Statistical comparisons for cardiac dimension data were made before and after normalization for BW differences. The measured values were expressed as mean ± SEM. P-values of less than 0.05 were considered to be statistically significant.

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