Age-Related Changes in Lamin A/C Expression in Cardiomyocytes

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ABSTRACT

Lamin A and C (A/C) are type V intermediate filaments that form the nuclear lamina. Lamin A/C mutations lead to reduced expression of lamin A/C and diverse phenotypes such as familial cardiomyopathies and accelerated aging syndromes. Normal aging is associated with reduced expression of lamin A/C in osteoblasts and dermal fibroblasts but has never been assessed in cardiomyocytes. Our objective was to compare the expression of lamin A/C in cardiomyocytes of old (24 months) versus young (4 months) C57Bl/6J mice using a well validated mouse model of aging. Lamin B1 was used as a control. Immunohistochemical and immunofluorescence analyses showed reduced expression of lamin A/C in cardiomyocyte nuclei of old mice (proportion of nuclei expressing lamin A/C, 9% vs. 62%, p<0.001). Lamin A/C distribution was scattered peripherally and perinuclear in old mice, whereas it was homogeneous throughout the nuclei in young mice. Western blot analyses confirmed reduced expression of lamin A/C in nuclear extracts of old mice (ratio of lamin A/C:B1, 0.6 vs. 1.2, p<0.01). Echocardiographic studies showed increased left ventricular wall thickness with preserved cavity size (concentric remodeling), increased left ventricular mass, and a slight reduction in fractional shortening in old mice. This is the first study to show that normal aging is associated with reduced expression and altered distribution of lamin A/C in nuclei of cardiomyocytes.

KEYWORDS

aging, lamin, laminopathy, nucleus, cardiomyocyte, cardiomyopathy
INTRODUCTION

Lamin A and C (A/C) are type V intermediate filaments encoded by the LMNA gene that form the nuclear lamina (17). The functions of lamin A/C are to support the inner nuclear envelope and to participate in DNA repair, signal transduction, mesenchymal stem cell differentiation, mitosis, and apoptosis (9; 17; 24). LMNA mutations (“laminopathies”) lead to a reduction in lamin A/C expression and diverse phenotypes such as familial cardiomyopathy and the Hutchison-Gilford Progeria accelerated aging syndrome (5; 10; 33). Normal aging is associated with a reduction in lamin A/C expression in mouse osteoblasts and human dermal fibroblasts (9; 30). To date, the effect of normal aging on lamin A/C expression in cardiomyocytes remains unknown.

Age-related changes in lamin A/C expression in cardiomyocytes may be associated with the downstream changes in myocardial function and structure seen in aging hearts. This is suggested by observations of marked myocardial abnormalities in LMNA mutation carriers and in lamin A/C deficient mice (26; 35). We hypothesized that normal aging is associated with a reduction in lamin A/C expression in cardiomyocytes. We used lamin B1 expression (a non-developmentally regulated protein from the same family found in all nucleated cells independent of aging) as a control. Our primary objective was to compare the expression of lamin A/C in cardiomyocyte nuclei of old versus young mice using a well validated mouse model of aging (13; 19; 20).
MATERIALS AND METHODS

Animal tissue preparation

C57BL/6J mice (Jackson Laboratory, Bar Harbour, ME, USA) were housed in a limited access room restricted to aging mice (light:dark 12h:12h) as previously described (9). All animal manipulations adhered to the Canadian Council on Animal Care Standards and all protocols were approved by the McGill University and Lady Davis Institute Animal Care Utilization Committee. The colony was free of any parasitic, bacterial, or viral pathogens as determined by a sentinel program. Using CO2, animals were euthanized at 4 months of age (n=5) and at 24 months of age (n=5). Hearts were dissected under sterile conditions, rinsed in 0.01 M PBS, blotted dry, and weighed. Heart pieces were frozen at -80°C for further protein expression analyses. Tissue was also fixed in 10% formaldehyde for histological analysis. Several sections of heart (4–5 μm thick) were prepared and stained with hematoxylin and eosin (H/E) and visualized by light microscope.

Echocardiography

2D/M-mode echocardiography was performed in young (4 months) and old (20 months) C57Bl/6J male mice anesthetised with isoflurane. Mice were placed in an induction chamber, anesthetized in 2% isoflurane mixed with air then maintained in 0.5-0.7% isoflurane. After removal of the thoracic fur, mice were placed in a left cubital position. Echocardiographic parasternal short-axis views at the midventricular level were acquired using a i13L transducer and a digital ultrasound system (Vivid 7, GE Medical Systems) at a 1 cm depth and 100 frames/sec. Measurements were performed off-line with the use of a customized version of the EchoPac Software (GE Medical Systems). End-diastolic and end-
systolic dimensions of the left ventricular (LV) cavity as well as anterior and posterior thickness of the LV wall were measured in M-mode tracings according to the leading edge method (27). Measurements were averaged from three consecutive beats of three image acquisitions. Fractional shortening, relative wall thickness, and LV mass were derived as described previously (32; 34).

Quantification of lamin A/C and B1 expression by immunohistochemistry

After dissection and fixation, young and old hearts samples were embedded in low-melting-point paraffin in a Shandon Citadel 2000 automatic tissue processor (Shandon Scientific Limited, Runcorn, UK). Coronal and transverse sections (4 mm) were mounted on silane-coated glass slides (Fischer Scientific, Springfield, NJ, USA). Paraffin was removed with three washes of xylene and rehydrated with washes of graded ethanol (80%–50%–30%) and PBS. Non-specific binding was blocked by addition of goat serum for 1 hour. Sections were then incubated with mouse monoclonal IgM lamin A/C antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) for either 4 hours at room temperature or 8–24 hours at 4°C. Sections incubated with mouse monoclonal IgG lamin B1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) were used as controls. After washing with PBS, hydrogen peroxide complexed rabbit anti-mouse IgG were added to the sections at room temperature for 30 minutes, followed by a 30 minute incubation with 0.6% hydrogen peroxide ± chromogen. Immunohistochemical staining was performed using the human ABC staining system (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Lamin positive cells showed a brown nucleus with punctate brown staining from the peroxidase-labelled antibody and blue counterstaining from the hematoxylin. Lamin A/C expression was calculated as the
number of cardiomyocyte nuclei positively stained for lamin A/C (brown stained nuclei) divided by the total number of cardiomyocyte nuclei (brown and blue stained nuclei) in each of 10 randomly chosen high power fields (hpf).

**Quantification of lamin A/C by immunofluorescence**

Heart sections were treated as described above, omitting the final step involving treatment of cells with hydrogen peroxide. After fixation in 4% paraformaldehyde, sections were washed with PBS and then incubated in PBS with 10% blocking serum for 20 minutes to suppress non-specific binding of IgG. Sections were incubated with mouse monoclonal IgM lamin A/C antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) with 1.5% blocking serum overnight at 4°C and then incubated with fluorescein-conjugated secondary antibody (FITC-Santa Cruz, Santa Cruz, CA, USA) diluted to 2 µg/ml in PBS with 1.5% blocking serum for 45 minutes. Nuclei were counterstained using propidium iodine (2 µg/ml). Control slides were incubated with rabbit IgG according to manufacturer instructions, and triplicate tests and control slides were included in immunodetection. Lamin A/C positive cells showed intense nuclear green fluorescence while negative controls showed only faint nuclear or cytoplasmic fluorescence. The number of positive cardiomyocyte nuclei divided by the total number of cardiomyocyte nuclei in each field was calculated as described above.

**Quantification of lamin A/C and B1 expression by western blot**

Nuclear extracts were obtained after suspending the heart pieces in 2 volumes of buffer containing 10 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, and protease
inhibitor cocktail diluted according to the manufacturers instruction (Complete™ protease
inhibitor, Boehringer Mannheim, Laval, QC, Canada). The homogenate was clarified by
centrifugation at 25,000 × g for 20 minutes at 4°C and the nuclear pellet was resuspended in
20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM MgCl2, 0.2 mM EDTA, 0.5
mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. Following a further 20 minute
centrifugation at 25,000 × g, nuclear extracts (supernatant) were dialyzed for 5 hours against
20 mM HEPES, pH 7.9, 20% glycerol, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM
phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol. Protein content was determined
with a protein assay kit (Bio-Rad, Mississauga, ON, Canada) and samples were then
aliquoted and stored at -80°C. For western blot analyses, nuclear extracts were resuspended
in SDS electrophoresis buffer (Bio-Rad, Hercules, CA, USA), proteins were separated on
SDS-polyacrylamide gels and the proteins electrotransfered to Immobilon P polyvinylidene
difluoride membranes. After blocking with PBS containing 0.1% Tween 20 and 10% non-fat
dry milk, membranes were incubated overnight at 4°C using a monoclonal antibody directed
against lamin A/C and a second monoclonal antibody against lamin B1 (Santa Cruz, Santa
Cruz, CA, USA). Specific staining was revealed after washing and incubating with
horseradish peroxidase-conjugated IgG rabbit anti-mouse antibodies followed by enhanced
chemiluminescence using Lumi-GLO reagents (Kirkegaard & Perry, Gaithensburg, MA,
USA). The lamin A/C signals were quantified by densitometry and normalized according to
lamin B1 signals.

Statistical analysis

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All results are expressed as mean ± standard error of the mean of three replicate determinations, and statistical comparisons are based on one-way analysis of variance (ANOVA) or student t-tests. A p-value of <0.05 was considered significant and ≥0.05 was considered non-significant (NS).
RESULTS

The young (n=5) and old (n=5) C57BL/6J mice were representative of the litter and did not have identifiable diseases. Dissection of the murine hearts revealed that the mean mass of the isolated hearts was 189 ± 15 mg in old mice and 167 ± 15 mg in young mice (p<0.05). Microscopic inspection of the H&E stained sections did not reveal any gross pathology in the cardiac tissues (Figure 1 A and B). The number of cardiomyocytes per hpf was inferior in old mice compared to young mice (40-62 cells/hpf vs. 65-80 cells/hpf, p<0.05) whereas the cell size was similar (108 µm vs. 110 µm, p=NS).

Echocardiographic results are shown in Table 1. In comparison to young mice, old mice demonstrated increased LV wall thickness with preserved LV cavity size resulting in increased relative wall thickness (0.38 ± 0.05 vs. 0.29 ± 0.06, p<0.05) suggestive of concentric remodeling. Accordingly, LV mass was increased in old mice (121 ± 19 mg vs. 93 ± 16 mg, p<0.05). Fractional shortening was slightly reduced in old mice (46.3 ± 3.9% vs. 48.3 ± 3.8%, p<0.05).

Immunohistochemical analyses showed that the expression of lamin A/C but not lamin B1 was reduced in cardiomyocyte nuclei of old mice (Figure 1 C, D, E, and F). The proportion of nuclei positively stained for lamin A/C was 9 ± 6% in old mice and 62 ± 16% in young mice (p<0.001). In contrast, the proportion of nuclei positively stained for lamin B1 control remained constant (90 ± 6% in old mice and 96 ± 4% in young mice, p=NS). Immunofluorescence analyses also showed that the expression of lamin A/C was reduced in cardiomyocyte nuclei of old mice (Figure 2 A-D).

In addition to quantitative changes in lamin A/C expression, qualitative changes in lamin A/C distribution were observed. The distribution of lamin A/C was homogeneous
throughout the nuclei in young mice, whereas it was scattered towards the periphery and perinuclear with minimal contact between staining sites in old mice (Figure 2 E and F).

Western blot analyses confirmed and quantified the reduction in lamin A/C expression in cardiomyocyte nuclear extracts of old mice (Figure 3). The ratio of lamin A/C:B1 as measured by densitometry was 0.6 in old mice and 1.2 in young mice (p<0.01).

Finally, changes in lamin A/C expression were observed in other heart cells. Specifically, immunohistochemical analyses suggested that the expression of lamin A/C but not B1 was reduced in vascular endothelial cell nuclei of old mice compared to young mice (Figure 4).
DISCUSSION

Our study is the first to show that normal aging is associated with a reduced expression of lamin A/C in cardiomyocytes. Moreover, we found that normal aging is associated with a scattered perinuclear distribution of lamin A/C and may be associated with a reduced expression of lamin A/C in vascular endothelial cells. Thus, age-related changes in lamin A/C expression and distribution denote a novel aging mechanism previously described in the dermatologic and osteoarticular systems (9; 30) and now discovered in the cardiovascular system.

Our finding of reduced myocardial lamin A/C expression with aging is preceded by the well documented finding of reduced myocardial lamin A/C expression with inherited LMNA mutations (1; 39). LMNA mutations are among the most common causes of familial autosomal-dominant cardiomyopathy (18). Individuals with these mutations often have heart failure with increased LV wall thickness (37). Up to 88% of affected individuals have electrophysiological disturbances such as sick sinus syndrome, atrioventricular block, and atrial fibrillation or flutter (10; 16). This constellation of findings shares several features with the physio-pathological changes seen in the aging heart (22; 23). We speculate that LMNA-related familial cardiomyopathy and age-related senile cardiomyopathy may represent two entities in a spectrum of lamin A/C deficient heart disease.

The newly discovered association of aging and myocardial lamin A/C expression is a fundamental first step in lamin-cardiology research. It opens the door for further characterization of the age-related changes in myocardial lamin A/C expression and distribution. More importantly, it opens the door for mechanistic research to test whether these exists a causal link between the observed decline in lamin A/C and the parallel
abnormalities in myocardial structure and function. One potential link between lamin A/C and aging hearts may be that both are epitomized by an impaired cellular and nuclear response to stressors. The structural model of age-related changes in lamin A/C suggests that loss of lamin function causes nuclear fragility which leads to permanent damage or death in the face of mechanical or environmental stressors (33). Similarly, age-related changes in the heart are described as a state of fragility or reduced adaptation to acute and chronic stressors such as exercise or myocardial ischemia (11; 13-15; 21; 27; 29).

In agreement with prior studies conducted in animal models and in human subjects (2; 3; 6; 31; 40), old mice demonstrated increased LV wall thickness with preserved cavity size resulting in increased relative wall thickness (concentric remodeling) and mass. Systolic function was slightly, yet significantly, reduced as previously demonstrated by Yang et al (40). Although diastolic function was difficult to assess given the rapid heart rates of our mice under physiologic conditions (mean 548-563) and the challenge of transducer positioning for reliable parallel mitral inflow, our finding of concentric hypertrophy is consistent with the finding of a relaxation abnormality in senescent mice demonstrated by Taffet et al (36). We do not intend for the echocardiographic data to be causally explanatory; however, we believe that these data add an important dimension to our study by showing that the morphological and functional correlation of our histopathological findings are consistent with the expected changes of myocardial aging.

Clinically, lamin A/C has the potential to be a prognostic marker and a therapeutic target. Among 15 patients with nonischemic cardiomyopathy requiring left ventricular assist device support, lamin A/C expression over time was a strong predictor of myocardial recovery leading to explantation of the device (4). In presymptomatic LMNA mutation
carriers, lamin A/C expression may be used as a screening tool to identify high-risk subjects who may benefit from more aggressive therapy (28). Therapeutic agents such as farnesylation modulators have been shown to prevent or reverse some of the nuclear defects in Hutchison-Gilford Progeria Syndrome (7; 8; 12; 25; 38). These agents modulate post-translational conversion of precursor prelamin A to the active lamin A correcting the nuclear defect associated with lamin A depletion or prelamin A accumulation. To our knowledge, the effect of farnesylation modulators on normal aging or on the cardiovascular system has not been evaluated.

In conclusion, the expression of lamin A/C is substantially reduced and the distribution is scattered peripherally in nuclei of cardiomyocytes isolated from a validated mouse model of aging. Further research in this field may attempt to clarify the causal link between lamin A/C and aging hearts, and to explore the value of farnesylation modulators as novel therapeutic agents to counter the potentially negative effects of lamin A/C depletion on the heart.
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DISCLOSURES

The authors have not published or submitted any related papers from the same study and have no conflicts of interest or financial disclosures to report.
REFERENCE LIST


36. **Taffet GE, Hartley CJ, Wen X, Pham T, Michael LH and Entman ML.**


FIGURE LEGENDS

Figure 1:
Microscopic inspection of the H&E stained sections in young (A) and old (B) mice did not reveal any gross pathology in the cardiac tissues. The number of cardiomyocytes per hpf was greater in young mice compared to old mice whereas the cell size was similar.

(C) Lamin A/C expression in young cardiomyocytes, (D) Lamin B1 expression in young cardiomyocytes, (E) Lamin A/C expression in old cardiomyocytes, (F) Lamin B1 expression in old cardiomyocytes. Note that the expression of lamin A/C but not B1 is decreased in old cardiomyocytes. The scatter plot (G) shows the proportion of nuclei positive for lamin A/C in each of the 10 high power fields analyzed per mouse (9% vs. 62%, p<0.001).

Figure 2:
Imunofluorescence staining of lamin A/C in young cardiomyocytes (A) vs old cardiomyocytes (C). Young cardiomyocytes show bright fluorescence staining at the nuclei (A, white arrows) by lamin A/C antibody. Lamin A/C labeling is reduced in old cardiomyocytes (B, white arrows). Panels A and C show overlap between propidium iodine and green immunofluorescence. Panel B and D show PI counterstaining to determine the total number of nuclei in the field.

(E) Lamin A/C distribution in young cardiomyocytes, (F) Lamin A/C distribution in old cardiomyocytes. Note that the distribution of lamin A/C as shown by the red arrows is peripherally scattered and perinuclear in old vs. homogeneous and intranuclear in young cardiomyocytes.
Figure 3: Quantification of Lamin A/C and Lamin B1 (Control) by Western Blot

(Left) Western blot of lamin A/C and B1 expression in nuclear extracts of young cardiomyocytes, (Right) Western blot of lamin A/C and B1 expression in nuclear extracts of old cardiomyocytes. One representative blot of each protein (60 μg) is shown from three experiments that yielded similar results. The signals were quantified by densitometry and normalized according to the lamin B1 expression. The bar graph shows the ratio between lamin A/C : lamin B1 which is significantly decreased in old cardiomyocytes (0.6 vs. 1.2, p<0.01).

Figure 4: Vascular Expression of Lamin A/C

(A) Lamin A/C expression in the vascular endothelial cells of young cardiomyocytes, (B) Lamin A/C expression in the vascular endothelial cells. Note that the expression of lamin A/C as shown by black arrows is decreased in the vascular endothelial cells of old mice.
**Table 1. Echocardiographic data for young and old mice**

<table>
<thead>
<tr>
<th></th>
<th>Young</th>
<th>Old</th>
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<tbody>
<tr>
<td>HR (beats/min)</td>
<td>563±72</td>
<td>548±57</td>
</tr>
<tr>
<td>LVEDD (mm)</td>
<td>42.5±3.7</td>
<td>41.2±2.8</td>
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<tr>
<td>LVESD (mm)</td>
<td>22.0±2.6</td>
<td>22.0±1.9</td>
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<tr>
<td>SW (mm)</td>
<td>5.7±1</td>
<td>7.3±1.1*</td>
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<tr>
<td>PW (mm)</td>
<td>6.6±1.1</td>
<td>8.3±1.3*</td>
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<tr>
<td>RWT</td>
<td>0.29±0.06</td>
<td>0.38±0.05*</td>
</tr>
<tr>
<td>FS (%)</td>
<td>48.3±3.9</td>
<td>46.3±3.8*</td>
</tr>
<tr>
<td>LVM (mg)</td>
<td>93±16</td>
<td>121±19*</td>
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Significance is indicated by an * where *p* < 0.05 when young versus old values are compared.

Abbreviations: HR, heart rate; LVEDD, left ventricular end diastole dimension; LVESD, left ventricular end systole dimension; SW, septal wall thickness; PW, posterior wall thickness; RWT, relative wall thickness calculated as \((PW+SW)/LVEDD\); FS, fractional shortening calculated as \([(LVEDD-LVESD)/LVEDD] \times 100\); LVM, left ventricular mass calculated as \(1.055 \left[\left(PW+SW+LVEDD\right)^3-(LVEDD)^3\right]\).
Figure 1: Microscopic inspection of the H&E stained sections in young (A) and old (B) mice did not reveal any gross pathology in the cardiac tissues. The number of cardiomyocytes per hpf was greater in young mice compared to old mice whereas the cell size was similar. (C) Lamin A/C expression in young cardiomyocytes, (D) Lamin B1 expression in young cardiomyocytes, (E) Lamin A/C expression in old cardiomyocytes, (F) Lamin B1 expression in old cardiomyocytes. Note that the expression of lamin A/C but not B1 is decreased in old cardiomyocytes. The scatter plot (G) shows the proportion of nuclei positive for lamin A/C in each of the 10 high power fields analyzed per mouse (9% vs. 62%, p<0.001).
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