Eliminating the Synthesis of Mature Lamin A Reduces Disease Phenotypes in Mice Carrying a Hutchinson-Gilford Progeria Syndrome Allele*

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Hutchinson-Gilford progeria syndrome is caused by the synthesis of a mutant form of prelamin A, which is generally called progerin. Progerin is targeted to the nuclear rim, where it interferes with the integrity of the nuclear lamina, causes misshapen cell nuclei, and leads to multiple aging-like disease phenotypes. We created a gene-targeted allele yielding exclusively progerin (LmnaHG) and found that heterozygous mice (LmnaHG/+’) exhibit many phenotypes of progeria. In this study, we tested the hypothesis that the phenotypes elicited by the LmnaHG allele might be modulated by compositional changes in the nuclear lamina. To explore this hypothesis, we bred mice harboring one LmnaHG allele and one LmnaLCO allele (a mutant allele that produces lamin C but no lamin A). We then compared the phenotypes of LmnaHG/LCO mice (which produce progerin and lamin C) with littermate LmnaHG/+’ mice (which produce lamin A, lamin C, and progerin). LmnaHG/LCO mice exhibited improved body weight curves (p < 0.0001), reduced numbers of spontaneous rib fractures (p < 0.0001), and improved survival (p < 0.0001). In addition, LmnaHG/LCO fibroblasts had fewer misshapen nuclei than LmnaHG/+’ fibroblasts (p < 0.0001). A likely explanation for these differences was uncovered; the amount of progerin in LmnaHG/LCO fibroblasts and tissues was lower than in LmnaHG/+’ fibroblasts and tissues. These studies suggest that compositional changes in the nuclear lamina can influence both the steady-state levels of progerin and the severity of progeria-like disease phenotypes.

Mutations in LMNA yield a host of different human diseases, including muscular dystrophy, partial lipodystrophy, and Hutchinson-Gilford progeria syndrome (HGPS) (1–3). LMNA yields two principal proteins, prelamin A and lamin C (4). Prelamin A terminates with a CAAAX motif (i.e. CSIM), which triggers farnesylation and methylation of a carboxy-terminal cysteine (5, 6). Following these modifications, the carboxy-terminal portion of prelamin A (including the farnesylcysteine methyl ester) is clipped off by ZMPSTE24 and degraded, releasing mature lamin A (6, 7). Lamin C does not contain a CAAAX motif and is not farnesylated. Lamins A and C are important proteins of the nuclear lamina, a filamentous meshwork that lines the inner nuclear membrane (1–3). In the mouse, a complete absence of lamin A and lamin C leads to muscular dystrophy and death by 6 weeks of age (8). However, prelamin A and lamin A appear to be dispensable, as mice that are homozygous for a “lamin C-only” allele (LmnaLCO/LCO) are healthy and robust (9).

Hutchinson-Gilford progeria syndrome is a rare pediatric progeroid syndrome associated with the production of a mutant form of prelamin A (6, 10, 11). Patients with HGPS appear normal at birth but soon develop multiple disease phenotypes resembling premature aging (12). HGPS is almost always caused by a point mutation in LMNA that alters pre-mRNA splicing, resulting in a mutant form of prelamin A, commonly called progerin, with a 50-aminocacid internal deletion (10, 11). Progerin retains the carboxy-terminal CAAAX motif and is farnesylated and methylated (13). However, the 50-aminocacid deletion eliminates the site for the ZMPSTE24-mediated cleavage reaction, abrogating further processing to mature lamin A. Progerin accumulates at the nuclear rim and impairs the integrity of the nuclear lamina, resulting in misshapen nuclei and multiple disease phenotypes (10, 11). Importantly, rare LMNA mutations leading to particularly high levels of progerin synthesis are associated with particularly severe forms of progeria (14).

Yang et al. (15) created a gene-targeted Hutchinson-Gilford allele, LmnaHG, which yields exclusively progerin. Mouse fibroblasts heterozygous for the mutant allele (LmnaHG/+’) express large amounts of progerin and have an increased frequency of misshapen nuclei (15). LmnaHG/+’ mice appear normal at birth, but by 6–8 weeks of age they manifest hallmarks of progeria (slow growth, weight loss, loss of adipose tissue, and osteolytic lesions in ribs and other bones) (16). Osteolytic lesions in the ribs result in numerous spontaneous fractures. Most LmnaHG/+’ mice die by ~28 weeks of age. Mice with a double dose of progerin (LmnaHG/HG) are severely affected and die by 3 weeks of age (16).

Genetic studies in both humans and mice have shown that higher levels of progerin are associated with more severe dis-
ease phenotypes (14, 16). Thus far, however, no one has investigated whether alterations in the composition of the nuclear lamina could affect the severity of disease. Progerin is targeted to the nuclear rim and directly interacts with other nuclear lamins (17); hence, it seemed possible that changes in lamin composition might influence disease phenotypes elicited by the mutant allele. In this study, we hypothesized that compositional changes in the nuclear lamina might modulate disease phenotypes in mice harboring the LmnaHG allele. To explore this hypothesis, we took advantage of the existence of a lamin C-only allele, LmnaLCO, which produces lamin C but no lamin A. In our studies, we compared the severity of disease phenotypes in LmnaHG/LCO mice (which produce progerin and lamin C) and littermate LmnaHG/- mice (which produce mature lamin A, lamin C, and progerin). Our data indicate that the severity of disease phenotypes elicited by the LmnaHG allele is diminished in the setting of the LmnaLCO allele (i.e. in the setting of a nuclear lamina devoid of mature lamin A).

**EXPERIMENTAL PROCEDURES**

**Genetically Modified Mice**—Male chimeric mice, generated by injecting C57BL/6 blastocysts with LmnaHG/+ embryonic stem cells (9, 15), were bred with LmnaLCO/- female mice (9, 15), and Lmna+/-, LmnaHG/+ (n = 16), and LmnaHG/LCO (n = 26) offspring were analyzed. Genotyping was performed by PCR (9, 15) and by Western blotting with an antibody against lamin A/C (sc-6215, Santa Cruz Biotechnology) (15).

**Cell Culture, Protein Extraction, and Western Blotting**—Primary mouse embryonic fibroblasts were prepared from embryonic day 13.5 mouse embryos (18). To generate cell extracts for Western blots, early passage fibroblasts were washed with phosphate-buffered saline, and urea-soluble extracts were prepared as described (19). For tissue extraction, 150 mg of fresh mouse tissues were frozen with liquid nitrogen and ground into a powder with a mortar and pestle. The powder was then resuspended in 0.5 ml of urea solubilization buffer (19) and ground in a glass tissue grinder for an additional 2 min. Samples were then sonicated and centrifuged at 14,000 × g for 10 min. The supernatant fluid was collected and analyzed by Western blotting.

The protein extracts were electrophoresed on 4–12% gradient polyacrylamide BisTris gels (Invitrogen); the size-fractional gels were electrophoretically transferred to a sheet of nitrocellulose membrane for Western blotting. The antibody dilutions were 1:400 for anti-lamin A/C goat IgG (sc-6215, Santa Cruz Biotechnology), 1:1000 for anti-actin goat IgG, and 1:6000 for horseradish peroxidase-labeled anti-actin goat IgG (sc-2020, Santa Cruz Biotechnology). Antibody binding was detected with the ECL Plus chemiluminescence system (GE Healthcare) and exposure to x-ray film. Western blots were also analyzed with the Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE). In those experiments, the nitrocellulose membrane was rinsed in phosphate-buffered saline for 5 min before incubation in Odyssey blocking buffer. The membrane was incubated with the primary antibodies in blocking solution for 1 h at room temperature. The antibody dilutions were 1:400 for anti-lamin A/C rabbit IgG (sc-20681, Santa Cruz Biotechnology, Santa Cruz, CA) and 1:1000 for anti-actin goat IgG (sc-1616, Santa Cruz Biotechnology). After washing the membrane three times for 10 min in phosphate-buffered saline containing 0.1% Tween 20, it was incubated with 1:5000 IRDye 700 anti-goat IgG antibody and 1:5000 IRDye 800 anti-rabbit IgG antibody (both from Rockland Immunochemicals) in Odyssey blocking buffer. After washing, the membranes were scanned on an Odyssey infrared imaging system, and images were acquired and analyzed according to the manufacturer’s instructions.

**RNA Extraction, cDNA Synthesis, and RT-PCR**—Total RNA was extracted from primary fibroblasts and mouse tissues with the RNeasy mini kit (Qiagen, Valencia, CA) and quantified with a NanoDrop Spectrophotometer (NanoDrop Technologies, Wilmington, DE). The integrity of the RNA was verified with an ethidium bromide-stained 1.5% agarose gel. Reverse transcription reactions were carried out with 1 μg of RNA that had been treated with DNase (DNA-Free, Ambion, Foster City, CA), Superscript III (Invitrogen), and a mixture of oligo(dT) (Invitrogen) and random primers (Invitrogen). The cDNA samples were incubated with RNase (RNase H, Ambion, Foster City, CA) and then diluted to 10 ng/μl for PCRs.

The progerin and prelamin A cDNAs were amplified with primers corresponding to exon 10 and exon 12 sequences (forward, 5’-CTGACATGTTGGCAGGCAA-3’; reverse, 5’-TTCAGACACGGGGCAGAAG-3’). Arbp, used as the internal control, was amplified with primers 5’-ACTGAGATTCGGGATATGCTGT-3’ and 5’-TCCTAGACAGTGTTCTGACGCTG-3’. All primers were designed with Primer 3 software. The RT-PCR was performed with AmpliTaq (ABI Foster City, CA) and 50 ng of cDNA. The thermal cycling conditions were as follows: 95 °C for 3 min, 95 °C for 15 s, 60 °C for 30 s, and 72 °C for 15 s, for a total of 26 cycles. The RT-PCR fragment spanning exon 10–12 was 456 bp in the wild-type Lmna allele and 306 bp in the LmnaHG allele. The RT-PCR fragment for Arbp was 108 bp. 10 μl of Arbp PCR products were mixed with 25 μl of lamin A and progerin PCR products and loaded onto a 1.3% agarose gel containing SYTO 60 nucleic acid stain (Invitrogen) (1:16,000 dilution). Images of the gels were acquired with the Odyssey infrared imaging system, and band intensity was quantified according to the manufacturer’s instructions.

**Immunofluorescence Microscopy**—Early passage fibroblasts (matched for passage number) were grown on coverslips, fixed with 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, and blocked with bovine serum albumin (20). Cells were
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FIGURE 2. Phenotypes of Lmna\[^{HG/LO}\] and Lmna\[^{HG/LO}+\] mice. A, body weight curves in littermate Lmna\[^{HG/LO}\] (n = 26), Lmna\[^{HG/LO}+\] (n = 16), and Lmna\[^{HG/LO}+\] (n = 26). Body weight curves of Lmna\[^{HG/LO}\] mice were incubated for 60 min with antibody against lamin A (1:200, sc-20680, Santa Cruz Biotechnology). After washing, cells were stained with anti-rabbit Cy3-conjugated secondary antibody (Jackson Immunoresearch) and 4',6-diamidino-2-phenylindole (to visualize DNA). Images were obtained on an Axiovert 200 MOT microscope (Carl Zeiss, Thornwood, NY) with a 63X/1.25 oil immersion objective and processed with Axo-Vision 4.2 software (Zeiss). The number of misshapen nuclei (exhibiting blebs or folds) was scored by three independent observers blinded to genotype. Differences in the percentages of misshapen nuclei between cells with different genotypes were assessed with a \( \chi^2 \) test.

Mouse Phenotypes—Body weights of Lmna\[^{+/+}\], Lmna\[^{HG/+}\], and Lmna\[^{HG/LO}\] mice were monitored weekly. Body weight curves for different groups of mice were compared with repeated measures analysis of variance and the log-rank test. For survival analysis, Kaplan-Meier analyses were performed, and the log-rank test was used to compare the survival curves. At the time that each mouse died, the heart and lungs were removed, and the thorax was photographed with a digital camera, and the number of rib fractures was counted. The number of rib fractures in different groups of mice was compared with a two-tailed Student’s \( t \) test.

RESULTS

To determine whether the absence of lamin A synthesis would affect the disease phenotypes elicited by the Lmna\[^{HG}\] allele, we compared the phenotypes of Lmna\[^{HG/LO}\] and littermate Lmna\[^{HG/+}\] mice. As expected, the Lmna\[^{HG/LO}\] mice synthesized lamin C and progerin, whereas Lmna\[^{HG/+}\] mice produced lamin A, lamin C, and progerin (Fig. 1).

Lmna\[^{HG/LO}\] and Lmna\[^{HG/+}\] mice appeared similar at weaning. By 10–15 weeks of age, however, the body weight curves in Lmna\[^{HG/LO}\] mice were significantly better than those of Lmna\[^{HG/+}\] mice (\( p < 0.0001 \)) (Fig. 2A). These differences were apparent in both males and females (\( p < 0.0001 \) for both groups). Kaplan-Meier survival curves revealed that Lmna\[^{HG/LO}\] mice lived longer than Lmna\[^{HG/+}\] mice (\( p < 0.0001 \)) (Fig. 2B). Again, this difference was significant in both males and females (\( p < 0.0001 \)). Also, the number of rib fractures was lower in Lmna\[^{HG/LO}\] mice than in Lmna\[^{HG/+}\] mice (\( p < 0.0001 \)) (Fig. 2C), despite the fact that the Lmna\[^{HG/LO}\] mice were older at the time of death (27 ± 1.65 weeks versus 23 ± 0.70 weeks in Lmna\[^{HG/+}\] mice).

A hallmark of Lmna\[^{HG/+}\] fibroblasts is misshapen cell nuclei. Given the less severe disease phenotypes in Lmna\[^{HG/LO}\] mice, we hypothesized that the frequency of misshapen nuclei in Lmna\[^{HG/LO}\] fibroblasts might be lower than in Lmna\[^{HG/+}\] fibroblasts. To test this possibility, we scored nuclear shape abnor-

significantly improved, compared with Lmna\[^{HG/+}\] mice (\( p < 0.0001 \)). The body weight curves of Lmna\[^{LO/+}\] mice (n = 20) were indistinguishable from Lmna\[^{+/+}\] mice (n = 26) (not shown). B, Kaplan-Meier survival plots for Lmna\[^{HG/LO}\] mice (n = 26) and littermate Lmna\[^{HG/+}\] mice (n = 16). The number of surviving male and female mice was recorded weekly and expressed as a percentage of the total number of mice. Differences in the curves were assessed with a log-rank test (\( p < 0.0001 \)). C, rib fractures in Lmna\[^{HG/LO}\] and Lmna\[^{HG/+}\] mice. The number of rib fractures in Lmna\[^{HG/LO}\] mice was lower than in Lmna\[^{HG/+}\] mice (\( p < 0.0001 \)), as determined by the two-tailed \( t \) test.
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Figure 3. Frequency of misshapen nuclei in primary fibroblasts from Lmna<sup>HG/LCO</sup>, Lmna<sup>HG/+</sup>, Lmna<sup>LCO/+</sup>, and Lmna<sup>LCO/+</sup> embryos. A, Immunofluorescence microscopy of Lmna<sup>HG/+</sup>, Lmna<sup>LCO/-</sup>, and Lmna<sup>LCO/+</sup> fibroblasts. Cells were stained for lamin A (red) and 4',6-diamidino-2-phenylindole (blue). Nuclear blebs in Lmna<sup>HG/+</sup> and Lmna<sup>LCO/+</sup> cells are indicated by a white arrowhead; folds are indicated by a white arrow. B, frequency of misshapen nuclei in Lmna<sup>HG/LCO</sup> fibroblasts (n = 5) was lower than in Lmna<sup>HG/+</sup> fibroblasts (n = 3) (p < 0.0001), as judged by a χ² test. Each bar chart shows the frequency of nuclear blebbing with an independently isolated fibroblast cell line. Ratios above each bar represent the number of cells with misshapen nuclei divided by the total number of cells evaluated (from all cell lines). Similar results, with identical levels of statistical significance, were observed with two other observers blinded to genotype.

Figure 4. Western blotting with the Odyssey infrared imaging system to document levels of progerin expression in Lmna<sup>HG/LCO</sup> and Lmna<sup>HG/+</sup> fibroblasts and tissues. A, Western blot analysis (with antibodies against lamin A/C and actin) showing progerin, lamin A, lamin C, and actin in Lmna<sup>HG/+</sup> and Lmna<sup>HG/LCO</sup> fibroblasts. B, quantitative analysis of progerin expression in the Lmna<sup>HG/LCO</sup> (n = 8) and Lmna<sup>HG/+</sup> fibroblasts (n = 8), showing reduced progerin levels, relative to actin, in Lmna<sup>HG/LCO</sup> fibroblasts (p < 0.0001 by a two-tailed t test). Each sample was analyzed on eight separate Western blots; the variation in the progerin to actin ratio in the different Western blots averaged 2.3%. C and D, Western blot analysis (with antibodies against lamin A/C and actin) showing progerin, lamin A, lamin C, and actin in the heart (C) and skin (E) of Lmna<sup>HG/+</sup> and Lmna<sup>HG/LCO</sup> mice. D and F, quantitative analysis of progerin expression in the tissues of Lmna<sup>HG/LCO</sup> and Lmna<sup>HG/+</sup> mice. Two comparisons of Lmna<sup>HG/LCO</sup> and Lmna<sup>HG/+</sup> mice were made, each with one Lmna<sup>HG/LCO</sup> mouse and one Lmna<sup>HG/+</sup> mouse. Each sample was analyzed on eight separate Western blots; the variation in the progerin to actin ratio in the different Western blots averaged 3.03%. In both of the two comparisons with a Lmna<sup>HG/LCO</sup> mouse and a Lmna<sup>HG/+</sup> mouse, the progerin/actin ratio was lower in the Lmna<sup>HG/LCO</sup> mouse, both in the heart (D) and the skin (F). MEFs, mouse embryo fibroblasts.

DISCUSSION

Previous studies have established, quite unequivocally, that progerin is the culprit molecule in HGPS (22–24). Higher levels of progerin produce more severe nuclear shape abnormalities than lower levels of progerin (22, 23). Also, LMNA mutations that yield particularly high levels of progerin result in particularly severe forms of progeria (14). In this study, we studied an entirely new issue, whether changes in the composition of the nuclear lamina might modulate the phenotypes associated with the synthesis of a fixed amount of progerin. To explore this issue, we compared disease phenotypes in Lmna<sup>HG/+</sup> and
The quantitative RT-PCR experiments revealed equal amounts of progerin transcripts in \( Lmna^{HG/+} \) and \( Lmna^{HG/LCO} \) cells and tissues, so the reduced levels of progerin protein in \( Lmna^{HG/LCO} \) cells and tissues cannot be ascribed to changes in progerin transcript levels. It is a formal possibility that the lower progerin levels in \( Lmna^{HG/LCO} \) cells and tissues are because of reduced translation of the \( Lmna^{HG} \) transcript. However, we believe that it is more likely that the altered composition of the nuclear lamina in \( Lmna^{HG/LCO} \) cells and tissues (i.e. a complete absence of mature lamin A) is responsible for the alterations in progerin levels. We suspect that the cellular machinery responsible for the turnover of progerin could be more efficient when that protein is surrounded by lamin C than when it is surrounded by a mixture of lamin A and lamin C. Part of this may relate to the abilities of lamin A, lamin C, and progerin to interact with each other. More than 15 years after a molecular understanding of lamin A and lamin C biogenesis (4), no one has conclusively determined whether lamins A and C form heterodimers in the cell or exist solely in homodimers. Recently, however, Delbarre et al. (17) examined the polymerization of green fluorescent protein-tagged and red fluorescent protein-tagged wild-type and mutated lamins in the nuclear envelope by measuring fluorescence resonance energy transfer. They concluded that both wild-type lamin A and lamin B1 form homopolymers that interact further in the nuclear lamina. In contrast, their studies suggested that progerin co-assembles with both lamin B1 and lamin A to form a heteropolymer, so that the usual segregation of A-type and B-type lamins is lost (17). Assuming that these fluorescence resonance energy transfer studies with green fluorescent protein-tagged and red fluorescent protein-tagged lamins accurately portray the ability of native untagged progerin to interact with other lamin proteins, it is not at all farfetched to imagine that changes in the composition of the lamina could influence the steady-state levels of progerin in the cell.

The phenotypic differences between \( Lmna^{HG/+} \) and \( Lmna^{HG/LCO} \) mice were unequivocal and highly significant, but we emphasize that the reduction in disease phenotypes in \( Lmna^{HG/LCO} \) mice was only partial. The \( Lmna^{HG/LCO} \) mice still had markedly abnormal body weight curves, still developed spontaneous bone fractures, and still died prematurely. A similar situation was observed in \( Lmna^{HG/+} \) mice that had been treated with a farnesyltransferase inhibitor. Treatment of \( Lmna^{HG/+} \) mice with a farnesyltransferase inhibitor significantly improved disease phenotypes but that treatment fell far short of a complete cure (15, 25, 26).

This study shows that the absence of mature lamin A affects steady-state progerin levels and progerin-induced disease phenotypes. These studies lay the foundation for a new concept that compositional changes in the nuclear lamina have secondary effects on progerin levels and progeria disease phenotypes. This concept can now be explored in more detail. For example, it would be intriguing to determine whether transgenic overexpression of lamins A and C (27) would affect the severity of disease. Similarly, it would be interesting to determine whether a lamin B1 knock-out allele (28) would influence the severity of progeria. Ultimately, these genetic studies could yield clues regarding the functional relevance of different lamin proteins in the pathogenesis of progeria.

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**FIGURE 5.** Measurement of progerin mRNA in \( Lmna^{HG/LCO} \) and \( Lmna^{HG/+} \) fibroblasts by quantitative RT-PCR analysis. A and B, Syto60-stained agarose gels of progerin RT-PCR products, generated from RNA purified from the fibroblasts and tissues of \( Lmna^{HG/LCO} \) and \( Lmna^{HG/+} \) mice. The RT-PCR spanned exons 10–12 of \( Lmna \); trace amounts of a longer prelamin A transcript were observed in \( Lmna^{HG/+} \) samples. In these studies, Arbp was used as an internal control. A, comparison of the expression of progerin transcripts in the fibroblasts from \( Lmna^{HG/LCO} \) and \( Lmna^{HG/+} \) mice (\( n = 8 \) independent fibroblast cell lines, each from a different embryo). B, comparison of the expression of progerin transcripts in the heart and skin of \( Lmna^{HG/LCO} \) and \( Lmna^{HG/+} \) mice (\( n = 2 \) independent embryo; each sample was loaded in duplicate). C, quantitative analysis of the progerin mRNA levels, corrected for Arbp expression, showing no difference in progerin transcript levels in \( Lmna^{HG/LCO} \) and \( Lmna^{HG/+} \) fibroblasts (\( n = 8 \) genotype) and in the heart and skin of \( Lmna^{HG/LCO} \) and \( Lmna^{HG/+} \) mice (\( n = 2 \) genotype). Progerin mRNA levels in \( Lmna^{HG/LCO} \) samples were expressed relative to those in \( Lmna^{HG/+} \) samples (which were set at a value of 1). MEFs, mouse embryo fibroblasts; WT, wild type; NTC, non-template control.
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