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Splicing-Directed Therapy in a New Mouse Model of Human Accelerated Aging

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Hutchinson-Gilford progeria syndrome (HGPS) is caused by a point mutation in the LMNA gene that activates a cryptic donor splice site and yields a truncated form of prelamin A called progerin. Small amounts of progerin are also produced during normal aging. Studies with mouse models of HGPS have allowed the recent development of the first therapeutic approaches for this disease. However, none of these earlier works have addressed the aberrant and pathogenic LMNA splicing observed in HGPS patients because of the lack of an appropriate mouse model. Here, we report a genetically modified mouse strain that carries the HGPS mutation.

The variant in splincing site 1824 changes the site involved in splincing and induce the deletion of 150 nucleotide. This deletion corresponds with the loss of the protease cleavage site, so the synthesis of progerin form.

__**Description of progeria animal model:**__

These **mice accumulate progerin, present histological and transcriptional alterations characteristic of progeroid models, and phenocopy the main clinical manifestations of human HGPS, including shortened life span and bone and cardiovascular aberrations**.

Using this animal model, the authors have developed an **antisense morpholino–based therapy** that prevents the pathogenic Lmna splicing, markedly reducing the accumulation of progerin and its associated nuclear defects. Treatment of mutant mice with these morpholinos led to a marked amelioration of their progeroid phenotype and substantially extended their life span, supporting the effectiveness of

antisense oligonucleotide–based therapies for treating human diseases of accelerated aging.

To generate a knock-in mouse strain carrying the HGPS mutation, the authors designed a strategy for replacement of the wild-type mouse Lmna gene with a mutant allele that carried the 1824C>T nucleotide variant- Gly609Gly mutation, which is equivalent to the HGPS c.1824C>T;p.Gly608Gly mutation in the human LMNA gene.

How transgenic mice is making.

The locus of lamin A is constituted of 12 exons (red boxes). Lamin C is derived from the LMNA gene using an alternative splice site located in intron 10; Lamin C thus differs C-terminally from the lamin A.

Transgenic mice principles.

In order to modify a specific genomic regions, it is used a vector that contains the target genomic sequences, two selection reagents, neomycin and thymidine kinase for homologous recombinant clones selection. Neomycin is flanked with loxP sequences that is used for Cre-Lox systems. The genomic sequence deleted in progerin is between loxP sequences.

In the first step is obtained the mice that expressed laminin C.

Crossing this mice with Cre-mice, it is obtained the deletion of progerin production sequence.

In an attempt to avoid the breeding problems present in other mouse models that express progerin, **a conditional mutant allele with a neomycin resistance gene flanked by two loxP sites inserted in Lmna intron 10. This cassette was able to prevent the formation of prelamin A transcripts by blocking lamin A‑specific splicing**. Therefore, this allele, which we refer to as LmnaLCS (Lamin C‑Stop), directs only the expression of lamin C and allows study of the potential effects of lamin A deficiency . LmnaLCS/+mice and crossed them with a Cre-deleter mouse strain to

obtain germline removal of the neomycin resistance cassette. As a result, we obtained offspring that carried the LmnaG609G knock-in allele, which expressed lamin C, lamin A, and progerin.

Testing the generation of transgenic mice.

To ensure the correct translation of the mutant allele, we analyzed protein extracts from cultures of mouse fibroblasts of each genotype by Western (immuno) blot with a specific antibody that recognizes lamin A/C. Genomic analysis of Lmna exon 11 confirmed the genotypes of Lmna+/+, LmnaG609G/+, and LmnaG609G/G609G mice (fig. S1). Likewise, semiquantitative transcriptional analysis and direct sequencing of RNA samples from several tissues confirmed that the aberrant Lmna splicing in LmnaG609G mice was equivalent to the LMNA splicing error that occurs in HGPS patients. Finally, several tissue samples from Lmna+/+, LmnaG609G/+, and LmnaG609G/G609G mice were analyzed further by Western blotting, which confirmed the expected lamin A/C and progerin expression patterns.

Mice that carried the LmnaG609G allele expressed lamin C, lamin A, and progerin, reproducing the same molecular situation as is present in HGPS patients. Homozygous mice with the c.1827C>T;p.Gly609Gly mutation were infertile but seemed healthy until 3 weeks of age. Subsequently, they showed a reduction in growth rates (Fig. 2A), with a progressive loss of weight (Fig. 2B) and the acquisition of an abnormal posture and a marked curvature of the spine (cervicothoracic lordokyphosis) (Fig. 2D). These multiple alterations finally resulted in premature death of these mutant mice, which have an average life span of 103 days (Fig. 2C) compared to more than 2 years for wild-type mice. Heterozygous LmnaG609G/+ mice had normal weight, size, and fertility until about 8 months of age. At that point, the mice started to lose weight, exhibiting a process similar to that observed in homozygous mice and causing their premature death at an average of 242 days. Both heterozygous and homozygous mice that carried the c.1827C>T;p.Gly609Gly mutation showed profound nuclear abnormalities as a consequence of progerin accumulation (Fig. 2E).

LmnaG609G/G609G mice of advanced age exhibited a generalized loss of the principal fat deposits. Microscopy analysis of the skin revealed loss of the subcutaneous fat layer and a general attrition of hair follicles (Fig. 2F). **Senescence associated b-galactosidase staining was increased in liver and kidney sections** from 3-month-old LmnaG609G/G609G mice when compared with age-

matched wildtype animals (Fig. 2F), reflecting a premature aging process in these animals.

The tibias of mutant mice showed a reduction in bone density and cortical thickness as well as an increased porosity.

Mutant mice exhibited important cardiovascular alterations that could be related to their premature death and that also occur in HGPS patients as well as during normal aging (28). Because **vascular smooth muscle cell (VSMC)** depletion has been reported in another progeroid mouse model (12) and in some HGPS patients (29), we focused on the study of these cells. The number of VSMCs in the medial layer of the thoracic aorta was similar in wild-type and LmnaG609G/G609G mice (fig. S6A), but **LmnaG609G/G609G mice displayed a significant loss of VSMCs in the aortic arch, a region that exhibits extensive branching and is subjected to high hemodynamic stress** (Fig. 3B). Notably, the severity of this phenotype in one HGPS patient correlated with hemodynamic stress around the site of branching (29). Blood pressure appeared normal in LmnaG609G/G609G mutant mice (fig. S6B), but they progressively **developed bradycardia between 9 and 15 weeks of age** (Fig. 3B). Moreover, electrocardiographic (ECG) studies revealed prolonged QRS waves in LmnaG609G/G609G mice without changes in the PR interval (Fig. 3B) relative to wild-type mice, which indicates an alteration of heart ventricular depolarization. Finally, we assessed heart function by transthoracic echocardiography. Both M-mode and Simpson's method two-dimensional (2D) echocardiography of left ventricular function revealed no differences in systolic function (ejection fraction and fractional shortening) or diastolic function (mitral valve inflow velocity E to A ratio) in LmnaG609G/G609G mutant versus wild-type mice (fig. S6C).

LmnaG609G/G609G mice also showed, relative to wild-type mice, altered circulating plasma concentrations of various hormones and other biochemical markers. For example, at 2 months of age, the **LmnaG609G/G609G mutant mice showed a decrease in serum glucose concentrations, relative to wild-type mice**, an alteration that worsened with age, leading to extreme hypoglycemia at 3 months of age (Fig. 3C). We also observed a decrease of serum glucose concentrations in heterozygous LmnaG609G/+ mutant mice of advanced age (8 months), indicating that hypoglycemia might also contribute to the cardiovascular compromise and early death of mice that carried the c.1827C>T;p.Gly609Gly mutation.

In addition, LmnaG609G/G609Gmice showed **decreased serum levels of insulin-like growth factor 1 (IGF-1), insulin, and leptin and increased levels of growth hormone (GH) and adiponectin relative to wild-type mice** (Fig. 3C).

As a measure of genotoxic stress, we analyzed **histone gH2AX levels—a marker for the amount of nuclear DNA double-strand breaks—in the nuclei** of cultured fibroblasts from mice of the various genotypes (Fig. 3D). Most (81%) LmnaG609G/G609G fibroblasts at passage 5 (P5) contained large and abnormally shaped nuclei with foci that were highly stained with anti-gH2AX antibodies. Similar abnormal nuclei were identified in 24% of LmnaG609G/+ fibroblasts and in 3% of Lmna+/+ fibroblasts (Fig. 3D).

To elucidate the molecular pathways that underlie the phenotypic alterations described in LmnaG609G/G609G mice, we analyzed the **transcription profiles of livers from LmnaG609G/G609G mutant mice and wild-type littermates using complementary DNA (cDNA) hybridization to DNA microarrays**. A large number of genes showed reproducible changes in their expression levels in the mutant mice, consistent with the critical roles of nuclear lamins in chromatin structure and function (30) (table S1). From these data, **gene set enrichment analysis (GSEA) was used for an unbiased identification of molecular pathways that were significantly altered in these mice** (31). This analysis revealed a very strong correlation between the transcriptional alterations detected in these LmnaG609G/G609G and Zmpste24-deficient mice (32), supporting the existence of a common transcriptional signature in two different models of progeroid laminopathies. Functionally, most of the pathways that were significantly enriched in LmnaG609G/G609G samples are associated with stress responses.

Thus, **the p53 tumor suppressor pathway** (Fig. 3D) **and the ATM** (ataxia telangiectasia mutated)–related pathway are significantly **up-regulated in LmnaG609G/G609G mice**, probably as a consequence of unrepaired DNA damage caused by progerin accumulation (33). We previously hypothesized that these alterations, including chronic activation of the p53 pathway, are drivers of the senescent phenotype displayed by these mice (32). Moreover, **the list of genes that were overexpressed in the LmnaG609G/G609G mutant mice relative to wild-type animals contains a significant number of components of the hypoxia response pathway mediated by induction of the HIF-1a (hypoxia-inducible factor 1a) transcription factor** (fig. S7). This pathway recently was associated with metabolic changes in a mouse model of progeria and could be involved in the serum

glucose alterations seen in these mice (34). Other pathways that were down-regulated in LmnaG609G/G609G mice are related to metabolic processes such as fatty acid metabolism, oxidative phosphorylation, and mitochondria biogenesis (figs. S7 and S8).