INTRODUCTION

Hutchinson-Gilford progeria syndrome (HGPS) is a rare autosomal dominant segmental premature aging disorder that occurs in approximately 1 in 4–8 million births (Hennekam, 2006). Progressively developing features of HGPS include growth deficiency, alopecia, loss of subdermal fat, sclerodermatous skin, and musculoskeletal abnormalities including relative mandibular and clavicular hypoplasia, joint contractures, and osteoporosis (Ullrich & Gordon, 2015). The most serious aspects of the disease involve loss of vascular smooth muscle cells (VSMC) in the medial layer of large arteries which are replaced by proteoglycan-rich extracellular matrix and development of generalized features of atherosclerosis with focal areas of calcification (Gerhard-Herman et al., 2012). In the majority

Abstract

Hutchinson-Gilford progeria syndrome (HGPS) is a rare accelerated aging disorder most notably characterized by cardiovascular disease and premature death from myocardial infarction or stroke. The majority of cases are caused by a de novo single nucleotide mutation in the LMNA gene that activates a cryptic splice donor site, resulting in production of a toxic form of lamin A with a 50 amino acid internal deletion, termed progerin. We previously reported the generation of a transgenic murine model of progeria carrying a human BAC harboring the common mutation, G608G, which in the single-copy state develops features of HGPS that are limited to the vascular system. Here, we report the phenotype of mice bred to carry two copies of the BAC, which more completely recapitulate the phenotypic features of HGPS in skin, adipose, skeletal, and vascular tissues. We further show that genetic reduction of the mechanistic target of rapamycin (mTOR) significantly extends lifespan in these mice, providing a rationale for pharmacologic inhibition of the mTOR pathway in the treatment of HGPS.

KEYWORDS
lamin A/C, laminopathies, mTOR, progeria, S6 Kinase
of HGPS cases, death occurs as a result of complications of severe cardiac disease (myocardial infarction or heart failure) or cerebrovascular disease (stroke) at an average age of 14.6 years.

Classic HGPS is caused by a de novo mutation (c.1824C→T, p.G608G) in the LMNA gene, encoding the A-type lamins A, C, and A10 (Eriksson et al., 2003; De Sandre-Giovannoli et al., 2003). Along with B-type lamins, A-type lamins comprise the nuclear lamina underlying the inner nuclear membrane. Prelamin A is subject to several post-translational processing events that include farnesylation of the cysteine in the C-terminal CAAX sequence, cleavage of the terminal AAX sequence with addition of a methoxy group to the terminal cysteine, and subsequent cleavage of the terminal 15 amino acid residues by the metalloprotease ZMPSTE24. Although the G608G mutation does not alter the LMNA coding sequence, activation of an exonic cryptic splice donor site produces an in-frame protein with an internal deletion, termed progerin, that excludes the ZMPSTE24 cleavage recognition site. Permanently farnesylated progerin functions in a dominant-negative manner to disrupt the roles of the nuclear lamina in regulating nuclear shape, DNA replication, transcription, cell division, and chromatin organization (Kubben & Misteli, 2017).

Development of a transgenic model that reproduces the human HGPS phenotype should be useful in testing therapeutic approaches to target either the mutation or mutant gene product in the context of the human sequence. We previously reported the creation of a transgenic mouse harboring the G608G mutated human LMNA on a 164-kb bacterial artificial chromosome (BAC), designated as C57BL/6-TgLMNA*G608G*HChns/J, and henceforth referred to as LMNA<sup>G/G</sup> (Varga et al., 2006). Although the LMNA transgene expresses human lamin A, lamin C, and progerin in all tissues tested, mice carrying a single copy of the human BAC (LMNA<sup>G/G</sup>) exhibit normal growth and external phenotypic features compared to wild-type littermates or mice carrying a normal human LMNA transgene. The most dramatic finding was the progressive loss of VSMC, particularly in descending aorta and carotid artery, that was associated with thickening of the adventitia and medial layer, and proteoglycan accumulation by 5 months of age. Arterial calcification was observed in older mice with severe VSMC loss and extracellular matrix deposition. Thus, while single-copy transgenic mice (LMNA<sup>G/G</sup>) lack the external phenotype seen in patients, this model demonstrates the progressive vascular abnormalities that closely resemble the most lethal aspect of HGPS (Varga et al., 2006).

Aging-related disorders such as cancers, diabetes, cardiovascular, and neurodegenerative diseases, as well as normal aging, have been linked to dysregulated signaling of the mechanistic target of rapamycin (mTOR) pathway (Dazert & Hall, 2011). While genetic reduction of mTOR, mLST8, or S6K1 in mice increases lifespan and inhibits the onset of aging-related diseases, inhibitors of mTOR signaling components have drawn interest as potential treatments for disorders associated with deposition of insoluble protein aggregates by means of enhanced clearance through autophagic-lysosomal pathway activation (Selman et al., 2009; Wu et al., 2013). In cell culture experiments, treatment with rapamycin, or its analogs everolimus and temsirolimus, has been demonstrated to reduce progerin aggregates and nuclear blebbing, enhance cell proliferation, and delay the onset of cellular senescence, thereby providing evidence that chemical inhibition of mTOR signaling might restore cellular homeostasis in HGPS (Cao et al., 2011; Cenni et al., 2011; DuBose et al., 2018). These findings have led to a clinical trial of everolimus in children with HGPS, with results expected within a year. Here, we present the phenotypic characterization of double-copy LMNA G608G transgenic mice, which faithfully recapitulate vascular, dermal, adipose, and skeletal features that develop in HGPS patients. Furthermore, we report the beneficial effects of genetic mTOR reduction in these mice as a rationale for pharmacologic intervention in HGPS therapy.

### RESULTS

We have previously developed a transgenic mouse model of HGPS by using a 164Kb circular BAC containing 4 functional genes (UBQLN4, RAB25, MEX3A, and a recombined LMNA) in addition to incomplete sequences of two additional genes, SSR2 and SEM4A4 (Varga et al., 2006). Initial efforts to characterize the structure of the transgene expressed in our mouse model utilized hybridization capture and short-read NGS sequencing. Although this analysis was useful for obtaining the genomic location of the insertion sites on murine chromosome 4, it lacked the resolution required to resolve the actual transgene configuration and any recombination events that occurred during integration (Dubose et al., 2013). We therefore employed whole-genome sequencing of transgenic mouse DNA and comparison analyses with BAC sequence to determine the precise structure.
and contents (Figure S1a). Fine mapping of the transgene identified three separate copies of human LMNA, the first of which includes sequence covering exon 1 and portions of the first intron, a second copy that includes exons 2–12, and a third copy consisting of the full gene sequence with the G608G mutation. Partial gene sequences were identified for SSR2 and SEMA4A, as expected. A third gene, MEX3A, localized to an inversion junction and underwent multiple recombination events resulting in several fragments averaging ~400–500 bp each. Since UBQLN4 sequence was entirely absent from WGS reads we concluded that only two functional genes are encoded within the transgene, including the mutant LMNA and normal RAB25 genes.

Similar to HGPS patients, mice carrying either one (LMNA<sup>G/+</sup>) or two copies (LMNA<sup>G/G</sup>) of the human LMNA G608G transgene are indistinguishable from wild-type littermates at birth. We noted, however, that the genotype distribution of offspring generated by matings between mice harboring one copy of the transgene slightly
deviated from the predicted Mendelian ratio, with no observed lethality in LMNA<sup>G</sup>/<sup>G</sup> mice but 12% lethality in LMNA<sup>G</sup>/<sup>+</sup> (Figure S1b). Although no difference was observed between male and female mice (n = 150 per gender), these numbers suggest a moderate level of either embryonic or perinatal lethality in LMNA<sup>G</sup>/<sup>G</sup> offspring.

We previously reported that LMNA<sup>G</sup>/<sup>G</sup> transgenic mice lack pathologic changes outside of the vascular system prior to 20 months of age (Varga et al., 2006). In contrast to single-copy mice, LMNA<sup>G</sup>/<sup>G</sup> mice developed phenotypic features consistent with HGPS following the first month after birth, relative to wild-type (LMNA<sup>+/+</sup>) littermates. By 5 weeks of age, both male and female LMNA<sup>G</sup>/<sup>G</sup> mice were growth deficient (Figure 1a). From 5 to 15 weeks of age, double-copy transgenic mice weighed 10–20% less than LMNA<sup>+/+</sup> and LMNA<sup>G</sup>/<sup>+</sup> littermates. After 3.5–4 months of age, no additional weight gain was observed in LMNA<sup>G</sup>/<sup>G</sup> mice, with progressive wasting resulting in a 20–45% decrease in weight compared to LMNA<sup>+/+</sup> and LMNA<sup>G</sup>/<sup>+</sup> mice (p < 0.01 versus both genotypes) (Figure 1a, Figure S1c).

The decreased weight observed in LMNA<sup>G</sup>/<sup>G</sup> mice relative to wild-type (LMNA<sup>+/+</sup>) and single-copy transgenic (LMNA<sup>G</sup>/<sup>+</sup>) littermates largely reflected growth deficiency, combined with the loss of adipose tissue and bone mass. Consistent with the lipodystrophic phenotype of HGPS patients, histopathologic analysis of skin from 6-month-old LMNA<sup>G</sup>/<sup>G</sup> mice demonstrated decreased dermal and subcutaneous adipose tissue resulting in tightened skin (Figure 1b). Although mild hair loss occurred infrequently in these mice, no obvious abnormalities of hair follicles were evident.

We also noted that LMNA<sup>G</sup>/<sup>G</sup>, but not LMNA<sup>G</sup>/<sup>+</sup> mice, began to develop hindlimb contractures and moved with a waddling gait beginning at 6–7 months of age. To assess whether transgenic mice exhibited skeletal features similar to those observed in HGPS patients, bone morphology was evaluated in murine femurs at 6 months of age by μCT analysis (Figure 1c). All trabecular and cortical structural parameters in long bones of LMNA<sup>G</sup>/<sup>G</sup> mice were comparable to wild-type (Lmna<sup>+/+</sup>) littermates, reflecting the normal weights and the absence of pathologic features outside of the vascular system. In contrast, bone structural parameters were altered in LMNA<sup>G</sup>/<sup>G</sup> mice relative to wild type. Trabecular bone volume (BV/TV) was half of wild-type values (p < 0.01), due to decreased trabecular thickness rather than a decrease in trabecular number, while cortical thickness (CT Th) was modestly reduced (~11%) in LMNA<sup>G</sup>/<sup>G</sup> femurs (p < 0.001). Additional musculoskeletal features observed in LMNA<sup>G</sup>/<sup>G</sup> mice included kyphosis, which developed by 4 months and progressed until mice reached endpoint (Figure 1d, Figure S1d).

Similar to LMNA<sup>G</sup>/<sup>+</sup> mice but even more extensive, progressive loss of VSMCs, elastic fiber breakage, proteoglycan, and collagen accumulation in the media, and dramatic thickening of the adventitia occurred in vascular tissues of 5-month-old LMNA<sup>G</sup>/<sup>G</sup> mice (Figure 2a–c). Arterial and arteriolar pathology was prominent in the aorta, but also included the carotid arteries with focal pathology in arteries and arterioles of the heart and kidneys, skeletal muscles, and adipose tissues. These vascular abnormalities were characterized by decreased numbers of smooth muscle nuclei, increased amounts of ground substance in the tunica media, and increased amounts of adventitial connective tissue. Adventitial expansion was associated with deposition of collagenous matrix, as noted by picrosirius red staining (Figure S1e).

Most notable, however, was the generalized severe phenotype exhibited in LMNA<sup>G</sup>/<sup>G</sup> mice, characterized by reduction of activity around 6 months of age and lasting until death by age 7–8 months (Figure 1e). The average lifespan of LMNA<sup>+/+</sup> and LMNA<sup>G</sup>/<sup>G</sup> mice was 897 and 485 days, respectively, compared to 212 days for LMNA<sup>G</sup>/<sup>G</sup> mice (p – 10<sup>-137</sup> vs LMNA<sup>+/+</sup>; p – 10<sup>-101</sup> vs LMNA<sup>G</sup>/<sup>+</sup>). Furthermore, there was no significant difference in average lifespan of male (216 ± 36 days) and female (208 ± 35 days) LMNA<sup>G</sup>/<sup>G</sup> mice. Despite the severe vascular alterations observed in LMNA<sup>G</sup>/<sup>G</sup> mice; however, post-mortem analyses were unable to identify a specific acute cause of death.

At the tissue level, molecular and biochemical analyses of endogenous and transgene-derived A-type lamin expression were performed. As expected, quantitative PCR of endogenous and transgene-derived transcripts demonstrated only murine lamin A/C transcripts in wild-type (LMNA<sup>+/+</sup>) tissues, including skin, inguinal fat, heart, and aorta (Figure S2a). In all tissues that were tested, the levels of endogenous Lmna transcripts were comparable across all genotypes. In both LMNA<sup>G</sup>/<sup>+</sup> and LMNA<sup>G</sup>/<sup>G</sup> tissues, however, we found variable levels of transgene expression.

Transgene expression levels correlated with copy number in skin and heart tissue. Conversely, transgene expression was significantly decreased in inguinal fat and aorta tissue of LMNA<sup>G</sup>/<sup>G</sup> versus LMNA<sup>G</sup>/<sup>+</sup> mice which may reflect loss of viable adipocytes and VSMCs, respectively, that express high levels of A-type lamins (Uhlen et al., 2015).

Immunoblots of tissue-derived A-type lamins also revealed variable levels of total lamin A, progerin, and lamin C (Figure S2b). We found that, only in skin and aorta, total levels of A-type lamins correlated with endogenous and transgene copy number. In heart and inguinal adipose tissue derived from LMNA<sup>G</sup>/<sup>G</sup> mice, total A-type lamins were reduced approximately 25% and 50% in fat and heart, respectively, compared to LMNA<sup>G</sup>/<sup>+</sup> mice. However, total levels of endogenous and transgene-derived A-type lamins relative to the reference protein were consistently higher in tissues derived from transgenic mice compared to wild type (Figure S2b).

Having established by phenotypic and biochemical analyses that mice harboring two copies of the human LMNA G608G transgene represent a relevant model of classic HGPS, we turned our attention to ways in which this model might be used to identify possible therapeutic interventions to treat children with HGPS. Two treatments currently being tested include farnesyltransferase inhibitors to reduce production of the toxic progerin protein and

**FIGURE 2** Severe vascular phenotype in HGPS mice can be partially rescued by genetic reduction of Mtor. (a) Movat’s pentachrome-stained ascending aorta sections from 5-month-old mice. (b) Hematoxylin and eosin (H&E)-stained aortae from LMNA<sup>G</sup>/<sup>+</sup> and LMNA<sup>G</sup>/<sup>G</sup> mice. (c) Quantitation of adventitial area and VSMC number in aortae. Genetic reduction of Mtor partially restores the loss of vascular smooth muscle cells but does not rescue the adventitial expansion of aortae in LMNA<sup>G</sup>/<sup>G</sup> mice. *, p < 0.05; **, p < 0.01; ***, p < 0.001
autophagy activation with rapamycin analogs to promote its clearance (Evangelisti et al., 2016; Harhouri et al., 2018; Young et al., 2013). While the former approach has proven clinically beneficial, the latter is currently the subject of a clinical trial (Gordon et al., 2012, 2018). We reasoned that, using our mouse model, a genetic approach could be undertaken to investigate mTOR inhibition as a potential strategy to alleviate HGPS pathology. Mice carrying an Mtor hypomorphic allele (MtorΔ/+ ) were bred into the G608G transgenic mouse line, followed by matings of doubly heterozygous mice (MtorΔ/+LMNAΔ/+). The offspring distribution from these matings was significantly skewed toward Mtor+/+, regardless of LMNA genotype, and the presence of the hypomorphic allele increased embryonic lethality from 12% to 61% in LMNAΔ/Δ offspring (Figure S3). As demonstrated previously, Mtor+/+LMNAΔ/Δ mice consistently weighed significantly less than their wild-type littermates by 5 weeks (Figure 3a). Although MtorΔ/+LMNAΔ/Δ mice failed to attain weights similar to wild-type littermates, after 20 weeks of age both male and female mice harboring one copy of the Mtor hypomorphic allele retained 10–15% more of their weight compared to Mtor+/+LMNAΔ/Δ mice (p < 0.05).

Histologic examination of vascular tissue revealed quantifiable alterations in these mice (Figure 2). By five months of age, aortas of both Mtor+/+LMNAΔ/Δ and MtorΔ/+LMNAΔ/Δ mice exhibited vascular alterations, including loss of vascular smooth muscle cells, deposition of proteoglycan and elastin disorganization within the medial layer, and expansion of the adventitial matrix, compared to wild-type aortas (Figure 2a–c). However, LMNAΔ/Δ mice carrying the Mtor hypomorphic allele (MtorΔ/+LMNAΔ/Δ) retained twice as many VSMC within the medial layer of ascending aorta compared to LMNAΔ/Δ mice with normal Mtor expression (Mtor+/+LMNAΔ/Δ). In contrast,

**FIGURE 3** Genetic reduction of Mtor extends lifespan in LMNA G608G transgenic mice. (a) Growth curves of wild-type (Mtor+/+LMNA+/+) and double-copy transgenic mice containing two wild-type Mtor alleles (Mtor+/+LMNAΔ/Δ) or heterozygous for the Mtor hypomorphic allele (MtorΔ/+LMNAΔ/Δ). Growth deficiency in LMNAΔ/Δ mice is not rescued by reduced expression of mTOR in male (n = 6 Mtor+/+LMNAΔ/Δ, 7 MtorΔ/+LMNAΔ/Δ, 13 MtorΔ/-LMNAΔ/Δ) or female (n = 6 Mtor+/+LMNAΔ/Δ, 6 MtorΔ/+LMNAΔ/Δ, 12 MtorΔ/-LMNAΔ/Δ) mice. (b) Kaplan-Meier plot demonstrates the 30% extension in lifespan of double-copy transgenic mice heterozygous for the Mtor hypomorphic allele (MtorΔ/+LMNAΔ/Δ) compared to transgenic mice harboring two wild-type Mtor alleles (Mtor+/+LMNAΔ/Δ), p < 0.001.
Mt or expression levels made no difference in the expansion of adventitial tissue in LMNA<sup>G/G</sup> mice. Importantly, the improved retention of VSMC in Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> mice was associated with a 30% increase in lifespan versus Mt or<sup>+/+</sup>LMNA<sup>G/G</sup> (Figure 3b). These data suggest that genetic reduction of Mt or expression can partially rescue the VSMC loss that is a hallmark feature of vascular pathology in HGPS mice.

To determine the mechanism by which genetic reduction of Mt or elicited an increase in lifespan in LMNA<sup>G/G</sup> mice, fibroblast cultures were generated from dermal tissue of two independent Mt or<sup>+/+</sup>LMNA<sup>G/G</sup>, Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup>, Mt or<sup>+/+</sup>LMNA<sup>G/G</sup>, and Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> newborn mice for biochemical characterization of A-type lamins and Mt or signaling pathways. As expected, no progerin was detected in cell lines expressing wild-type Lmna by Western analysis (Figure 4a). In both Mt or<sup>+/+</sup>LMNA<sup>G/G</sup> and Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> cell lines, the presence of progerin was associated with autophagic activation determined by the LC3-I/II ratios. Furthermore, increased levels of autophagic cargo receptor p62/SQSTM1 in Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> cultures were normalized to wild-type levels in cells with genetically reduced Mt or expression suggesting autophagic elimination of this protein. However, cultures expressing the G608G transgene contained equimolar amounts of lamin A, lamin C, and progerin proteins regardless of Mt or allele status (Figure 4a,b). Despite the 50% reduction in intracellular Mt or protein levels and upregulation of autophagic activity in Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> versus Mt or<sup>+/+</sup>LMNA<sup>G/G</sup> cell lines, no difference in the total level of A-type lamins, or progerin as a fraction of total lamin A/C, was observed. Neither the increased basal autophagic activity nor the reduction of Mt or protein levels were associated with any reduction of nuclear blebbing, a hallmark feature of HGPS cells, in Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> fibroblasts (Figure S4).

Further characterization of Mt or signaling in newborn fibroblast cultures revealed equivalent levels of phosphorylated AKT and 4EBP1 in all cell lines tested, demonstrating no difference in the extent of their activity (Figure 4a,b). In contrast, phosphorylation of S6K was increased twofold in Mt or<sup>+/+</sup>LMNA<sup>G/G</sup> compared to Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup>, Mt or<sup>+/+</sup>Lmna<sup>+/+</sup>, and Mt or<sup>Δ/Δ</sup>Lmna<sup>+/+</sup> cells (p < 0.05), consistent with normalization of increased S6K activity in LMNA<sup>G/G</sup> fibroblasts by genetic reduction of Mt or. Normalized S6K activity in Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> cells was associated with improved indices of cell growth and proliferation in comparison to Mt or<sup>+/+</sup>LMNA<sup>G/G</sup> fibroblasts; in sub-confluent cultures, the fraction of Mt or<sup>+/+</sup>LMNA<sup>G/G</sup> cells in G<sub>1</sub>/M phase was increased 23%, with a corresponding 44% decrease in G<sub>S</sub>/G<sub>2</sub> phase, when compared to Mt or<sup>Δ/Δ</sup>Lmna<sup>+/+</sup> wild-type cells (Figure 4c). Genetic reduction of Mt or in Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> cells, however, doubled the fraction of cells in G<sub>S</sub>/G<sub>2</sub> phase while reducing the fraction of cells in G<sub>1</sub>/M phases by 26%, thereby altering the cell cycle distribution to more closely resemble that of Mt or<sup>+/+</sup>Lmna<sup>+/+</sup> wild-type cells. Delayed cell growth was observed in Mt or<sup>+/+</sup>LMNA<sup>G/G</sup> fibroblasts, with an approximate doubling time of 2.5 days versus a 2-day doubling time for wild-type (Mt or<sup>+/+</sup>Lmna<sup>+/+</sup>) cells (Figure 4d). Genetic reduction of Mt or had no effect on growth of wild-type cells, but further decreased proliferation in progerin-expressing (Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup>) fibroblasts to a doubling time of 3.5 days.

Based on our initial hypothesis that reduced levels of Mt or expression would upregulate autophagy and, therefore, decrease levels of progerin in vivo, we analyzed tissues from Mt or<sup>+/+</sup>Lmna<sup>+/+</sup>, Mt or<sup>Δ/Δ</sup>Lmna<sup>+/+</sup>, Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup>, and Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> littermates. Using an antibody specifically recognizing human LMNA (JoL2), no difference in the levels of transgene-derived lamin A, progerin, or lamin C relative to alpha-smooth muscle actin (aSMA) or beta-actin were found by Western analyses of immunoprecipitated A-type lamins from heart and liver, respectively, of Mt or<sup>+/+</sup>LMNA<sup>G/G</sup> and Mt or<sup>Δ/Δ</sup>LMNA<sup>G/G</sup> mice (Figure 5a,b). Subsequent detection with an alternative antibody (4c11) reactive to both human and mouse A-type lamins also failed to demonstrate any discernible quantitative differences in tissue homogenates derived from transgenic mice with genetically reduced Mt or. We therefore considered the possibility that, in vivo, autophagic elimination of progerin might be affected by transgene-derived RAB25 expression due to its putative role in regulating autophagy by activation of AKT (Cheng et al., 2012).

Quantitative RT-PCR demonstrated that human RAB25 expression was 2.5- to 60-fold greater than endogenous mouse RAB25, depending on the tissue analyzed, in mice harboring two transgene copies (Figure S5a). To determine if high levels of transgene-derived RAB25 were inhibiting autophagy, we next generated murine embryonic fibroblasts from the offspring of Mt or<sup>Δ/Δ</sup> mice crossed with either Lmna<sup>G608G</sup> knock-in mice, which lack human RAB25 expression, and LMNA<sup>G608G</sup> transgenic mice. Western analyses from cell lysates demonstrated a modest reduction in total A-type lamins in both LMNA<sup>G608G/G608G</sup> transgenic (G/G) and Lmna<sup>G608G/G608G</sup> knock-in (KI/KI) cell lines treated with everolimus (RAD001), as well as in cell lines harboring the Mt or hypomorphic allele (Figure S5b). The decrease in A-type lamins relative to ACTB levels, which was not specific to progerin, was associated with increased LC3-I/LC3-II ratios and decreased p62 levels, indicating autophagic activation in cell lines where Mt or was either chemically inhibited or genetically reduced (Figure S5c).

Analysis of Mt or signaling in tissues collected from adult mice contrasted with the results from cell cultures. Although Western immunoblots of tissue homogenates collected from 5 month-old LMNA<sup>G/G</sup> mice revealed significantly increased levels of phosphorylated Mt or in hearts (Figure 6a), and to a lesser extent in livers (Figure 6b), compared to wild-type littermates, downstream targets of Mt or signaling were found to be hypophosphorylated. In heart tissue from adult mice, relative phosphorylation of S6 was significantly reduced in LMNA<sup>G/G</sup> versus wild type, with further reduction in phosphorylation occurring in transgenic mice with genetically reduced Mt or (Figure 6a). A similar, but nonsignificant, trend in reduced S6 phosphorylation was found in livers of adult mice, where genetic reduction of Mt or was associated with hypophosphorylation of 4EBP1 (Figure 6b). In both tissues, we found that AKT activation was suppressed in the presence of progerin. From these data, we conclude that Mt or signaling is hyperactivated in LMNA<sup>G/G</sup> newborn and embryo-derived cell cultures, but in tissues of mice where the HGPS phenotype has progressed Mt or signaling is inhibited.
Here, we describe a double-copy LMNA*G608G transgenic mouse model of HGPS that replicates most of the features of the human disorder. Several other mouse models have been created to characterize the pathogenic mechanisms underlying HGPS, and the comparison of phenotypes is useful. Yang and colleagues generated a knock-in model, LmnaHG/+ , created by deletion of intron 10, intron 11 and the last 150 nucleotides of exon 11, so that only the mutant progerin transcript is produced without any normal lamin A or C (Yang et al., 2005). LmnaHG/+ mice, which have an average lifespan of approximately 27 weeks, develop growth deficiency, bone dysplasia, and kyphosis by 4 months of age. However, somewhat surprisingly these mice do not exhibit vascular smooth muscle cell loss and the intima, media, and adventitial layers of aortas appear normal out to 7 months of age (Yang et al., 2005). In contrast, mice homozygous for the progerin-only allele (LmnaHG/HG) rapidly develop a more severe phenotype, characterized by spontaneous fractures and complete absence of adipose tissue with death at 3–4 weeks of age (Yang et al., 2006).

A second, well-characterized, knock-in model that carries the murine equivalent of the common human mutation (LmnaG609G, c.1827C>T) produces progerin via abnormal splicing as occurs in HGPS patients. Heterozygous LmnaG609G/+ mice have normal weight and size until approximately 24 weeks of age and then start to show symptoms of aging until death at an average age of 35 weeks. LmnaG609G/GG mice show a more pronounced and earlier progeroid phenotype than heterozygous LmnaG609G/+ mice and, prior to premature death at 14–15 weeks of age, display the main clinical manifestations of human HGPS such as osteoporosis, loss of principal fat deposits, and VSMC depletion, as well as aberrant hormonal profiles that manifest as hypoglycemia and IGF deficiency (Osorio et al., 2011).

In cell cultures from patients with HGPS, a minor fraction of transcripts derived from the mutant LMNA allele encode progerin, while the majority of transcripts yields mostly wild-type lamin A and lamin C (Eriksson et al., 2003). In contrast, we found that a significant proportion of murine transcripts derived from the mutant human LMNA transgene encode progerin. Nevertheless, the dominant-negative effects of progerin accumulation progress with age in both patients and murine models of HGPS. Despite the possibility that mice require a higher level of progerin expression to elicit the HGPS disease phenotype (Yang et al., 2005), it is apparent that the severity of the progeroid phenotype in LMNA*G609G transgenic mice, as well as other mouse models of HGPS, correlates with gene dosage. Comparison to other mouse models of HGPS shows that LMNA*G609G transgenic mice present with a less severe phenotype than LmnaG609G/GG and LmnaHG/HG mice, which may be attributed to altered molecular interactions between human LMNA/progerin and endogenous murine nuclear proteins, or alternatively, retention of normal functioning of endogenous Lmna in transgenic mice.

We also noted that at the transcript level, inguinal fat and aorta from LmnaG609G mice expressed lower levels of progerin compared to LMNA*G609G, which may reflect the loss of specific cell populations with higher transgene expression in these tissues (Uhlen et al., 2015). While a dramatic absence of subcutaneous adipose tissue was observed, LMNA*G609G transgenic mice do not develop alopecia or glandular and follicular abnormalities as occurs in LmnaG609G mice (Osorio et al., 2011).

Although LMNA*G609G mice appear to lack any detectable bone phenotype at 6 months of age, micro-CT analysis of LMNA*G609G mouse femora detected a modest decrease (~5%) in BMD with significant reductions in structural parameters of trabecular and cortical bone, consistent with previous reports (Cubria et al., 2020). This aspect of the HGPS phenotype appears to be due to defective modeling of bone tissue, since bone- and cartilage-specific Oxs-driven expression of progerin leads to skeletal abnormalities associated with impaired osteoblast differentiation and loss of the osteocyte population in bone tissue (Schmidt et al., 2012). While there is no current evidence of increased resorption, the potential for reduced cortical and trabecular bone volumes in LMNA*G609G femurs resulting from abnormal bone remodeling cannot be ruled out. Despite key differences existing between rodent and human skeletal systems, the LMNA*G609G transgenic mouse model will be a useful model for further investigating the effects that progerin expression has on bone cell populations in HGPS bone dysplasia and, potentially, its role in aging-related osteoporosis.

As was seen in LMNA*G609G mice, LMNA*G609G mice were found to have a progressive and dramatic defect of large arteries, particularly the aorta. These pathologic alterations in the medial layer of large vessels of transgenic mice recapitulate findings in HGPS. Since SM2a-Cre-mediated VSMC-specific expression of progerin is sufficient to accelerate pathologic vascular alterations and death in atheroprene Apolipoprotein E-null mice (Hamczyk et al., 2018), progerin-induced
vascular pathology may be directly related to the cause of death in patients. Although the precise mechanism(s) underlying the vascular changes remains to be clarified, HGPS VSMCs and endothelial cells are more sensitive to mechanical stress, have been shown to be particularly susceptible to progerin accumulation and are exposed to the higher hemodynamic forces (pressure and shear stress) exerted on arterial walls (Varga et al., 2006; Verstraeten et al., 2008), which may be influenced by dysregulation of the glycosylcalyx components involved in flow shear stress sensing (Pitre et al., 2020). Progerin-induced VSMC vulnerability has also been attributed to dysregulation of the DNA damage response pathway, hyperactivation of ER stress (ER), and the unfolded protein response (UPR) (Hamczyk et al., 2017; Zhang et al., 2014). Furthermore, evaluation of the DNA damage response pathway, hyperactivation of ER stress (ER), and the unfolded protein response (UPR) (Hamczyk et al., 2017; Zhang et al., 2014).

In a genetic strategy to test the effect of mTOR inhibition in our mouse model, mice carrying an Mtor hypomorphic allele (MtorΔ/Δ) were bred into the G608G transgenic mouse line. We found no significant improvement of nuclear membrane structure in newborn fibroblasts derived from offspring of these matings, nor could we verify any reduction of progerin in tissues of MtorΔ/ΔLMNAG/G mice in the presence of activated autophagic activity in adult tissues. Importantly, however, genetic reduction of Mtor in HGPS transgenic mice led to a 30% increase in lifespan versus MtorΔ/ΔLMNAΔ/Δ littermates.

Analysis of mTOR signaling in fibroblasts derived from newborn mice demonstrated that increased activation of the downstream effector S6 protein kinase (S6K) in MtorΔ/ΔLMNAΔ/Δ was normalized in MtorΔ/ΔLMNAΔ/ΔG/G mice, consistent with lifespan extension observed in S6K-null mice (Selman et al., 2009). Genetic reduction of Mtor has previously been reported to normalize increased mTOR/S6K1 activity, partially restore autophagy and postpone the premature aging phenotype in Zmpste24-null mice (Pan et al., 2020). Hyperactivation of S6K has also been observed in adipocyte precursor and osteosarcoma cell lines expressing LMNA mutations associated with Type 2 Familial Partial Lipodystrophy (FPLD2) and Mandibuloacral Dysplasia (MADA), respectively (Evangelisti et al., 2015; Pellegrini et al., 2019). Moreover, there is evidence to suggest that S6K mediates the pathogenesis and premature death of Lmna-null mice, and reducing its activity improves muscle function as well as lifespan (Liao et al., 2017).

In contrast to cells from embryos and newborn mice, however, signaling downstream of mTOR was found to be inhibited in LMNAΔ/ΔG/G adult tissues, particularly in the heart. These findings suggest that hyperactivation of mTOR signaling may be an early event in the onset of HGPS pathology in MtorΔ/ΔLMNAΔ/ΔG/G mice, but its activity is age-, phenotype-, and tissue-dependent, similar to what has been observed for Zmpste24Δ−/− mice (Pan et al., 2020), and which may be informative to the rational design of a therapeutic approach involving chemical inhibition of mTOR signaling.

The mechanism by which reduction of S6K activity increases lifespan in our mouse model of HGPS remains to be determined.
Interaction of S6K1 with various substrates promotes several broad cellular processes such as protein production, cell growth and size, cell survival, and gene transcription. For example, S6K1 regulates protein production by phosphorylating substrates that function in translational initiation, elongation, and protein folding, including elf4B, PDCD4, SKAR, CBP80, eEF2K, CCTβ, and RPS6, a component of the 40S ribosome. S6K1 also regulates transcription by activating CREM, estrogen receptor, and SREBP (Duvel et al., 2010; De Groot et al., 1994; Yamnik et al., 2009). Furthermore, S6K1 participates in the DNA damage response by phosphorylating MDM2, thereby blocking its nuclear import and ability to ubiquitinate the tumor suppressor p53 (Lai et al., 2010). Finally, S6K1 promotes negative feedback on PI3K signaling through IRS1, thus suppressing insulin and IGF sensitivity (Tremblay et al., 2007). It will be important, therefore, to determine which specific signaling events downstream of S6K1 are altered in response to genetic reduction of Mtor in our mouse model of HGPS.

These data provide further evidence, based on an animal model carrying the human progeria mutation, that inhibitors of mTOR might provide benefit for children with HGPS. It should be kept in mind, however, that the mTOR protein takes part in two different complexes, mTORC1 and mTORC2. Only the first of these is classically stated to be sensitive to rapamycin and its homologs. So the genetic approach we describe here, which reduces function of both S6K1 are altered in response to genetic reduction of Mtor and Mtorα/αLMNA/G/G, relative phosphorylation of S6 decreased versus wild type. (b) Western analyses of liver homogenates. Genetic reduction of Mtor significantly reduces levels of p62 isoforms (p62α/α, full length; p62+, variant (Kageyama et al., 2018)) and phosphorylated 4EBP1 in LMNA/G/G mice. N = 4 mice per genotype (2 males, 2 females); *, p < 0.05; **, p < 0.01, Mtorα/αLMNA/G/G and Mtorα/αLMNA/G/G significance versus Mtorα/αLMNA/G/G.

4 | EXPERIMENTAL PROCEDURES

4.1 | Mouse strains and animal care

A transgenic mouse model of HGPS was developed by retrofitting a human bacterial artificial chromosome (BAC) harboring the LMNA gene containing the classic G608G mutation (c.1824C > T). The 164 kb transgene was incorporated into the germline of C57BL/6J mice as previously described (Varga et al., 2006). Single-copy mice, designated LMNA/G/G, were bred within the C57BL/6J line for twenty generations prior to generation of double-copy mice, designated as LMNA/G/G, for experimental use. Mice harboring the Mtor hypomorphic allele (Mtorα/α) were a generous gift from Beverly Mock, National Cancer Institute, and have been previously characterized (Wu et al., 2013). Mice harboring the LmnaG609G knock-in mutation were a generous gift from Carlos López-Otín, Universidad de Oviedo (Osorio et al., 2011). Murine growth curves were determined by weekly weights. Kaplan-Meier plots were generated from in-house data combined with published wild-type C57BL/6J data from Jackson Laboratories (https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-000664). Animal care and experiments were performed in accordance with a protocol approved by the NHGRI Animal Care and Use Committee.

4.2 | Cell cultures

Primary fibroblast cultures (FB) were derived from dermal tissue dissected from the abdomens of newborn mice. FB were allowed to grow out from dermal samples for 2 weeks before releasing by trypsin digestion. Murine embryonic fibroblasts (MEFs) were isolated from day 14–15 (E14-E15) embryos generated by crossing G608G transgenic or G609G knock-in mice with mice expressing the Mtor hypomorphic allele. After skin and viscera were removed, embryos were minced and digested in 0.25% trypsin for 15 min. Cells were plated and allowed to adhere for 24 h at 37°C. The following day, non-adherent cells were removed, and the adherent cell population was expanded for collection as MEFS. Cells were cultured in DMEM containing 10% fetal bovine serum, 2 mM glutamine, and 1% pen-strep at 37°C in 5% CO2. Cells cultured with everolimus (RAD001) were treated at 0.1 μM for 4 h prior to collection.

4.3 | Proliferation assays

Cells were plated at 10,000 cells per well in 24-well culture plates (day 0) in triplicate. Culture medium was replaced daily during the course of the assay. Each cell line was collected by trypsinization at 24-h increments. Cell concentrations were determined in Via-1 by weekly weights. Kaplan-Meier plots were generated from in-house data combined with published wild-type C57BL/6J data from Jackson Laboratories (https://www.jax.org/jax-mice-and-services/strain-data-sheet-pages/body-weight-chart-000664). Animal care and experiments were performed in accordance with a protocol approved by the NHGRI Animal Care and Use Committee.

4.4 | Western blot analyses

Cells cultures and tissues were homogenized in high-salt RIPA buffer (20 mM Tris-HCl, pH7.4; 0.5 M NaCl; 1mM EDTA; 0.1% SDS, 1% Triton X-100, 1x protease inhibitor (Sigma, P8340), 100 mM AEBSF (Sigma, SBR00015), 20 μM caspase inhibitor VI (Sigma, 219007)) and
quantitated by BCA assay. Initial loads used 20 μg of total protein before balancing according to reference proteins. Lysates were elec-
trophoresed on 3–8% Tris-acetate (mTOR), 4–12% Bis-Tris (LC3A/B),
or 8% Bis-Tris gels (Thermo Fisher), transferred to nitrocellulose
membranes, which were blocked in 5% milk or 5% BSA overnight
at 4°C. Blots were incubated with primary antibody for 4 h at room
temperature, followed by 2 h incubation with IRDye® 680CW
donkey anti-mouse IgG or IRDye® 680RD donkey anti-rabbit IgG
fluorescent secondary antibodies (LI-COR Biosciences) and imaged
on an Odyssey CLx Imaging system with Image Studio software (LI-
COR Biosciences). A list of all antibodies used in this study is avail-
able in Supplementary Methods.

4.5 | Cell cycle analysis

Subconfluent fibroblast cultures were collected by trypsinization
and pelleted at 500 × g for 10 min. Cells were then washed once
with PBS and centrifuged again at 500 × g for 10 min. Cell pellets
were resuspended in PBS and fixed in a final concentration of 70%
EtOH overnight at 4°C. The following day cells were centrifuged at
500 × g for 5 min, resuspended in PBS for 1 min to rehydrate, then
centrifuged again for an additional 5 min. Cells were gently resus-
pended in propidium iodide staining solution (20 μg/ml PI, 0.1% tri-
ton X-100, 200 μg/ml RNase A in PBS) and incubated at 37°C for
30 min. Stained cells were analyzed using the Fixed Cell Cycle-PI
Assay on a NucleoCounter NC-3000 (Chemometec).

4.6 | Histology

Tissues were fixed in 2% paraformaldehyde for 24 h prior to dehydra-
tion with graded alcohols and embedding in paraffin. Cross-sections
(4-μm thick) were cut and mounted on charged slides and visualized
by Masson’s trichrome, hematoxylin/eosin, Movat’s pentachrome,
or Picrosirius red staining (CV Path Inc). Images were captured on a
Axioscan imaging system (Zeiss) at 20× magnification and were
further processed by trimming using ZenBlue microscopy (Zeiss) and
Photoshop CC (Adobe) software.

4.7 | Bone structural analysis

Left femora of 6-month-old wild-type (LMNA+/+, 10 males, 9 fe-
males), single- (LMNA−/−, 10 males, 9 females), and double-copy
transgenic (LMNAG/G, 10 males, 10 females) littersmates were an-
alyzed by micro-computed tomography (μCT40, Scanco Medical AG).
Sequential transaxial images were generated using an integration
time of 250ms and a tube voltage and current of 70kVp and 114mA,
respectively. Images were reconstructed at 10 μm isotropic voxel
size, then assessed for volumetric bone mineral density (vBMD,
mg/cm³) using calibrated hydroxyapatite (HA) phantoms to convert
X-ray attenuation to a known mineral density. Morphometric analy-
yses were performed using native Scanco software. The trabecular
region of interest (ROI) was located proximal to the distal femoral
growth plate and extended 150 slices upward toward the proximal
end. The diaphyseal cortical ROI started on the 55 percentile femur
height from the proximal end, spanning 50 slices toward the distal
end.

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CONFLICT OF INTEREST

The authors declare no competing interests.

AUTHOR CONTRIBUTIONS

WAC, MRE, and FSC designed the experiments, which were
conducted by WAC, IB, DY, UT, and YDB. Data were analyzed by
WAC, IB, DY, UT, YDB, MAE, AN, and MRE. Manuscript was written
by WAC, MRE, and FSC with significant input from MAE and AN.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from
the authors upon request.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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