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LMNA-Associated Cardiocutaneous Progeria: a Novel Autosomal Dominant Premature Aging Syndrome with Late Onset

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Abstract

Hutchinson-Gilford Progeria Syndrome (HGPS) is a well-characterized premature aging disorder caused by mutations in *LMNA*, the gene encoding the nuclear scaffold proteins lamin A and C. In HGPS and related progerias, processing of prelamin A is blocked at a critical step mediated by the zinc metalloprotease ZMPSTE24. Emerging evidence indicates that *LMNA*-linked progerias can be grouped into two classes: 1) the processing-deficient, early onset “typical” progerias (*e.g.* HGPS), and 2) the processing-proficient “atypical” progeria syndromes (APS) that are later in onset. Here we describe a novel progeria syndrome with prominent cutaneous and cardiovascular manifestations belonging to the second class. We suggest the name *LMNA*-associated cardiocutaneous progeria syndrome (LCPS) for this disorder. Affected patients are normal at birth but undergo progressive cutaneous changes in childhood and die in middle age of cardiovascular complications, including accelerated atherosclerosis, calcific valve disease, and cardiomyopathy. In addition, the proband demonstrated cancer susceptibility, a phenotype rarely described for *LMNA*-based progeria disorders. The *LMNA* mutation that causes LCPS is a heterozygous missense mutation resulting in an amino acid substitution (D300G) in the coiled-coil domain of lamin A/C. In skin fibroblasts isolated from the proband, the processing and levels of lamin A and

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C are normal. However, nuclear morphology is aberrant and rescued by treatment with farnesyltransferase inhibitors (FTIs), as is also the case for HGPS and other laminopathies. Our findings advance knowledge of human *LMNA* progeria syndromes, and raise the possibility that typical and atypical progerias may converge upon a common mechanism to cause premature aging disease.

Keywords

Progeria; prelamin A; lamin A/C; LMNA; cancer; ZMPSTE24; Atypical Werner syndrome; FTI; laminopathy; atypical progeria; HGPS; MAD-A; farnesyl; RD

INTRODUCTION

Genetic diseases that recapitulate aging at an accelerated pace can provide insight into the normal human aging process [Burtner and Kennedy, 2010]. One of the most severe premature aging diseases is Hutchinson-Gilford Progeria Syndrome (HGPS, OMIM #176670). HGPS presents within the first year of life and generally results in death by the mid-teens due to cardiovascular disease [Hennekam, 2006; Merideth et al., 2008]. HGPS is caused by mutations in the *LMNA* gene, which encodes the A-type nuclear lamins: lamin A and lamin C [De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003]. Lamins A, B and C are intermediate filament proteins that polymerize to form a structural framework that underlies the nuclear envelope [Aebi et al., 1986; Butin-Israeli et al., 2012; Dittmer and Misteli, 2011]. Lamin A and lamin C can also be found in the nuclear interior where their role is not well understood [Dechat et al., 2010; Prokocimer et al., 2009]. The lamin A precursor, prelamin A, terminates with a CAAX motif (C is cysteine, A represents an aliphatic residue, and X is any amino acid) and undergoes a series of posttranslational modifications including farnesylation, endoproteolytic removal of the last three residues (or AAX'ing), and carboxymethylation. An additional processing step distinguishes prelamin A from lamin B and other CAAX proteins. This step is cleavage of the final 15 residues, including the post-translationally modified C-terminal tail, and is mediated by the zinc metalloprotease ZMPSTE24 to yield unmodified mature lamin A [Barrowman and Michaelis, 2009; Davies et al., 2009].

The *de novo* dominant mutation in *LMNA* that is common to most individuals with HGPS prevents the ZMPSTE24-mediated processing of prelamin A. That mutation, a silent codon change (p.G608G), activates a cryptic splice donor site leading to the in-frame internal deletion of 50 amino acids, which includes the ZMPSTE24 cleavage site [De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003]. The resulting mutant isoform of prelamin A retains the farnesylated tail and is called progerin. Notably, recessive mutations in *ZMPSTE24* also disrupt lamin A proteolytic processing and cause the progeroid disorders mandibuloacral dysplasia (MAD-B, OMIM #608612) and restrictive dermopathy (RD, OMIM #275210), due to accumulation of the full-length prelamin A processing intermediate that retains a farnesylated tail [Moulson et al., 2005; Navarro et al., 2005]. These three diseases (MAD-B, HGPS, and RD) represent a spectrum of progeroid disorders ranging from least to most severe, respectively, with a common dysfunction in lamin A processing. Within this spectrum of disorders, the cellular dose of persistently prenylated lamin A correlates with disease severity [Barrowman et al., 2012b; Davies et al., 2009]. Although the mechanism whereby persistently farnesylated lamin A causes premature aging phenotypes is unknown, changes in chromatin architecture and/or the activity of certain transcription factors may lead to altered gene expression in affected tissues, thus contributing to pathogenesis [Butin-Israeli et al., 2012; McCord et al., 2012]. Other proposed models of progeria pathogenesis have focused on the role of the nuclear lamina in regulating

mechanotransduction and nuclear stability [Philip and Dahl, 2008; Verstraeten et al., 2008], regulation of signaling pathways such as Wnt and Notch [Hernandez et al., 2010; Meshorer and Gruenbaum, 2008; Scaffidi and Misteli, 2008], defective DNA repair [Liu et al., 2006], perturbations in nuclear proteins such as Sun1 [Chen et al., 2012], and general metabolic dysfunctions observed in progeria mouse models [Kubben et al., 2011; Peinado et al., 2011].

A small, but growing, number of progeria cases described in the literature result from *LMNA* mutations that do not alter Lamin A processing, representing a second class of *LMNA*-linked progeria disorders [Al-Haggar et al., 2012; Caux et al., 2003; Chen et al., 2003; Csoka et al., 2004; Doubaj et al., 2012; Eriksson et al., 2003; Garg et al., 2009; Huang et al., 2005; Jacob et al., 2005; Kirschner et al., 2005; Madej-Pilarczyk et al., 2009; McPherson et al., 2009; Mory et al., 2008; Renard et al., 2009; Rodriguez et al., 2008; Van Esch et al., 2006; Verstraeten et al., 2006; Zirn et al., 2008]. The mutations that cause these disorders can be either dominant or recessive and can alter residues throughout the protein structure with no clear clustering in a single region of lamin A. The disorders have variably been called non-classical progeria [Hennekam, 2006], atypical Werner Syndrome (OMIM #277700) [Chen et al., 2003], or atypical progeria syndrome (APS) [Csoka et al., 2004; Doubaj et al., 2012; Garg et al., 2009] and are here referred to as the latter. APS disorders have differing ages of onset and symptom severity, with some nearly as severe as HGPS, but most far less severe. How mutations affecting different domains of the lamin A protein can lead to similar premature aging phenotypes is puzzling. However, it is likely that gaining an understanding of common downstream effects of both processing-deficient (i.e. HGPS) and processing-proficient (i.e. APS) classes of progeroid *LMNA* mutations will help us to understand the common pathways involved in premature aging disorders, and possibly even in normal physiological aging.

It is important to note that not all *LMNA* mutations cause progeria. The so-called laminopathies comprise over 450 different mutations in *LMNA* known to give rise to multiple diseases with a range of phenotypic overlap [Bertrand et al., 2011; Jacob and Garg, 2006; Worman and Bonne, 2007]. The laminopathies include different forms of cardiomyopathy, muscular dystrophy, lipodystrophy, neuropathy and the already discussed progeria syndromes (*LMNA*, OMIM #150330). Interestingly, progerias, both typical and atypical forms, encompass phenotypes such as lipodystrophy, skeletal muscular dystrophy and cardiomyopathy that overlap with these other laminopathies.

In this report we describe a family with a novel, dominantly inherited, premature aging syndrome that includes prominent cardiovascular and cutaneous manifestations, for which we propose the eponym *LMNA*-associated cardiocutaneous progeria syndrome (LCPS). The disease has a later onset than HGPS, with cutaneous manifestations of aging appearing in the third decade of life, rather than the first year of life as in HGPS. Affected individuals develop cardiac valve calcification and dysfunction, prominent atherosclerosis, and cardiomyopathy, leading to death on average in their fourth decade. One long-lived affected individual in this family was found to have four distinct primary malignancies by the age of 44 years, an unprecedented finding in *LMNA*-linked progeria disorders. We have identified a new mutation in *LMNA* (p.D300G) that cosegregates with LCPS, alters an evolutionarily conserved residue in the lamin family of proteins, and is associated with abnormalities of nuclear membrane architecture in patient fibroblasts and tissue culture cells. We show here that the dominant mutation in LCPS does not alter lamin A processing and represents another of the growing number of APS cases in which a *LMNA* mutation distal from the lamin A tail presents with premature aging disease.

MATERIALS AND METHODS

Clinical Examination

Written informed consent was obtained from all family members from whom blood was collected and analyzed. This work was reviewed and approved by the Johns Hopkins Medicine Institutional Review Board. All individuals participating in the study received a comprehensive clinical evaluation including medical history, physical examination, and echocardiogram. Information regarding deceased individuals was derived from family members, medical records, and autopsy reports. Signed permission was obtained for publication of photographs of the individual in Figure 1A.

LMNA molecular analysis

DNA was isolated from whole blood by standard methods (Qiagen). Mutation screening was performed by direct sequencing of genomic DNA polymerase chain reaction (PCR) amplicons, generated using intronic primers flanking each exon in the *LMNA* gene. Subsequent screening for the D300G mutation was performed by TaqI restriction enzyme digestion of amplicons spanning exon 5. Products were electrophoretically separated on a 2% agarose gel. The D300G mutation abolishes a cleavage site, resulting in a diagnostic uncut 437 kb restriction fragment in mutation carriers, in addition to the cleaved 175 kb and 262 kb products.

Culture Conditions for Patient Cells

Skin biopsy from the proband was collected and primary fibroblasts were isolated using standard techniques. HGPS (AG01972) and WT (GM01651) control cells were obtained from the NIA Aging Cell Culture and NIGMS Human Genetic Cell repositories, respectively (both housed by Coriell Institute). Primary patient fibroblasts from LCPS, HGPS and WT cells were grown in Modified Essential Medium (MEM, Gibco) supplemented with 15% fetal bovine serum (FBS, Gibco), penicillin and streptomycin (1X, Gibco), and 1X Non-essential amino acids (NEAA, Gibco). Where indicated, cells were treated with farnesyltransferase inhibitor (FTI) R11577 (a kind gift of M. Gelb, University of Washington) at 1 μ M for 48 hours prior to harvest. Early passage cells were between pass 8 and 11 and high passage cells were between pass 17 and 20.

Immunofluorescence of Patient Fibroblasts

For indirect immunofluorescence, primary skin fibroblasts grown on glass cover slips were fixed in 4% paraformaldehyde/phosphate buffered saline (PBS) for 10 minutes at 4° C, rinsed with 1X PBS and permeabilized in 0.5% Triton-X 100 in PBS for 10 minutes at room temperature. After blocking in 4% bovine serum albumin (BSA) in PBS, cover slips were incubated in primary antibodies in 4% BSA either for one hour at room temperature or overnight at 4°C. Fluorophore-conjugated secondary antibodies in 4% BSA were applied to cover slips for 1 hour at room temperature. Antibodies used for immunofluorescence were anti-Lamin A (Santa Cruz, #sc-20680), anti-Lamin A/C (Santa Cruz #sc-6215 or Chemicon International #MAB3211), anti-rabbit (Cy3-conjugated, Jackson ImmunoResearch #111-165-003), and anti-goat (Cy3-conjugated, Jackson ImmunoResearch). Slides were mounted in Prolong Gold (Molecular Probes).

Immunoblot Analysis

Patient cells were lysed in 1% Triton-X 100 in PBS with protease inhibitor cocktail (Roche). Protein concentrations were assessed by BCA protein assay (Thermo Scientific) and equal amounts of total protein were mixed with Laemmli sample buffer before resolution on a 4–15% SDS-PAGE gel (BioRad TGX). Following transfer to nitrocellulose, proteins were

detected by immunoblotting with primary antibody to Lamin A/C (Santa Cruz, sc-6215) followed by anti-goat HRP conjugated secondary antibody (Santa Cruz, sc-2020). The membrane was incubated with ECL reagent Femto (Thermo Scientific) and signal was detected with HyBlot CL autoradiography film (Denville Scientific).

Plasmids

Inducible plasmid constructs were created in the pSM2277 retroviral pMX vector [Barrowman et al., 2008]. GFP-tagged D300G prelamin A (pSM2461) was created by Quickchange mutagenesis (Agilent Technologies) of GFP-WT prelamin A (pSM2278, [Barrowman et al., 2008]). GFP-Progerin (pSM2457) was generated by subcloning the progerin lamin A allele from pSM2034 [Mallampalli et al., 2005] into pSM2277 by cut and paste ligation using BamHI and PmeI restriction enzymes.

Mouse Fibroblast Cell lines

Generation of stable, inducible cell lines was performed as previously described [Barrowman et al., 2008] using pSM2278 (GFP-lamin A), pSM2457 (GFP-progerin), and pSM2461 (GFP-D300G Lamin A). Briefly, retroviral constructs were co-transfected with pCL-Eco packaging vector into HEK293f cells and recovered virus was applied to Tet-Off NIH3T3 mouse fibroblasts [Barrowman et al., 2008]. Selection of cells with stable, random integration of the inducible constructs was performed using Hygromycin B (Clontech #631309) at 200ug/mL. Mouse fibroblasts were cultured in high glucose (4.5g/L) Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% FBS and antibiotics. NIH3T3 fibroblasts expressing GFP-tagged lamins were prepared for fluorescence microscopy as described above with the exclusion of antibody staining.

Statistical Analysis of Nuclear Images

Quantification of abnormal nuclear morphology was performed by averaging three or four blinded counts of over 100 nuclei per cell line. Statistical significance was calculated using Student's t-test with a one-tailed distribution. Paired t-tests were implemented for comparison within a single cell line (i.e., with or without farnesyltransferase inhibitors); unpaired t-tests were employed for comparison of mutant to wild-type cell lines.

RESULTS

Case Report

A 44-year-old woman was seen for evaluation of congestive heart failure (Fig. 1A). She was normal at birth and in early childhood, with the exception of loss of eyebrows and eyelashes by age 10 years. In her twenties, she developed taut skin, loss of subcutaneous fat stores, thinning and graying of scalp hair, and a prematurely aged appearance. She was otherwise well until approximately 27 years of age, when she began to develop exertional dyspnea. Subsequent evaluation revealed calcific mitral annular calcification, mitral regurgitation, and aortic valve stenosis. She underwent mitral valve replacement at age 29 years for severe mitral valve regurgitation. She did well until five years later, when she developed recurrent exertional dyspnea and underwent aortic valve replacement due to calcific aortic stenosis with accompanying regurgitation. Coronary angiography performed preoperatively showed no significant epicardial coronary artery stenosis. Symptoms improved, although four years after aortic valve replacement exertional dyspnea recurred. Echocardiography demonstrated normal prosthetic valve function and normal left ventricular (LV) size. Systolic LV function appeared normal.

At the age of 38 years, basal cell carcinomas were identified and resected from her neck and leg. At the age of 41 years, a focus of squamous cell carcinoma was resected from her nose.

Later that year, she developed sudden-onset chest discomfort. Coronary angiography demonstrated subtotal occlusion of the left circumflex coronary artery. This lesion was opened by angioplasty, with successful deployment of a stent, and no residual stenosis. There were 30–40% stenoses in the left anterior descending and right coronary arteries. At this point, she had a three-year history of hypertension, well controlled on moderate doses of a beta-blocker and an ACE-inhibitor, as well as elevation of her plasma triglycerides (350 mg/dl) and reduction of her HDL (30 mg/dl) with total cholesterol/HDL ratio of 5.3. She was a non-smoker with no evidence of glucose intolerance or diabetes mellitus.

The proband died at age 44 from acute myocardial infarction. On autopsy, her coronary arteries were severely calcified with 50–90% stenosis of all epicardial vessels. She had several areas of remote myocardial infarction, as well as multiple areas of acute and subacute ischemia in the left ventricle. She also had moderate atherosclerosis of her aorta with multiple complicated plaques, and mild atherosclerosis of her pulmonary arteries. In addition, multifocal papillary renal cell carcinoma was found on the capsular surface of her kidneys, and a small carcinoid tumor was identified in the right middle lobe of her lung. She was 165.1 centimeters (cm) tall and weighed 47.7 kilograms (kg). An eye exam performed during clinical evaluation revealed no ocular cataracts or other abnormal findings.

The proband had three siblings, two of whom were unaffected. Her younger sister (Fig. 1B, III-6) had similar symptoms. She was normal at birth and developed loss of eyebrows and eyelashes in childhood. In her early twenties, she was found to have a severely calcified mitral annulus, with both stenosis and insufficiency of the mitral valve. Attempted mitral valve replacement was aborted at age 26 years due to small annular size and impaired ventricular function. At age 29 years, she successfully underwent replacement of the mitral valve, though her exertional dyspnea persisted. Histologic analysis of a left ventricular biopsy obtained during mitral valve replacement surgery was unremarkable. Four years later, she developed heart failure. Coronary angiography demonstrated no significant obstruction, and pressure-volume loop analysis of the left ventricle demonstrated restrictive dysfunction. At the age of 34 years, she died from intracranial hemorrhage. Malignancies in III-6 include a basal cell carcinoma, diagnosed in her 20s and a bile duct adenoma discovered during autopsy. She was 167.6 cm tall and weighed 44.1 kg. Similar to the proband, no ocular abnormalities, such as cataracts, were found during her clinical evaluation.

The father of the proband (Fig. 1B, II-2) had progressive cardiac valve dysfunction that was attributed to rheumatic heart disease, though he had no history of rheumatic fever. In addition, he had a similar pattern of cutaneous manifestations of premature aging. He died suddenly of presumed cardiac disease at age 29 years. Autopsy demonstrated emphysema and severe calcific sclerosis and stenosis of both the mitral and aortic valves. He was 172.7 cm tall and weighed 77.3 kilograms kg at death. His older sibling (Fig. 1B, II-1) also had both cardiac and cutaneous manifestations of premature aging. He died at age 33 years from heart failure. His autopsy describes cardiac hypertrophy, calcific mitral and aortic valve stenosis, and moderate to severe coronary atherosclerosis. Individual II-1 was 182.9 cm tall and weighed 75.0 kg at death. Reported symptoms of the grandfather (Fig. 1B, I-1) include cardiac valve calcification, premature graying of the hair, skin wrinkling and a loss of body hair.

Individuals within this family were assigned affected status on the basis of overt cardiac and cutaneous manifestations of premature aging (Fig. 1B). Apparently unaffected children under age 12 were not included in analysis due to the age-dependence of the phenotype. Children over age 12 with a normal clinical evaluation and specifically without evidence of loss of eyelashes or eyebrows, a consistent feature among affected family members, were

coded as unaffected. Individuals III-2 and III-6 were coded as affected on the basis of direct clinical evaluation, and individuals I-1, II-1, and II-2 on the basis of history and medical records (Fig. 1B). All other family members over age 12 were coded as unaffected.

LMNA Molecular Analysis

Report of mutations in *LMNA* in HGPS and APS patients made this an attractive candidate gene for LCPS. Sequence analysis of *LMNA* was performed using genomic DNA from an affected individual (III-2) and an unaffected family member (II-3). We identified a heterozygous A-to-G transition at nucleotide 899 (c.899A>G) in the *LMNA* coding sequence that was unique to the patient sample (Fig. 2A). This mutation substitutes glycine (G) for aspartic acid (D) at codon 300 (p.D300G). Heterozygosity for D300G cosegregates with the LCPS phenotype in the family (Fig. 2B), and the mutation was not observed in over 100 chromosomes derived from unrelated and unaffected control individuals (data not shown). Variants at this site were not reported in Phase 1 of the 1000 Genomes project as available through the UCSC Genome Browser (2009 Assembly) [Abecasis et al., 2012; Kent et al., 2002] nor was this variant reported in over 13,000 *LMNA* alleles in the exome variant browser (<http://evs.gs.washington.edu/EVS/>).

Aspartic acid at codon 300 of lamin A/C is highly conserved throughout evolution. With the exception of zebrafish (*Danio rerio*) and Nile Tilapia (*Oreochromis niloticus*), other species for which lamin A/C coding sequence has been annotated in GenBank show aspartic acid at the corresponding position (selected species are shown in Fig. 2C). Zebrafish and Nile Tilapia have glutamic acid (E) at this position, showing conservation of negative amino acid charge. Lamin B isoforms have a similar structure to lamin A and largely show conservation of glutamic acid at this position through vertebrate evolution, with aspartic acid observed in frogs (*Xenopus laevis*). Through this analysis, one can infer extreme selective evolutionary pressure to maintain a negatively charged residue (D or E) at this position within the lamin family of proteins. The D300 residue lies within the second coiled-coil domain of lamin A/C proteins, called Coil 2 (Fig. 2D). This domain, along with Coils 1A and 1B, mediate lamin protein dimerization that in turn promotes filament formation, both of which are important in the formation of the nuclear lamina meshwork. These data support the hypothesis that the D300G mutation impairs a fundamental protein function in a dominant fashion and initiates the pathogenesis of LCPS. Additionally, substitution of glycine with a different residue, asparagine, at codon 300 (D300N) has been reported in two independent families showing progeroid disorders, also with significant cardiac involvement, as discussed below [Renard et al., 2009; Rodriguez et al., 2008].

Analysis of nuclear morphology and lamin A processing in patient fibroblasts

Abnormal nuclear morphology is a hallmark of *LMNA*-based progerias and other laminopathies [Garg et al., 2009; Mallampalli et al., 2005; Verstraeten et al., 2006]. Indirect immunofluorescence analysis of nuclei in primary skin fibroblasts derived from the LCPS proband was performed using lamin A antibodies to visualize the nuclear lamina. For comparison, HGPS patient and control healthy fibroblasts (WT) were also examined. Figure 3A shows representative examples of the predominant nuclear morphology seen in each cell line. Abnormal morphology in LCPS cells included single or multiple blebs, lobulation and occasional ringed or donut shaped nuclei (Fig. 3A, LCPS; donut nucleus represented by lower right panel; note lack of lamin A staining, marking a channel through the nucleus). These features of abnormal nuclear morphology in LCPS cells were similar to those in HGPS cells, with the exception that only LCPS cells had donut nuclei (Fig. 3A, HGPS). Representative WT nuclei, which are not blebbed, are shown for comparison (Fig. 3A, WT).

The rate of LCPS abnormal nuclear morphology was significantly greater than WT (62.6% for LCPS versus 27.1% for the control WT cells, passage 17–20) (Fig. 3B, $p < 0.05$). Likewise in HGPS cells, a pronounced increase in the frequency of abnormal morphology was observed as compared to WT cells (Fig. 3B, Fig. 76.2% for HGPS, $p < 0.01$ for passage 8–11 HGPS & WT cells). Thus, the abnormal nuclear morphology phenotype observed in LCPS patient fibroblasts is similar to that of HGPS. It should be noted that at early passages (8–11), LCPS cells showed no statistically significant differences from WT cells in the frequency of abnormal nuclear morphology and HGPS cells did not propagate to later passages.

As described above, farnesylation of the C-terminus of prelamin A is the first step of prelamin A posttranslational modification, followed by proteolytic processing and carboxymethylation. The final processing step, cleavage by ZMPSTE24, produces mature lamin A protein, which migrates more rapidly in an SDS-PAGE gel than prelamin A. To examine the processing status of lamin A in LCPS, patient and WT fibroblasts were prepared for immunoblotting. Protein samples were collected and run simultaneously; all lanes in figure 3C are from the same gel to allow for direct comparison of electrophoretic mobility. Lysates from LCPS primary fibroblasts show normal electrophoretic mobility for lamin A and lamin C, as compared to WT control fibroblasts. No significant accumulation of prelamin A is detected in LCPS fibroblasts versus WT fibroblasts (Fig. 3C). Treatment of WT cells with farnesyltransferase inhibitor (FTI), which blocks farnesylation and subsequent ZMPSTE24 cleavage, provides a marker for the prelamin A species (Fig. 3C, WT+FTI). Also, no alternative splice forms of lamin A, such as progerin observed in the HGPS patient fibroblasts (Fig 3C, progerin), were observed in LCPS fibroblasts. We note that a faint band is present above lamin A in all samples (Fig. 3C). This species is not detected by prelamin A antibody (data not shown) and is present in equal abundance in the WT and mutant cell lines, discounting the possibility that it contributes to the progeroid phenotype.

Farnesyltransferase Inhibition in LCPS fibroblasts

Treatment of cultured HGPS or RD fibroblasts with FTIs has been shown to improve nuclear morphology and other markers of the cellular progeroid phenotype [Capell et al., 2005; Glynn and Glover 2005; Mallampalli et al., 2005; Toth et al., 2005]. These findings are thought to reflect the beneficial effects of reducing the amount of persistently farnesylated progerin or prelamin A in the cell. Interestingly, we find that treatment with FTI improves LCPS nuclear morphology (14 percentage points increase in frequency of normal nuclei as compared to no treatment, $p=0.008$), comparable to the improvement observed for HGPS nuclear morphology (13.2 percentage points increase, $p=0.026$) (Fig. 3D). In comparison, WT nuclear morphology was only modestly improved (3.6 percentage points increase, $p=0.055$, Fig. 3D). Thus, FTI treatment improves nuclear morphology in LCPS even though lamin A processing is not defective. Similar results of FTI improvement of nuclear morphology have been reported for other APS mutations [Toth et al., 2005; Verstraeten et al., 2006].

D300G lamin A overexpression

To test if the D300G mutation acts in a dominant negative manner to alter nuclear morphology in cells with a full complement of WT lamin A, we transduced NIH 3T3 fibroblasts [Barrowman et al., 2008; Barrowman et al., 2012a] with inducible GFP-lamin A fusion constructs and generated stable transductants (Fig. 4). Induction of the GFP-tagged D300G lamin A construct led to abnormal nuclear morphology such as blebbing, membrane invaginations and irregularities, and micronuclei in 30.2% of cells ($p=0.035$ vs. WT induction). This is similar to the level of abnormal nuclear morphology seen upon progerin

induction (33.6% of cells, $p=0.029$ vs. WT induction). Only 17.3% of cells induced to express GFP-tagged WT lamin A showed aberrant nuclear morphology. Thus, induced expression of D300G, like progerin, dominantly causes abnormal nuclear morphology. It should be noted that the frequency and severity of abnormalities in NIH 3T3 fibroblasts induced to express mutant forms of lamin A (Fig. 4) were lower overall than in late-passage cultured primary patient cells (Fig. 3). This is likely due to the short time frame of induction of mutant lamin A (four days) in NIH3T3 cells as compared to the long-term expression in patient cells.

DISCUSSION

In “typical progeria” syndromes (e.g. HGPS), the ZMPSTE24-mediated proteolytic processing of prelamin A is defective. However, a small but growing number of progeria cases involve *LMNA* mutations that do not result in defective prelamin A processing. The latter comprise a second class of progeria disorders called “atypical progeria” syndrome (APS) [Csoka et al., 2004; Doubaj et al., 2012; Garg et al., 2009]. It should be noted that the designation of progeria cases as “classical” *versus* “non-classical” has also been used in the literature (see [Hennekam 2006] for a detailed review of classic and non-classic progeria phenotypes). However, this classification was largely based on patient phenotypes, and preceded the standard availability of genetic and molecular analysis. Recent reports have employed the term “atypical progeria syndromes” to collectively refer to non-classical progeroid syndromes that harbor mutations in *LMNA* other than p.G608G [Doubaj et al., 2012; Garg et al., 2009]. A comprehensive list of all APS cases reported to date is presented by Doubaj and colleagues [Doubaj et al., 2012].

APS cases are generally less severe and later in onset than HGPS. This is particularly true for patients carrying dominant mutations [Garg et al., 2009]. Yet, it is important to point out that individuals with recessive *LMNA* mutations can be severely affected [Madej-Pilarczyk et al., 2009; Plasilova et al., 2004], however, the severity of the phenotype clearly depends on the particular *LMNA* mutation involved [Doubaj et al., 2012; Van Esch et al., 2006].

We report here a new *LMNA* missense mutation, p.D300G, which causes an autosomal dominant familial APS that shows both similarities to and differences from HGPS. Individuals with LCPS are normal throughout childhood and young adult life, with the exception of mild cutaneous features, including loss of eyebrows and eyelashes in early adolescence. Beginning around the third decade of life, cardiac and cutaneous manifestations become evident. The former include aggressive cardiac valve calcification, atherosclerosis, and cardiomyopathy and the latter include loss of subcutaneous fat, tightening of the skin, thinning and graying of scalp hair. The average age at death for LCPS was 37.5 years and fertility is intact, as documented by 10 offspring of affected individuals. The longest-lived LCPS patient showed a clear predisposition for cancer, including four different primary malignancies; her affected sister was also found to have two primary malignancies. In contrast, in HGPS patients premature aging manifests much earlier, within the first year of life, and progresses rapidly with loss of subcutaneous fat, alopecia and decreased bone density within the first decade. HGPS patients are not fertile and die from cardiovascular disease at an average age of 13 [Merideth et al., 2008]. Thus, although LCPS is a milder form of progeria than HGPS, there is substantial overlap in the manifestations of premature aging.

The D300 residue that is altered in LCPS lies in the coiled-coil domain of lamin A (Fig. 2D), a region critical for lamin protein dimerization [Dechat et al., 2008; Dittmer and Misteli 2011]. Dimerization of lamin A, followed by head-to-tail oligomerization of dimers contributes to the formation of the nuclear lamina. Interestingly, mutations at the D300

residue have previously been reported in two unrelated cases [Renard et al., 2009; Rodriguez et al., 2008]. Those mutations, both D300N, cause phenotypes that have significant overlap with those of LCPS (D300G). In one report, father and son with a heterozygous D300N mutation were diagnosed with the premature aging disorder designated “atypical Werner Syndrome” [Renard et al., 2009]. Their symptoms were late onset (age 31 for the proband) and included calcific valve disease, peripheral vascular disease, aortic atheromas, downward curving nose, aged skin, premature graying and lipoatrophy. The father, but not son of this pedigree, also suffered from cataracts, a cardinal sign of classic Werner Syndrome. In the second report, published as an abstract, an individual with a heterozygous D300N mutation presented with late onset progeria (age 27), featuring severe cardiac dysfunction. This disease was termed “isolated cardiac progeria” [Rodriguez et al., 2008]. The conservation of the D300 residue in the *LMNA* genes of multiple species (Fig. 2C) and the phenotypic overlap seen in the different individuals with dominant missense mutations at this site suggest that the D300 residue plays a key role in lamin A/C function. This residue likely influences proper lamin A/C dimerization dynamics; improper lamin A dimerization may affect specific protein-protein interactions within the nuclear lamina that, in turn, could result in premature aging phenotypes.

Among APS mutations other than D300G or D300N, genotype-phenotype correlations can be complex. Indeed, alterations in a single lamin A codon can lead to diseases with strikingly different phenotypes. Mutant alleles that show this pleiotropy include R133L [Caux et al., 2003; Chen et al., 2003; Vigouroux et al., 2003], R527C [Cao and Hegele 2003; Liang et al., 2009] and R644C [Genschel et al., 2001; Rankin et al., 2008]. For instance, individuals with an R644C mutation can be completely unaffected or suffer from diseases ranging from cardiomyopathy to atypical progeria. In contrast, the D300G mutation featured in this work and the D300N mutations discussed above consistently lead to similar premature aging phenotypes with significant cardiac manifestations.

Laminopathies comprise a group of more than 10 diseases, in addition to the progerias discussed above. Many of these non-progeroid laminopathies have phenotypes that overlap with one another, and also with LCPS. For instance, the loss of subcutaneous fat and altered lipid profile phenotypes in LCPS are similar to phenotypes seen in Familial Partial Lipodystrophy, Dunnigan-type (FPLD, OMIM #151660) [Capanni et al., 2005; Garg et al., 2001; Vigouroux and Capeau 2005]. Additionally, the cardiomyopathy that occurs in LCPS and in several other APS disorders is a major feature in many non-progeroid laminopathies, including dilated cardiomyopathy (DCM, OMIM #115200), autosomal dominant Emery-Dreifuss muscular dystrophy (AD-EDMD, OMIM #181350) and limb-girdle muscular dystrophy (LGMD, OMIM #159001) [Hermida-Prieto et al., 2004; Muchir et al., 2007; Perrot et al., 2009]. One common manifestation broadly shared among patients with *LMNA* mutations is a distinctive set of facial features including a downward curving nose, prominent eyes and a small mandible; these features are particularly remarkable in APS, including LCPS (Fig. 1A).

The LCPS proband and her affected sister appeared to show a predisposition to cancer, unlike individuals with most other *LMNA*-associated progerias, but similar to individuals with the non-*LMNA* based premature aging disorders Rothmund-Thompson (OMIM #268400), Bloom (OMIM #210900), Cockayne (OMIM # 216400) and classic Werner Syndrome, in which DNA repair is defective. For *LMNA*-associated progerias, only two other cases of cancer have been reported to date, highlighting the rarity of cancer in these patients. The first patient harbored a rare lamin A processing-defective allele resulting in a smaller in-frame deletion than classical HGPS (deletion of 35 residues as compared to 50 residues in HGPS) and was treated for osteosarcoma at age 9 [Shalev et al., 2007]. The second patient, whose genotype is not known, presented with many classical progeria signs

and developed an osteosarcoma of the right chest wall; she succumbed to pneumonia at age 15 [King et al., 1978]. The predisposition to cancer in these two other progeria cases and LCPS may or may not share a common mechanism. However, it is important to note that no evidence of tumors was reported in the affected LCPS individuals in generations I and II, although this may be attributed to limited medical records available for these individuals. It is possible that the multiple malignancies in the LCPS proband and her sister were unrelated to the p.D300G *LMNA* mutation.

Calcification of the cardiac valves is a prominent, early feature of LCPS. Thickening and calcification of cardiac valves has been reported in many progeria patients to date, but is not a consistent finding in either typical or atypical progeria (see [Hennekam 2006] for a review of historic cases, also [Doubaj et al., 2012; Garg et al., 2009; Hanumanthappa et al., 2011; Merideth et al., 2008; Salamat et al., 2010]). Other clinical entities also present with aggressive calcification of cardiac valves and blood vessels, including Singleton-Merten syndrome (SMS, OMIM entry #182250) [Feigenbaum et al., 2013]. While LCPS shows intriguing overlap with SMS, including aggressive cardiovascular calcification causing early death, phenotypic differences include the lack of growth retardation or psoriasiform skin lesions in LCPS. The lack of genetic information at this time regarding the etiology of SMS precludes detailed speculation regarding the possible convergence of disease mechanisms.

Compared to the processing-defective class of “typical” progerias, the APS class of processing-proficient *LMNA* mutations to which D300G belongs is less well understood in terms of molecular mechanism. While HGPS is dominant, APS *LMNA* mutations can be either dominant or recessive. Mutations in the APS class are spread throughout the lamin A/C coding sequence, mapping to multiple domains and often adjacent to mutations associated with non-progeroid laminopathies. Notably, many APS mutations alter both lamin A and lamin C isoforms. Indeed, most APS mutations reported to date, including D300G, fall in the 566 amino acid coding sequence shared by both the lamin A and lamin C isoforms. Only three of the 26 reported APS mutations alter residues contained exclusively in lamin A: S573L, E578V, and C588R [Csoka et al., 2004; Garg et al., 2009; Van Esch et al., 2006]. For LCPS, whether it is the aberrant form of lamin A only or both lamin A and C that causes disease remains to be determined.

It is notable that another adult onset premature aging disease resembling Werner syndrome was recently reported [Hisama et al., 2011]. The patients described by Hisama and colleagues harbor rare, heterozygous mutations near the 3' end of *LMNA* that alter the splicing efficiency of the prelamin A transcript and result in a low dose of progerin protein accumulation, much lower than in HGPS. In contrast, the D300G mutation lies roughly in the middle of the *LMNA* coding sequence, far away from the lamin A C-terminus, and does not result in prelamin A accumulation (Fig. 3C).

At the cellular level, D300G (and other APS mutations) cause aberrant nuclear morphology similar to the well-documented nuclear blebbing seen in HGPS fibroblasts (Fig. 3). It is known that rescue of abnormal nuclear morphology in HGPS fibroblasts can be achieved by FTI treatment, which reduces the amount of the persistently farnesylated progerin form of lamin A [Capell et al., 2005; Glynn and Glover 2005; Mallampalli et al., 2005; Toth et al., 2005]. At the organismal level, a decrease in farnesylated lamin A isoforms is also associated with an improvement in overall phenotypes in progeroid mice and humans [Denecke et al., 2006; Worman et al., 2009; Yang et al., 2010]. It is notable that despite proficient prelamin A processing in LCPS patient fibroblasts (Fig. 3C), we find a significant improvement in the nuclear morphology of LCPS cells upon FTI treatment (Fig. 3D). Interestingly, other processing-proficient APS patient cells, harboring mutations T528M, M540T, E578V or R644C, were also reported to show improved nuclear morphology upon

FTI treatment [Toth et al., 2005; Verstraeten et al., 2006]. However, alterations in nuclear morphology have also been described for non-progeroid laminopathies [Parnaik and Manju 2006]. Thus, while aberrant nuclear morphology is not sufficient to cause progeria, nuclear abnormalities may intensify disease severity and ameliorating these nuclear defects could be potentially beneficial.

It is important to note, however, that improvement of aberrant nuclear morphology does not always correlate with rescue of all cellular phenotypes seen in progeria, including defects in DNA damage repair [Liu et al., 2006]. Additional therapeutic approaches to inhibit accumulation of farnesylated lamin A isoforms have been implemented, including co-administration of statins and aminobisphosphonates which resulted in improvement of nuclear morphology and other cellular defects, as well as increased longevity in a mouse model of progeria [Varela et al., 2008]. Whether this combination therapy would improve LCPS or APS patient cells remains to be investigated.

Emerging data derived from APS, including LCPS, suggest that altered lamin A processing *per se* (as seen in HGPS, RD and MAD-B), is not an exclusive driver of the pathogenic sequence for progeria. While the manner by which processing-proficient mutant forms of lamin A recapitulate events seen with processing-deficient forms remain to be elucidated, it seems likely that farnesylated and nuclear envelope-targeted wild-type lamin A could both contribute to disease by a common mechanism – perhaps serving as nidus for the accumulation of abnormal protein aggregates, through physical interaction between WT and mutant forms of lamin A [Candelario et al., 2011; Hubner et al., 2006]. While we find no evidence of lamin A aggregates or elevated levels of mature lamin A or prelamin A in LCPS versus WT cells, the possibility remains that other APS or laminopathy mutations may affect the expression of the product from the WT *LMNA* allele. Given the phenotypic improvement in HGPS mouse models with a targeted reduction in expression of wild-type lamin A [Yang et al., 2008], and improvement in nuclear morphology upon treatment of APS/LCPS cells with FTIs, it is reasonable to propose that altering the composition of the nuclear lamina in cells expressing an APS mutant form of lamin A could ameliorate disease phenotypes. Inhibiting farnesylation affects not only prelamin A, but also lamin B1 and B2 and other proteins, with potential impact on the stability and functions of all of these proteins in the nuclear envelope. The benefit of inhibiting farnesylation by FTI treatment in atypical progerias, such as LCPS, or in other laminopathies is an interesting possibility to consider.

Ultimately, it will be important to determine whether different progeria mutations, both typical and atypical, such as D300G, drive premature aging phenotypes by altering the same molecular pathway(s) and whether FTI or statin and bisphosphonate administration impacts these pathways. The nuclear lamina plays a key role in the maintenance of chromatin architecture [Shimi et al., 2008]. Mutant lamin A may disrupt interactions between the nuclear lamina and chromatin or specific protein-binding partners [Bruston et al., 2010; Pegoraro et al., 2009]. Systematic analysis of both classes of progerias will provide insight not only into premature aging but may also identify cellular alterations in the normal aging process.

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Figure 1.

A) Individual III-2 with LCPS, age 44 years. Note her loss of eyebrows and eyelashes and premature graying. **B)** Pedigree of LCPS family. Squares indicate males, circles indicate females. Solid figures indicate affected individuals with LCPS. Diagonal lines signify death, and age of death is indicated below.

Figure 2.

A) Chromatogram of sequence from exon 5 in control individual II-3 (top) and LCPS individual III-2 (bottom). Heterozygous adenosine to guanine at nucleotide 899 is indicated in the bottom chromatogram. **B)** *LMNA* exon 5 amplicons from individuals indicated, digested with TaqI, and separated on 2% agarose gel. Undigested 437 bp amplicon is indicated with an arrow, the digested 262 and 175 bp fragments are below. **C)** Amino acid homology for lamins A and C, B1, and B2 among several species listed in GenBank. In comparison with *Homo sapiens*, identical residues are shaded black, similar residues are shaded grey, and those that are different are not shaded. **D)** Schematic of the prelamins A

protein structure with various domains shown to scale. Coils 1A (amino acids 33–70), 1B (a.a. 80–218) and Coil 2 (243–383) and the immunoglobulin (Ig)-like fold (a.a. 432–541) are indicated. The nuclear localization signal (NLS, a.a. 417–422) is indicated by a narrow orange line next to the Ig Fold. The lamin A tail that is proteolytically removed by ZMPSTE24 is shown in grey. The location of the D300 residue in Coil 2 and the location of the classic HGPS mutation (G608G, indicated by a red star) are also shown.

Figure 3.

LCPS patient fibroblasts show aberrant nuclear morphology, no lamin A processing defect, and nuclear phenotypic rescue with FTI treatment. **A)** Indirect immunofluorescence of representative morphology of 4 nuclei from WT, LCPS and HGPS primary patient fibroblasts stained with anti-lamin A/C antibodies. **B)** Quantification of abnormal nuclear morphology of WT and LCPS cells at late (p17–20) passage and HGPS cells at early passage (p8–11) as discussed in the text. Error bars represent standard error, single asterisks indicate $p < 0.05$, double asterisks indicate $p < 0.01$. **C)** LCPS cells do not accumulate prelamin A or misspliced lamin A isoforms. SDS-PAGE of protein lysates, immunoblotted with anti-lamin A/C antibody. The protein species are labeled. As a marker for prelamin A migration, WT cells were treated with 1 μ M FTI for 48 hours. **D)** Quantification of improved nuclear morphology with FTI treatment of WT and LCPS cells (late passage) and HGPS cells (early passage) as discussed in the text. The percentage plotted indicates the increased frequency of normal nuclei from paired analysis of cells treated without versus with FTI, measured by difference in percentage points. Single asterisks indicate $p < 0.05$, double asterisks indicate $p < 0.01$ (Student's t-test).

Figure 4.

D300G is a dominant negative allele capable of altering nuclear morphology in cultured mouse fibroblasts. NIH 3T3 cells stably transduced with the inducible GFP-tagged lamin A constructs indicated were induced by withdrawal of tetracycline for four days.

Representative nuclei in cells expressing GFP-tagged WT lamin A, D300G lamin A and progerin are shown. Bar graph indicates the frequency of abnormal nuclear morphology in three cell lines. Error bars represent standard error, asterisks indicate p-value < 0.05 (Student's t-test).