Hutchinson–Gilford progeria mutant lamin A primarily targets human vascular cells as detected by an anti-Lamin A G608G antibody

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Hutchinson–Gilford progeria syndrome (HGPS; Online Mendelian Inheritance in Man accession no. 176670) is a rare disorder that is characterized by accelerated aging in children. The LMNA gene encoding the A-type lamins A and C is the causative gene of HGPS (1–3). Approximately 80% of HGPS cases are caused by a G608 (GGC→GGT) mutation within exon 11 of LMNA, which elicits a deletion of 50 aa near the C terminus of prelamin A. In this article, we present evidence that the mutant lamin A (progerin) accumulates in the nucleus in a cellular age-dependent manner. In human HGPS fibroblast cultures, we observed, concomitantly to nuclear progerin accumulation, severe nuclear envelope deformations and invaginations preventable by farnesyltransferase inhibition. Nuclear alterations affect cell-cycle progression and cell migration and elicit premature senescence. Strikingly, skin biopsy sections from a subject with HGPS showed that the truncated lamin A accumulates primarily in the nuclei of vascular cells. This finding suggests that accumulation of progerin is directly involved in vascular disease in progeria.

Results and Discussion

Mutant Lamin A G608G Accumulates Within the Nucleus in a Cellular Age-Dependent Manner as Detected by a Specific Ab. A rabbit polyclonal Ab was raised against a short peptide overlapping the 50-aa deletion region in the mutant lamin A G608G (progerin; see Fig. 6, which is published online as supporting information on the PNAS web site) (17). The immune sera, denoted anti-LMNA G608G, were tested on primary dermal fibroblast cultures at early and late population doublings (PPDs; 20–50) by using four different HGPS fibroblast cultures (see Materials and Methods). All four cultures behaved similarly in vitro; therefore, results reported herein are derived from HGPS human fibroblasts HGAFN127. Surprisingly, only a few cells were positively labeled with the anti-LMNA G608G Ab at early PPDs (~25; Fig. 1A), whereas all nuclei were labeled with anti-emerin Ab that detects an inner-nuclear membrane protein (Fig. 1A) (18). Strikingly, the number of nuclei positively stained with anti-LMNA G608G increased with the number of cellular generations in vitro. Signal intensity varied; in some cells, it was faint, and in others, it was very bright, indicating that the HGPS fibroblasts contained different amounts of progerin (Fig. 1A). Primary dermal fibroblast cultures are asynchronous, suggesting that heterogeneity of anti-LMNA G608G staining reflected cellular age (19). Indeed, Western blot analysis indicated that the progerin product was increased in cultures at late PPDs (Fig. 1B).

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Abbreviations: HGPS, Hutchinson–Gilford progeria syndrome; PPDs, population doublings; NE, nuclear envelope; β-gal, β-galactosidase.

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as reported by using anti-A-type lamin Abs (16). Anti-LMNA G608G serum gave no signal in control dermal fibroblasts by indirect immunofluorescence or by Western blotting (Fig. 1 A and B).

Progerin Accumulation Induces NE Invaginations. Careful analysis of brightly labeled nuclei with anti-LMNA G608G revealed that the truncated lamin A was distributed in dot-like or cable-like structures (Fig. 1 C). The intra- and transnuclear cable-like structures detected with anti-LMNA G608G were also labeled with emerin or nucleoporin 414 Abs, suggesting that they associated with the membrane compartment. Furthermore, DAPI staining of mutant nuclei showed severe chromatin rearrangement with the formation of interchromatin folds that coincided with anti-LMNA G608G staining (Fig. 1 C). These results indicated increased NE invaginations. In nuclei containing low levels of progerin, the mutant protein colocalized with wild-type lamin A and lamin B1 within the nuclear lamina, and emerin and nucleoporin 414 Abs showed a normal distribution pattern (data not shown).

To verify that the cable-like structures were indeed NE invaginations, we performed EM of HGPS cultures at 38 PPDs, where a significant number of cells (25–30%) harbored progerin cable-like distribution as detected by indirect immunofluorescence. EM of ultrathin HGPS sections revealed that 57% of the nuclei showed drastic NE deformations altering the nucleus from a spherical to a lobulated shape; some nuclei remained roughly ovoid but harbored significant intranuclear invaginations (Fig. 2 C). Those numerous invaginations were composed of a double membrane containing nuclear pores, with some regions exhibiting high pore density; they also remained in close apposition with the nuclear lamina and the chromatin (Fig. 2 D). In contrast, ultrathin sections of control fibroblast cultures at similar or higher PPDs had ≈22% of the nuclei exhibiting NE invaginations, none as deep or as numerous as in HGPS cells (Fig. 2 A).

Increased NE invaginations have been reported in cells permanently overexpressing prenylated lamins or components of the NE (lamin B receptor or nucleoporin 153), which resembled the ones observed in HGPS fibroblasts at late PPDs (20, 21).

Farnesyltransferase Inhibitor Normalized Nuclear Morphology in HGPS Dermal Fibroblasts. Normal prelamin A processing involves an orchestrated sequence of modifications: farnesylation, carboxyl methylation of the cysteine residue within the CSIM motif, proteolytic cleavage of SIM, and finally cleavage of 15 residues giving rise to a strong nuclear rim staining (Fig. 3 C). After a 48-h treatment, the percentage of HGPS nuclei with abnormal shapes decreased from 31% to 8% (Fig. 3 A). The intranuclear repartition of prelamin A and the localization of LMNA G608G differed greatly between treated and untreated cells; prelamin A was not detectable in untreated control or HGPS cells (Fig. 3 B). When farnesylation was inhibited in control cells, the lamin A precursor was present in nuclear foci but was mostly accumulated in the lamina where it is assembled, giving rise to a strong nuclear rim staining (Fig. 3 B), as reported in refs. 23–25. In HGPS fibroblasts treated with FTI-277, the nuclei appeared more ovoid overall, and wild-type prelamin A localization resembled that in control cells (Fig. 3 B). After a 24-h
treatment, anti-LMNA G608G stained the nuclear rim in HGPS fibroblasts, indicating that progerin was still incorporated into the nuclear lamina. Progerin was also detected in nuclear foci similarly to wild-type prelamin A (Fig. 3B). After 48 h FTI-277 treatment, however, progerin was progressively depleted from the NE and lamina. Progerin formed large aggregates within the nucleoplasm that sometimes overlapped with the wild-type prelamin A. Seventy-two hours after treatment, whereas wild-type prelamin A was still predominantly detected at the NE and in some nucleoplasmic foci (Fig. 3B), progerin was totally depleted from the NE in most nuclei and was localized within nucleoplasmic aggregates (Fig. 3B). These results suggest that unfarnesylated progerin lost its ability to bind the NE and to assemble into the lamina, thus restoring a more regular nuclear shape for the majority of the HGPS cells (Fig. 3), as also reported recently in refs. 13, 14, and 26.

**Mutant Lamin A Induces Decreased Cellular Proliferation, Premature Senescence, and Altered Motility.** During the spatiotemporal expression studies of mutant LMNA G608G, we noticed that the HGPS fibroblasts HGAFN127 grew at the same rate as control cells until they reached ~40–45 PPDs (Fig. 4A). Between 40 and 50 PPDs, as HGPS cells accumulated progerin, their growth rate decreased rapidly until they ceased dividing, between 52 and 57 PPDs under our culture conditions. Late passage control fibroblasts continued to grow and exhibited only a slow decline in their proliferative index when compared with early PPDs (Fig. 4A). These observations prompted us to examine whether HGPS cells were entering premature senescence.

Primary cultured cells undergoing cellular senescence in vitro express increased β-galactosidase (β-gal) activity when assayed at pH 6 (27). Indeed, when senescence β-gal-positive cells were scored at late PPDs in HGPS cells after 1 week in culture, 3% of the cells were positive, whereas none were detected in control cultures at the same PPDs (Fig. 4C). Furthermore, the number of senescent HGPS cells increased with time in vitro, reaching 43% after 4 weeks (Fig. 4C). In control cells after 4 weeks, one to two senescent β-gal-positive cells could be detected on the entire coverslips. These results indicate that senescent cells accumulated prematurely in HGPS fibroblast cultures. Moreover, when senescence-associated β-gal activity and LMNA G608G levels were assayed on late PPDs of HGPS cells, we observed that senescent cells (β-gal-positive) were brightly labeled with anti-LMNA G608G Ab and harbored nuclear cable-like structures reminiscent of NE invaginations (Fig. 4D). This finding indicates that HGPS fibroblasts, concomitantly with the accumulation of mutant lamin A, withdraw from the cell cycle either via the apoptotic pathway as reported in ref. 28 or senescence (Fig. 4D).

To gain insight on the cellular effects of progerin, we examined cellular migration, an important function in a variety of physiological aspects. We used a migration assay in which fibroblasts...
could demonstrate both inherent and directional motility in response to a chemotactic stimulus (29). Although the overall number of migrant HGPS cells was similar to controls, none of the migrant HGPS cells were strongly labeled with anti-lamin A G608G Ab (Fig. 4E). Indeed, all LMNA G608G brightly positive cells remained on the upper face of the filters where they had been seeded (Fig. 4E), associating high progerin levels with reduced migration.

Vascular Cells Are the Primary Targets for Progerin Build-Up, Linking the Mutation to the Pathogenesis of Atherosclerosis in HGPS. Understanding the cellular mechanism underlying the clinical sequelae of HGPS requires the identification of the cellular targets of progerin in vivo. Immunohistochemistry was performed on serial frozen sections from a skin biopsy derived from a 9-year-old subject with HGPS (HGADFN143). Hematoxylin/eosin staining showed a well-organized epidermis, a large blood vessel, sweat glands, an arrector pili muscle, and absence of hair follicles (Fig. 5A). Connective tissue was very dense with thick fibrous structures throughout the dermis, s.c. fat tissue was absent in this sample. Lamin A/C was detected in the nuclei of most cells from the epidermis and dermis (Fig. 5A). The anti-LMNA G608G Ab showed a very restricted pattern of distribution, with positively labeled nuclei localized within the blood vessels, some cells surrounding the sweat glands and in arrector pili muscle. In the epidermis, very few keratinocytes were positively stained with anti-LMNA G608G in the uppermost layer of the epidermis (data not shown). Immunodetection with the LMNA G608G preimmune serum gave no signal, indicating that the signal obtained with anti-LMNA G608G Ab was specific. The anti-LMNA G608G Ab also gave no signal on skin sections from an 86-year-old unaffected individual (Fig. 5C). To identify the cell type(s) positively labeled in the blood vessels, we double-labeled HGPS skin sections with anti-LMNA G608G Ab and with anti-SMA directed against the smooth muscle cell surface antigen CD31. We confirmed that the brightest signal obtained with anti-LMNA G608G Ab was indeed located within the vascular system and that the positively labeled nuclei were primarily smooth muscle and endothelial cells (Fig. 5B). At high magnification, smooth muscle cell nuclei with a strong LMNA G608G signal adopted similar cable-like structures as HGPS fibroblasts reminiscent of the NE invaginations (data not shown). Thus, we have provided in vivo evidence for the presence of progerin in vascular cells. We also have provided support for a direct relation between progerin and atherosclerosis in HGPS.

Previously, autopsies of a few HGPS cases demonstrated severe smooth muscle cell depletion in atherosclerotic aorta media (30, 31). The rare remaining smooth muscle cells had aberrant cellular shape, ballooning mitochondria, and increased...
cytoplasmic density (30). Those observations suggested that smooth muscle cells in HGPS were hypersensitive to hemodynamic and ischemic stress and may become defective in restoring vascular integrity after injury (30). In uninjured blood vessels, smooth muscle and endothelial cells are mostly quiescent. In HGPS subjects, vascular smooth muscle cells may be subjected to increased mechanical stress, forcing them to undergo several cellular divisions to regenerate vascular tissue. The build-up of nuclear progerin may occur as cells reach a high number of PPDs. This phenomenon could be the cause of the detected progerin accumulation in vascular cell nuclei of HGPS skin sections. In such a setting, cells would progressively lose their capacity to grow and migrate and would either become apoptotic or senescent. All those progerin-dependent cellular changes might contribute to the vascular deterioration responsible for the progression of atherosclerosis in HGPS subjects.

In conclusion, this article addresses the in vivo cellular and tissue localization of a mutant lamin A (progerin) responsible for severe, premature atherosclerosis in HGPS. Progerin accumulates primarily in vascular cells and can be regarded as a key player in the onset of atherosclerosis, the primary cause of death for HGPS patients. Normalization of cellular function by preventing progerin accumulation, expression, or posttranslational modification by using treatments such as farnesyltransferase inhibitor or genetic therapies (32) show promise as treatments that could significantly reduce disease progression in HGPS children.

**Materials and Methods**

**Characterization of the Anti-Lamin A G608G Ab.** The lamin A G608G amino acid sequence reading frame was determined in refs. 1 and 17. To generate a specific anti-Lamin A G608G Ab, we have chosen a short peptide (eight residues; GAQSPONC) overlapping the region where the 50-residue truncation occurred in the lamin A mutant G608G (see Fig. 6). The peptide was purchased from BioSynthesis (Lewisville, TX); Cocalico Biologicals (Reamstown, PA) immunized two rabbits. Preimmune and immune sera were characterized by Western blot analysis and by indirect immunofluorescence on HGPS and control fibroblasts.

**Primary Dermal Fibroblast Cells.** We obtained three primary cultures of dermal fibroblasts derived from HGPS patients carrying the LMNA mutation G608G: HGADFN001, HGADFN003, and HGAFFN127 from the Progeria Research Foundation (Peabody, MA) and PT001 recently identified in ref. 17. We used in parallel three previously established control dermal fibroblast cultures. All results shown in this article correspond to HGPS fibroblasts HGADFN127.

**Indirect Immunofluorescence and Western Blot Analysis.** Primary cultures of dermal fibroblasts were processed for indirect immunofluorescence as described in ref. 17. Rabbit Abs directed against A-type and B-type laminas were kindly provided by N. Chaudhary (Ridgeway Biosystems, Inc., Cleveland) (33, 34). Goat anti-lamin B1 M-20 and anti-prelamin A C-20 (Santa Cruz Biotechnology), mAbs anti-emerin 4G5 (Novocastra), anti-nucleoporin Nup153 clone 414 (Covance, Richmond, CA), anti-lamin A Jol4 (Serotec, Raleigh, NC), and anti-Ki-67 (BD Bioscience) were purchased. Secondary Abs were affinity purified Alexa Fluor 488 goat or donkey IgG Abs (Molecular Probes) and Cy3-conjugated IgG Abs (Jackson ImmunoResearch). All samples were counterstained with DAPI (Sigma–Aldrich).

When cells were treated daily for 3 days with the farnesyltransferase inhibitor FTI-277 (Calbiochem), the culture medium was processed by using ADOBE PHOTOSHOP (Adobe Systems, San Jose, CA). For Western blot analysis, total cellular protein...
extracts were isolated from dermal fibroblast cultures at different PPDs (25–55) and processed as described in ref. 17.

In Vivo Detection of Lamin A G608G on HGPS Skin Biopsy Sections. Immunohistochemistry was performed on frozen skin sections of a biopsy of a 9-year-old donor with HGPS carrying the LMNA G608G mutation (HGADFN143), provided by the Progeria Research Foundation, and on normal skin sections from an 86-year-old unaffected individual (Columbia University). Frozen sections (6 μm) were fixed by methanol/acetic acid (1 vol/1 vol) at −20°C for 10 min and processed for indirect immunofluorescence as described above. Rabbit anti-lamin A G608G serum and corresponding preimmune serum were used at a dilution of 1:400, and anti-lamin A/C Ab was used at a dilution of 1:600 (33). Anti-human α-smooth muscle actin, clone 1A4 (DAKO), and anti-CD-31/PECAM-1 Abs (Lab Vision, Fremont, CA) were used according to the manufacturer's instructions. One section was stained with hematoxylin/eosin for morphological comparison.

Cell Migration Assay. Cell migration assays were performed by using standard 8.0-μm pore size transwell inserts (BD Bioscience). Subconfluent HGPS and control dermal fibroblasts from 35–40 PPDs were harvested and resuspended in serum-free DMEM (5 × 10⁴ cells/ml). A cell suspension (500 μl) was added to the upper chamber of the inserts. The lower chamber was filled with 750 μl of DMEM containing 15% FCS. After a 22-h incubation at 37°C, the filters were removed, and the membranes were either processed for upper or lower membrane surfaces for indirect immunofluorescence analysis with anti-lamin A G608G and anti-emerin Abs.

Senescence-Associated β-gal Staining. Dermal fibroblast cultures from 40–45 PPDs were stained for senescence β-gal activity at pH 6 (27). Cells grown on coverslips were fixed in methanol/acetone at −20°C for 5 min, washed in PBS and incubated with anti-lamin A G608G Ab for 1 h, washed in PBS, and then incubated overnight in fresh senescence β-gal staining solution [1 mg of 5-bromo-4-chloro-3-indolyl β-D-galactoside per ml of buffer containing: 40 mM citric acid sodium phosphate, pH 6/0.5 mM potassium ferrocyanide/5 mM potassium ferricyanide/150 mM sodium chloride/2 mM magnesium chloride (27)]. After a 15-h incubation at room temperature, cells were washed in PBS and further processed for indirect immunofluorescence with the secondary Ab.

Quantification. The percentages of Ki-67-positive cells, of misshapen or blebbing nuclei, and senescence β-gal-positive cells were determined by a direct count of 300 cells per coverslip in triplicate and from three independent experiments. The data sets were plotted by using KALEIDAGRAPH (Synergy Software, Reading, PA).

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