Prelamin A Acts to Accelerate Smooth Muscle Cell Senescence and Is a Novel Biomarker of Human Vascular Aging

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Background—Hutchinson-Gilford progeria syndrome is a rare inherited disorder of premature aging caused by mutations in LMNA or Zmpste24 that disrupt nuclear lamin A processing, leading to the accumulation of prelamin A. Patients develop severe premature arteriosclerosis characterized by vascular smooth muscle cell (VSMC) calcification and attrition.

Methods and Results—To determine whether defective lamin A processing is associated with vascular aging in the normal population, we examined the profile of lamin A expression in normal and aged VSMCs. In vitro, aged VSMCs rapidly accumulated prelamin A coincidently with nuclear morphology defects, and these defects were reversible by treatment with farnesylation inhibitors and statins. In human arteries, prelamin A accumulation was not observed in young healthy vessels but was prevalent in medial VSMCs from aged individuals and in atherosclerotic lesions, where it often colocalized with senescent and degenerate VSMCs. Prelamin A accumulation correlated with downregulation of the lamin A processing enzyme Zmpste24/FACE1, and FACE1 mRNA and protein levels were reduced in response to oxidative stress. Small interfering RNA knockdown of FACE1 reiterated the prelamin A–induced nuclear morphology defects characteristic of aged VSMCs, and overexpression of prelamin A accelerated VSMC senescence. We show that prelamin A acts to disrupt mitosis and induce DNA damage in VSMCs, leading to mitotic failure, genomic instability, and premature senescence.

Conclusions—This study shows that prelamin A is a novel biomarker of VSMC aging and disease that acts to accelerate senescence. It therefore represents a novel target to ameliorate the effects of age-induced vascular dysfunction. (Circulation. 2010;121:2200-2210.)

Key Words: aging ■ arteriosclerosis ■ muscle, smooth ■ nuclear lamina ■ progeria

Hutchinson-Gilford progeria syndrome (HGPS) is a rare condition of premature aging caused by defects in the integrity of the nuclear lamina. It is caused by mutations in LMNA, the gene encoding A-type lamins, intermediate filament proteins that provide the structural scaffold for the nuclear lamina. In the majority of cases, a specific mutation (G608G) activates a cryptic splice site, resulting in the generation of a mutant prelamin A protein, referred to as progerin. Normally, prelamin A is posttranslationally modified by farnesylation to facilitate nuclear envelope (NE) targeting and is subsequently cleaved by the metallocproteinase FACE1/Zmpste24 to remove the farnesyl groups and produce mature lamin A, which can then insert into the nuclear lamina. Progerin lacks the conserved FACE1/Zmpste24 cleavage site and therefore remains permanently farnesylated. Thus, although it can enter the nucleus and associate with the NE, it cannot incorporate normally into the nuclear lamina. More rarely, HGPS is caused by mutations in Zmpste24, leading to the accumulation of permanently farnesylated prelamin A. In vitro, progerin and prelamin A accumulation in fibroblasts causes nuclear morphology defects, and the cells undergo premature...
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An important feature of HGPS is that aging symptoms appear to be tissue specific. Accumulating evidence suggests that vascular smooth muscle cells (VSMCs) are highly susceptible to progerin accumulation. In skin sections from HGPS patients, progerin was observed exclusively in arterial VSMCs, whereas transgenic mice, ubiquitously expressing human progerin from a BAC clone, exhibited VSMC calcification and degeneration as the only phenotype. Similarly, in HGPS patients, the most catastrophic defect is VSMC dysfunction. Affected individuals develop severe premature arteriosclerosis and die of myocardial infarction or stroke usually within the second decade of life. Large arteries and small arterioles show characteristic VSMC changes including calcification, lipid accumulation, fibrosis, and VSMC attrition. These VSMC defects are highly reminiscent of those occurring in common, age-associated vascular pathologies, including atherosclerosis and related arteriosclerotic processes such as medial calcification. Thus, an important question is whether the dysfunctional pathways in HGPS are relevant to normal aging.

To date, the role of nuclear lamina defects in normal aging processes is unclear. One study showed that the alternate splicing of LMNA to produce progerin is consistently utilized in a low percentage of cells in both young and old individuals but with no age-associated rise in expression. Accumulation of progerin in a very small fraction of skin fibroblasts in aged individuals has also been demonstrated. However, despite the prevalent vascular phenotype of HGPS, analyses of lamin A processing in human VSMCs have not been performed. Therefore, we sought to determine the contribution, if any, of lamin A dysfunction in human VSMC aging. We demonstrate that prelamin A accumulation is a novel and specific hallmark of VSMC aging and disease and may be a therapeutic target to ameliorate the effects of age-related vascular dysfunction.

Methods

An expanded Methods section appears in the online-only Data Supplement.

Cell Culture

Human VSMCs were cultured from explants as described previously. Human dermal fibroblasts were obtained from NIA Aging Cell Repository (Tables I through III in the online-only Data Supplement). Cells were seeded at a known density, and counts were performed on each passage until senescence. Senescence-associated β-galactosidase (SAβG) activity in cells and tissues was detected as described previously. Cells were treated for 48 hours with the farnesyl transferase inhibitor (FTI) α-hydroxyfarnesylphosphoric acid (H-9279; Sigma, St Louis, Mo), at a dose of 2.5 μmol/L, or atorvastatin, at a dose of 0.5 μmol/L. Oxidative stress was induced in VSMCs and arteries by incubation in the presence or absence of the indicated H2O2 concentration for 24 to 72 hours.

Reverse Transcription Polymerase Chain Reaction and Quantitative Polymerase Chain Reaction

Human FACE1 (Zmpste24) (Hs00195298 m1; Applied Biosystems, Foster City, Calif) and 18S (4333760F; Applied Biosystems) primers were coamplified in triplicate samples, and the fluorescence intensity of the polymerase chain reaction products was measured with the use of theRotor Gene RG-3000 (Corbett).

Antibodies, Immunofluorescence, and Immunocytochemistry

Primary antibodies used were as follows: prelamin A (SC-6214, C-20), lamin A/C (SC-6215, N-18), FACE1 (SC-34777) (Santa Cruz Biotechnology, Inc, Santa Cruz, Calif); p21 (2946), p16 (4824), phosphor-p38 (9215), γ-H2AX (2577), phosphor-ATM substrate (2851), 53BP1 (4937) (Cell Signaling Technology, Danvers, Mass); 8-oxo-dG (Japan Institute for Control of Aging); and FLAG (M2, F3165) (Sigma). Antibody specificity was tested with the use of adenoviral expression of different forms of lamin A (Figures I and II in the online-only Data Supplement). VSMCs were cultured on coverslips and were fixed in 50% methanol/acetone before processing for immunofluorescence. Human tissue samples for immunocytochemistry and SAβG studies were fixed and processed as described previously. Periodic acid–Schiff staining was performed according to the manufacturer’s instructions (Sigma).

Fluorescence-Activated Cell Sorting Analysis

The DNA content of cells was measured by propidium iodide staining, and γ-H2AX analysis was performed according to the manufacturer’s instructions (Cell Signaling Technology).

Small Interfering RNA–Mediated Interference

Dharmacon smart pool FACE1 and control small interfering RNA (siRNA) oligonucleotides were transfected into VSMCs with the use of HiPerfect transfection reagent (Qiagen). At 48 or 72 hours after transfection, samples were prepared for Western blotting, fluorescence-activated cell sorting analysis, or immunofluorescence.

Comet Assays

Comet assays were performed with the use of protocols obtained from the Comet Assay Interest Group Web site.

Electron Microscopy

VSMCs were fixed in 2% glutaraldehyde, postfixed in 1% osmium ferricyanide, and embedded. The 50-nm sections were cut and stained with uranyl acetate and viewed in a FEI Philips CM100.

Adenoviral Constructs

VSMCs at 70% to 80% confluence were infected with FLAG-tagged recombinant adenoviruses containing wild-type lamin A (Ad/WT); an uncleavable form of prelamin A mutated within the Zmpste24 cleavage site (L647R) (Ad/UC); or prelamin A (Ad/Pr). Multiplicity of infection was 10 to 50 particles per cell as stated, routinely achieving 80% transfection efficiency as assessed by the control Ad/EGFP.

Statistical Analysis

Cell counts for statistical analysis were performed on n = 100 to 500 cells in triplicate for each control and experimental group, and results were verified in at least 3 independent experiments performed in different VSMC isolates. Data are shown as mean ± SD. Statistical analysis was performed with GraphPad software, and comparisons were made with the Student paired or unpaired t test or Kruskal-Wallis test as indicated.

Results

Aged VSMCs Exhibit Nuclear Morphology Defects and Limited Growth Capacity

VSMCs showed limited growth potential in vitro and abruptly ceased proliferation; these growth characteristics are...
reminiscent of HGPS fibroblasts (Figure 1A).8 VSMCs from old donors (71.1 ± 5.8 years; n = 9) reached replicative senescence sooner (passage 5.2 ± 2.3) than those from younger donors (28.7 ± 14.8 years; n = 10) (passage 15.9 ± 6.4; P = 0.0004) and showed an increased frequency of senescent cells, exhibiting a large flattened appearance and SAβG positivity, at early passages (Table I in the online-only Data Supplement and Figure 1B). The frequency of cells exhibiting nuclear convolutions increased with passage, and VSMCs isolated from old donors (>70 years; n = 3) showed a greater frequency of dysmorphic nuclei compared with those from young donors (<16 years; n = 3) at equivalent passage numbers (Figure 1B and 1C). Statins and FTIs reduced the frequency of these nuclear morphology defects, suggesting that they were due to the accumulation of farnesylated forms of prelamin A23,24 (Figure 1D and Figure III in the online-only Data Supplement). This notion was supported by transmission electron microscopy showing an early-passage VSMC with an ovoid nucleus (left panel), few NE convolutions, and a layer of heterochromatin (arrows in enlargement in right panel). Late-passage VSMCs with a severely convoluted nucleus and NE (arrows in left panel) are shown. The NE has an electron-dense lamina (arrows in enlargement in left panel), and both the nucleus and NE have defects in heterochromatin organization. NE heterochromatin is lost and poorly organized, whereas areas of the nucleus have chromatin reorganization and areas deficient in electron-dense chromatin (asterisks). Bar = 1 μm. Nu indicates nucleus; Cyt, cytoplasm.

Figure 1. Aged VSMCs exhibit nuclear morphology defects. A, Proliferation of 2 VSMC isolates in vitro in a 54-year-old man (●) and a 77-year-old woman (□). B, Nuclear morphology defects (arrow) and senescent cells (shown by SAβG staining and typical flattened appearance) were increased in aged VSMCs. Shown are an 11-year-old male subject (11M), passage 9, and a 78-year-old female subject (78F), passage 6. C, Significant increase in the frequency of nuclear morphology defects in early-passage vs senescent and young vs old VSMCs. Mean ± SD values are shown for n = 500 cells counted in 3 VSMC isolates per group. Unpaired t tests were used. D, Treatment with 2.5 μmol/L FTI or 0.5 μmol/L atorvastatin for 24 hours significantly reduced the number of abnormal nuclei seen in HGPS fibroblasts and VSMCs. P indicates passage number. Experiments were performed in triplicate; independent experiments for 3 VSMC isolates are shown. Mean ± SD values are shown. Paired t test was used (control vs treatment). E, Transmission electron microscopy showing an early-passage VSMC with an ovoid nucleus (left panel), few NE convolutions, and a layer of heterochromatin (arrows in enlargement in right panel). Late-passage VSMCs with a severely convoluted nucleus and NE (arrows in left panel) are shown. The NE has an electron-dense lamina (arrows in enlargement in left panel), and both the nucleus and NE have defects in heterochromatin organization. NE heterochromatin is lost and poorly organized, whereas areas of the nucleus have chromatin reorganization and areas deficient in electron-dense chromatin (asterisks). Bar = 1 μm. Nu indicates nucleus; Cyt, cytoplasm.

**Human VSMCs Accumulate Prelamin A During In Vitro Aging**

Reverse transcription polymerase chain reaction in VSMCs failed to detect the use of the cryptic splice site utilized in
HGPS fibroblasts to produce progerin (Figure IV in the online-only Data Supplement). Western blotting, with the use of antibodies that recognize mature and immature forms of lamin A, also failed to detect progerin in VSMCs (Figure 2A and 2B). However, a significant accumulation of prelamin A in aged VSMCs was detected (Figure 2A and 2C). Immunofluorescence confirmed that the number of prelamin A–positive cells increased with passaging and was significantly higher in VSMCs from aged donors (Figure 2D and 2E). Prelamin A accumulated in association with the NE, with punctate cytoplasmic staining also observed in VSMCs with the highest levels of prelamin A (Figure 2F and 2G). This suggests that prelamin A accumulates before the onset of senescence and not as a consequence of it.

Figure 2. Aged VSMCs accumulate prelamin A. A, Western blotting using antibodies to lamin A/C (top, SC-6215, N-18) and prelamin A (middle, SC-6214, C-20) showing prelamin A accumulation in aged and senescent VSMCs. Normal and senescent VSMCs are paired (ie, from the same isolate). Abbreviations are as defined in Figure 1 legend. B, Western blotting demonstrating absence of progerin in aged VSMCs (77-year-old woman, 78-year-old woman) with the use of lamin A/C, N-18, which detects progerin in HGPS fibroblasts. C, Western blotting showing the specificity of the lamin antibodies. Lamin A/C N-18 detects lamins A and C and progerin as well as prelamin A when overexpressed at high levels (asterisk). The prelamin A antibody (C-20) detects only prelamin A. Cell lysates are as follows: Ad/UC, VSMCs expressing uncleavable form of prelamin A; Ad/Pr, fibroblasts expressing progerin; P6/P17, early- and late-passage VSMCs; C, control fibroblasts; HGPS fibroblasts. D, Confocal microscopy showing the absence of prelamin A in VSMCs from young donors and at early passages and its accumulation at the NE and in the cytoplasm (arrows) in aged VSMCs. Lamin A/C was detectable in all nuclei (data not shown). E, Significant increase in prelamin A–positive nuclei in aged VSMC cultures. Mean±SD values are shown for n=300 cells counted in triplicate, with 3 independent VSMC isolates per group. Unpaired t tests were used. F, Top panel shows proliferating VSMCs that are prelamin A negative (arrows). Bottom panel shows prelamin A–positive VSMCs with a highly convoluted nucleus (arrow). G, Percentage of proliferating VSMCs that are prelamin A positive (54-year-old man, passage 8).
Prelamin A Accumulation Is Triggered by Downregulation of FACE1 In Vitro and In Vivo

Quantitative reverse transcription polymerase chain reaction and Western blotting for the lamin A processing enzyme FACE1 showed a significant decrease in mRNA and protein levels in late-passage VSMCs, correlating with the accumulation of prelamin A (Figure 3A and 3B). Although more variable, FACE1 expression was also lower in aged donors (Figure 3A).

To determine whether these observations were relevant in vivo, the pattern of prelamin A and FACE1 expression was...
analyzed by immunocytochemistry in aortic and carotid artery samples from patients ranging in age from 14 to 83 years (Table III in the online-only Data Supplement). In the vessel media of young healthy donors, VSMCs contained high levels of FACE1 protein and undetectable levels of prelamin A (Figure 3C). In contrast, arteries from aged individuals showed reduced FACE1 staining and an increased frequency of prelamin A–positive cells in the media (Figure 3C and Table III and Data in the online-only Data Supplement). Prelamin A was also observed in VSMCs of the adventitial vasa vasorum in aged individuals but was never observed in adventitial fibroblasts or endothelial cells (Figure 3C). In advanced atherosclerotic lesions, the expression of FACE1 and prelamin A was heterogeneous. Some intimal patches of VSMCs were strongly FACE1 positive and prelamin A negative, whereas in other areas VSMCs were negative for FACE1 and showed prelamin A accumulation (Figure 3D). VSMCs with the highest levels of prelamin A were isolated and embedded within the matrix. Many of these cells showed nuclear fragmentation and aberrant nuclear morphologies or were anuclear and contained remnants of vesiculated/degenerate VSMCs within a thick matrix cage composed of proteoglycans as shown by periodic acid–Schiff stain (Figure 3E).

The relationship between prelamin A accumulation and VSMC senescence was determined with the use of costaining with SAβG. In arteries from young patients with no evidence of senescence, prelamin A was absent. In atherosclerotic lesions from older patients, senescent VSMCs were detected and were positive for prelamin A. However, there was a much larger subset of VSMCs that were positive for prelamin A but negative for SAβG (Figure 3F).

FACE1 Is Downregulated in Response to Oxidative Stress

Oxidative stress is a major factor implicated in VSMC aging and senescence; therefore, we tested whether it may be causal in reducing FACE1 levels. VSMCs exposed to either serum starvation or H2O2 treatment showed reduced FACE1 mRNA expression (Figure 4A and 4B). Short-term treatment with high-dose H2O2 also decreased FACE1 protein levels and upregulated p21, indicative of cell cycle arrest (Figure 4C). Longer-term treatment with low-dose H2O2 decreased FACE1 protein and increased prelamin A, and this was associated with activation of the cell stress marker phosphorylated p38 as well as an increase in p16 (Figure 4D), suggesting that reduced FACE1 and prelamin A accumulation may contribute to stress-induced premature senescence.

To investigate further the relationship between oxidative stress and prelamin A, vessel rings harvested from healthy children were exposed to H2O2 ex vivo. Nuclear staining for the oxidative damage marker 8-oxo-dG was detectable after exposure to H2O2. In addition, prelamin A accumulation was induced in >90% of medial VSMCs, and there was also evidence of reduced FACE1 staining (Figure 4E).

Prelamin A Accelerates Senescence and Disrupts Mitosis

Next, early-passage VSMCs were treated with siRNAs to FACE1, and Western blotting confirmed prelamin A accumulation (Figure 5A). After 3 days, nuclear morphology defects were induced in VSMCs but not in human umbilical vein endothelial cells or fibroblasts (Figure 5A to 5C and Figure V in the online-only Data Supplement). However, at this time point, SAβG staining showed that VSMC senescence was not induced (data not shown). However, longer-term adenoviral overexpression of prelamin A for 7 days induced a significant increase in SAβG staining (Figure 5D). This effect was dose dependent and suggested that prelamin A can act to accelerate VSMC senescence (Figure 5E).

Immunofluorescence analysis of the cultures 3 days after FACE1 siRNA or adenoviral transduction showed that prelamin A had profound effects on mitotic VSMCs that were strongly positive for prelamin A both in the cytoplasm and at the NE (Figure 5F). Mitotic cells displayed abnormalities including asymmetrical nuclear division, resulting in daughter nuclei with abnormal DNA contents, anaphase bridge formation, severe nuclear fragmentation, and giant polyploid nuclei (Figure 5F through 5I).

Prelamin A Accumulation Induces DNA Damage

Aged VSMCs with high levels of prelamin A also showed increased activation of DNA damage signaling compared with their early-passage counterparts (Figure 6A). To investigate a possible causal relationship, early-passage VSMCs were again exposed to FACE1 siRNA. The induction of prelamin A rapidly increased DNA damage signaling, shown by pATM/ATR foci in interphase nuclei and increased γ-H2AX levels (Figure 6A and 6B). Detection of increased DNA fragmentation on comet assay confirmed that DNA damage signaling was activated in response to actual DNA strand breakage (Figure 6C). The frequency of mitotic VSMCs with DNA damage was greatly increased (Figure 6D and 6E), and these cells displayed mitotic abnormalities.

Discussion

This study highlights a major role for nuclear lamina dysfunction in vascular aging and disease. We show that a hallmark of VSMC aging, in vitro and in vivo, is the spontaneous and rapid accumulation of prelamin A, which is due, at least in part, to stress-induced downregulation of the processing enzyme FACE1. Our data suggest that prelamin A–induced DNA damage and mitotic dysfunction act in concert to accelerate VSMC senescence. In vivo, these events are likely to severely limit VSMC reparative capacity, leading to plaque instability and rupture.

Normal VSMCs Reiterate the Aging Phenotype Shown by HGPS Fibroblasts In Vitro

This study has shown that the lifespan and growth characteristics of normal human VSMCs in vitro resemble those of HGPS fibroblasts, which exhibit a short replicative lifespan compared with their normal counterparts. In HGPS fibroblasts, premature senescence is due to the toxic accumulation of progerin, a mutant form of prelamin A.⑧ In VSMCs, these same growth characteristics were associated with the rapid...
and spontaneous accumulation of prelamin A as the cells aged in vitro. These observations may explain why VSMCs are particularly susceptible to the effects of progerin accumulation in both HGPS patients and animal models of the disease. Accumulating evidence suggests that mesenchymal stem cells are preferentially affected in HGPS, and progerin has been shown to enhance the osteogenic differentiation of mesenchymal stem cells. VSMCs show mesenchymal stem cell–like properties and can undergo both osteogenic and adipocytic differentiation, leading to calcification and lipid accumulation, pathologies also prevalent in the vasculature of children with HGPS and in transgenic mice overexpressing progerin. We found that prelamin A accumulation was greatest in aged, calcified arteries, suggesting that it may promote osteogenic differentiation of VSMCs. Indeed, the transcription factors that mediate osteogenic and adipocytic differentiation of VSMCs, Runx2 and SREBP1, both associate with the nuclear lamina, and therefore it will be important to investigate whether prelamin A can induce aberrant activation of these factors in VSMCs.

Prelamin A May Act to Accelerate VSMC Senescence by Inducing Mitotic Defects and DNA Damage
Prelamin A accumulated in VSMCs before senescence, and its overexpression accelerated senescence, suggesting that it may be causal in the induction of VSMC senescence rather than a consequence of it. There are at least 2 potential
mechanisms whereby prelamin A may act to accelerate VSMC senescence. First, overexpression of progerin in HeLa cells has been shown to induce its cytoplasmic aggregation and to disrupt mitosis, leading to binucleate cells and chromosomal aberrations.\textsuperscript{30,31} Similarly, we observed aberrant prelamin A accumulation in the cytoplasm during mitosis in normal fibroblasts or endothelial cells (human umbilical vein endothelial cells [HUVEC]). Mean±SD values are shown for n=500 cells counted in triplicate. Results are representative of 3 independent VSMC isolates. Paired t test was used. D, Adenoviral transfer of prelamin A (UC) (7 days) induced VSMC senescence shown by SA\textsuperscript{\textregistered}G staining in a dose-dependent manner. Prelamin A expression at different multiplicity of infection is shown by Western blotting with the use of the prelamin A antibody and a FLAG antibody to indicate levels of expression and transduction. Control was infected with Ad/EGFP at multiplicity of infection of 20. E, n=150 cells counted and compared with the use of the Kruskal-Wallis test. Results are representative of 3 independent experiments. F, Confocal images demonstrating the association of prelamin A with mitotic defects in VSMCs after FACE1 siRNA. i, Late-passage telophase cell showing unequal DNA partitioning in daughter nuclei (DAPI) and persistent prelamin A associated with the cytoplasm. ii, Severely fragmented prelamin A–positive nuclei indicative of mitotic failure. G, FACE1 siRNA induced a significant increase in the frequency of prelamin A–positive fragmented mitotic nuclei and in the number of aberrant mitotic VSMCs. Mean±SD values for n=100 mitotic cells and n=300 interphase cells from experiments performed in triplicate are shown. Unpaired t tests were used (control vs prelamin A). H and I, Three days after prelamin A adenoviral expression, there was a significant increase in nuclear size indicative of an increase in DNA content. Arrows indicate giant prelamin A–positive nuclei. Arrowhead indicates normal nucleus. n=80 cells counted for each group compared by Kruskal-Wallis test. Results are representative of 3 independent experiments.
In the context of vascular disease, it could be envisaged that by interfering with DNA damage repair and mitosis, prelamin A would accelerate senescence in atherosclerotic plaques. Moreover, the association of prelamin A in vitro and in vivo with nuclear fragmentation and VSMC loss/degeneration suggests that it may also contribute to the elimination of proliferating VSMCs during mitosis. Both VSMCs and HGPS fibroblasts undergo an abrupt loss of the proliferative cell fraction immediately before the onset of senescence, correlating with an increased frequency of cells with severely fragmented nuclei. Nuclear fragmentation is a hallmark of mitotic catastrophe, a form of cell death induced when cells carrying severe DNA damage enter mitosis. Such a mechanism of VSMC death is consistent with the almost complete absence of proliferation and the marked VSMC attrition observed in advanced atherosclerotic plaques. The deregulation of mitosis by prelamin A is also consistent with the well-documented increases in indicators of genomic instability, such as polyploidy and chromosomal aberrations, that occur in atherosclerosis and in animal models of vascular aging.

**FACE1 Is Downregulated by Oxidative Stress**

A crucial event in the accumulation of prelamin A was the downregulation of FACE1. Its sensitivity to oxidative stress, as well as the correlation of its loss with oxidative DNA damage, suggests that it may be a key factor in age-related vascular decline. Oxidative damage is detectable in >90% of cells in atherosclerotic plaques. Therefore, it could be envisaged that a vicious cycle of stress-induced FACE1 downregulation and prelamin A accumulation, leading to a potentiation of DNA damage, mitotic catastrophe, premature senescence, and aberrant differentiation, could be occurring in the vessel wall to accelerate VSMC aging and induce age-associated pathologies. FACE1 is clearly a novel candidate for genetic study because decreased levels may be associated with increased cardiovascular risk. The identification of novel targets of FACE1 will also be important because some of the effects attributed to prelamin A may be due to the accumulation of an as yet unidentified substrate.

**A Role for Statins in Treating Age-Induced Vascular Dysfunction**

The discovery that VSMC aging is accelerated by the toxic accumulation of prelamin A implies that therapeutic interventions may be designed to reduce age-associated vascular decline. Animal studies have shown that both FTIs and combined therapy with statins and bisphosphonates can ameliorate multiple premature aging phenotypes in progerin.
transgenics and FACE1−/− mice.39–43 In this study, statins, which act upstream of FTIs, reduced prelamin A–induced nuclear morphology defects in VSMCs, implying that they may potentially prevent age-associated VSMC dysfunction. Currently, FTIs are being tested in HGPS patients in a clinical trial that will shed further light on the therapeutic potential of these drugs in the treatment of vascular aging.

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Disclosures
None.

References
CLINICAL PERSPECTIVE

Age is the strongest risk factor for the development of vascular decline and associated mortality. Therefore, understanding the mechanisms of cell-specific forms of aging may lead to the development of novel targets for therapeutic intervention. Children with the genetic condition Hutchinson-Gilford progeria syndrome develop tissue-specific symptoms of premature aging and die of myocardial infarction within the second decade. This is due to rapid vascular decline caused by the severe loss and dysfunction of vascular smooth muscle cells (VSMCs). Hutchinson-Gilford progeria syndrome is caused by the toxic accumulation of a mutant form of the nuclear lamina protein lamin A. This mutant protein, called progerin, acts to deregulate mitosis and DNA damage signaling, leading to premature cell death and senescence. In this study, we show that VSMC aging and disease in the normal population are associated with the abnormal accumulation of prelamin A, which, like progerin, is an unprocessed form of lamin A. In normal aging, the accumulation of prelamin A is caused in part by the downregulation of the processing enzyme FACE1 by oxidative stress. Prelamin A accumulation accelerates VSMC DNA damage and senescence and may also impinge on VSMC phenotype to promote age-associated vascular pathologies such as calcification. Importantly, some of the toxic effects of prelamin A are thought to be due to permanent farnesylation, suggesting that drugs that interfere with this pathway, such as statins, may be useful for the treatment of age-related vascular dysfunction.
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Expanded Materials and Methods
Supplementary Material

Materials and Methods

Cell culture

Human aorta from transplant donors, carotid endarterectomy and renal arteries were obtained with appropriate ethical approval. The age, sex and disease state of the donor was noted. VSMCs were cultured by explant from areas of the media devoid of atherosclerosis as previously described\textsuperscript{1}. Normal and HGPS human dermal fibroblasts were obtained from the NIA Aging Cell Repository, Coriell Institute, (Supplementary Tables 1-2).

For proliferation studies cells were seeded at a known density and counts were performed on each passage until senescence. Cells that were deemed senescent had ceased proliferation in culture (assessed by cell counting and Ki67 staining) for at least 2 passages or 10 days. Senescence was confirmed using senescence associated beta-galactosidase (SA–\(\beta\)-gal) activity in cells and tissues which was detected as previously described with minor modifications\textsuperscript{2}. Cells were fixed at 37\(^\circ\)C for 10 minutes with 4\% paraformaldehyde and incubated overnight at 37\(^\circ\)C in the fresh SA–\(\beta\)-gal solution.

Cells were treated for 48 hours with the farnesyl transferase inhibitor (FTI), \(\alpha\)-Hydroxyfarnesylphosphonic acid (H-9279, Sigma) at a dose of 2.5 \(\mu\text{M}\) or Atorvastatin at a dose of 0.5\(\mu\text{M}\). Control cells were incubated with the appropriate vehicle, ethanol or DMSO. The most effective dose of FTI and statin were determined in dose response studies (data not shown). The predicted increase in prelamin A protein induced by both drugs was assessed by Western Blot (Supplementary Fig. 1).
Oxidative stress was induced in VSMCs and arteries by incubation in the presence or absence of the indicated H$_2$O$_2$ concentration for 24-72 hours.

**RT-PCR and qPCR**

RNA was isolated by lysis in STAT-60 (CS-110, AMS Biotechnology) and cDNA was generated from 1µg of RNA. To assess the level of expression of progerin, lamin A and FACE1 in VSMCs, lamin A/C, Prelamin A and FACE-1 specific primers (Sigma Genosys) were used for amplification with a high fidelity GC-rich PCR kit (2 140 306, Roche). GAPDH was used as an internal control (Supplementary Fig. 3).

Human FACE-1 (Zmpste24) primers and probes were purchased from Applied Biosystems (Hs00195298_m1), the 18S (4333760F, Applied Biosystems) gene was coamplified with FACE-1 as an internal standard. The PCRs were performed in 20µl reaction volumes with x10 PCR buffer, 25mM MgCl2, 25mM dNTPs, 1µl Taqman primers and probe and 0.5U AmpliTaq Gold (4311816, Applied Biosystems). Amplification was performed at 95°C for 10 minutes followed by 40 cycles of 95°C for 5 seconds and 60°C for 1 minute. The fluorescence intensity of the PCR products was measured using the Rotor Gene RG-3000 from Corbett. All samples were tested in triplicate and the value of the cycle threshold (CT) was determined by the RG-3000 software.

**Antibodies**

Antibodies used in Western Blots, FACs analysis and IF were; Prelamin A (SC-6214, C-20), Lamin A/C (SC-6215, N-18), FACE1 (SC-34777) (Santa Cruz); p21 (#2946), p16 (4824), phosphor-p38 (9215), γH2AX (#2577), phosphor-ATM
substrate (#2851), p53BP1 (#4937) (Cell Signaling Technology); 8-oxo-dG (Japan Institute for Control of Ageing). For WB, horseradish peroxidase-conjugated anti-mouse (NA931) or anti-rabbit (NA94V) antibodies from GE Healthcare and anti-goat (A9452) from Sigma were used. The ECL+Plus chemiluminescent kit (RPN2132, GE Healthcare) was used for signal detection according to manufacturer’s instructions. Invitrogen anti-mouse Alexa fluor 568 (A11031), anti-goat Alexa Fluor 568 (A11057) and anti-rabbit Alexa fluor 488 (A11034) were used as IF secondary antibodies. To verify the specificity of the anti-Prelamin A antibodies FLAG-tagged fusion constructs with wild type Lamin A, uncleaved Lamin A (Prelamin A), and progerin were expressed in vitro. Forty-eight hours after transfection, cells were fixed and stained with both anti-FLAG (A2220, Sigma) and anti-prelamin A antibodies (Supplementary Fig. 1-2).

**Immunofluorescence and Immunocytochemistry**

VSMCs were cultured on coverslips and were fixed in 50% methanol/acetone and processed as described previously. Cell counts for statistical analysis were performed on >500 cells in >3 separate experiments.

Human tissue samples are dissected and placed into 10% neutral buffered formalin and then embedded in paraffin wax. Sections are deparaffinised to water and then boiled in a microwave in Vector Antigen Retrieval Solution for 10 minutes. After cooling for 30 minutes sections are washed twice in ddH2O, then twice in PBS for 5 minutes each wash. Slides were then incubated for 10 minutes in 3% H2O2/ddH2O, washed as before and then incubated with 5% Rabbit serum/PBS (Prelamin A), 5% Goat serum/PBS (Lamin A/C) for 1 hour at room temperature. Slides were incubated with the primary antibodies at 4°C overnight (Prelamin A SC-
6214, Santa Cruz, 1/100 in 5% Rabbit serum and Lamin A/C #2032, Cell Signalling Technology, 1/200 in 5% Goat serum). Slides were washed twice in PBS for 5 minutes each wash, incubated with the appropriate secondary antibodies Rabbit anti Goat and Goat anti Rabbit (E0466 and E0432 Dako) at 1/400 for 30 minutes at room temperature and then washed in PBS as before. Slides were then incubated with Vector RTU ABC reagent (PK7100) for 30 minutes at room temperature before being washed again in PBS. The reactions were visualised with DAB solution (SK4100) and monitored closely under a microscope before the slides were washed well in ddH$_2$O counter stained and mounted. Slides were routinely counter-stained with a weak solution of hemotoxylin/eosin to visualize nuclei.

Periodic Acid Schiff (PAS) staining was performed according to manufacturer’ instructions using PAS staining system (Sigma-Aldrich).

Human tissue samples for immunohistochemistry and SA-β-G studies were fixed and processed as previously described$^2$.

**FACS analysis**

The DNA content of cells was measured by propidium iodide staining. Cells were trypsinised and washed 3 times in PBS then fixed in ice cold 90% methanol for 30 minutes at 4°C. DNA staining was achieved by addition of PI and RNase for 45 minutes at 37°C. For γH$_2$AX and annexin V staining cells were processed as per the manufacturers’ instructions (Cell Signaling Technology).

**siRNA mediated interference**

Dharmacon smart pool FACE1 and control siRNA oligos were transfected into VSMCs using HiPerfect transfection reagent, according to the manufacturers
instructions (Qiagen). At either 48 or 72 hours post transfection samples were prepared for western blotting, FACs analysis or IF. Each experiment was repeated 3 times.

**Comet assays**

Comet assays were performed as described\(^4\). Protocols were obtained from the Comet Assay Interest Group website.

**Electron microscopy**

Early and late passage VSMCs were fixed and processed as previously described\(^3\). Fifty nanometre sections were cut on a Leica Ultracut UCT, stained with saturated uranyl acetate in 50% ethanol and lead citrate and viewed in a FEI Philips CM100 operated at 80kv.

**Adenoviral constructs**

Adenoviral vectors (Ad) used in the study were Ad/WT, wild type lamin A; Ad/UC-LA, an uncleavable form of prelamin A mutated within the Zmpste24 cleavage site of prelamin A (L647R), Ad/Pr, progerin, a deleted form of prelamin A. The progerin construct was made from the pEGFP-LA Δ50 vector, which has the in-frame deletion of 50 amino acids (Δ607-656), producing a truncated prelamin A. i.e. progerin, was a kind gift from Dr. Francis S. Collins from NIH. The progerin cDNA was introduced into an adenovirus shuttle vector as a PCR product, using primers 5'ATTGGTACCATGGAGACCCCGTCCCAG3' and 5'TCTGGATCCTTACATGATGCTGCAGTTC3', digested with KpnI and BamHI. The Lamin A adenoviruses all carried a FLAG tag for visualization of transduction.
Ad/EGFP, expressing enhanced green fluorescence protein was used to assess virus infection efficiency and as a control virus. VSMCs at 70~80% confluency, were infected with recombinant adenovirus at a multiplicity of infection (MOI) of 10 to 50 particles/cell as stated, routinely achieving 80% transfection efficiency without cell toxicity.


Supplementary Figure 1: Western blot (WB) showing the specificity of the antibodies used for different lamin species. VSMCs were infected with adenoviruses containing wild type Lamin A (WT), an uncleavable form of lamin A that only expresses prelamin A (UC), Progerin, and a control adenovirus containing EGFP at MOI of 10 and 25, for 48 hours. Control VSMCs were exposed to mock treatment. Cell lysates were probed with Lamin A/C (SC-6215, N-18) and pre-Lamin A (SC-6214, C-20). Two exposures of the WB are shown for the Lamin A/C N-18 antibody that recognises Lamin A, progerin and Lamin C but only weakly prelamin A even at these overexpression levels. In contrast the prelamin A antibody (C-20) is clearly specific for prelamin A and recognises no other species. A clear dose response is also evident. Note that overexpression of WT lamin A also results in low levels of prelamin A probably due to overloading of the lamin A processing pathway. 

β-actin is the loading control.
**Supplementary Figure 2:** Immunofluorescence of U2OS transfected with adenoviral constructs expressing wildtype (WT) and uncleavable Lamin A (UC) i.e.prelamin-A, and progerin demonstrating the specificity of the prelamin A antibody (SC-6214, C-20). Transfected cells are identified by an anti-FLAG antibody. Even when expression of exogenous lamin A and progerin is high the prelamin A antibody only identifies cells expressing prelamin A. Conversely the Lamin A/C antibody (SC-6215, N-18) recognises all cells.
Supplementary Figure 3. WB showing treatment of VSMCs with either FTI or atorvastatin increased prelamin-A protein levels as previously described\(^1\) indicating that both were influencing prelamin-A processing. Farnesylation of prelamin-A is essential for its incorporation into the NE and subsequent cleavage into mature Lamin-A. Inhibition of farnesyltransferase, a pivotal component of the farnsylation pathway, blocks this step in the prelamin-A processing pathway, preventing its cleavage and results in increased non farnesylated prelamin-A protein levels.

**Supplementary Figure 4.** RT-PCR using primers to detect usage of the cryptic splice site activated by HGPS patient mutations. Note the obvious extra band present in cDNA derived from HGPS patient fibroblasts and its absence in human VSMCs from early and late passage cultures and old and young donors. This band was also absent in cDNA derived from atherosclerotic plaques from both young and old donors. This data supports the absence of progerin protein in VSMCs. Age and sex of donors indicated.
Supplementary Figure 5. Prelamin-A accumulation does not induce nuclear morphology defects in endothelial cells. Confocal IF microscopy was performed on HUVECs transfected with either control or FACE1 specific siRNAs. DAPI (blue) and Prelamin-A (red). This data shows that prelamin-A accumulation does not induce nuclear morphology defects in endothelial cells (HUVEC).
Supplementary Figure 6: VSMCs transfected with WT-lamin A (WT-LA) or prelamin A (UC-LA) showing that prelamin A induces defects in the nuclear lamina. In addition to nuclear morphology defects (not shown) VSMCs expressing lower levels of prelamin A showed mislocalization of the inner nuclear membrane protein emerin to nuclear aggregates and nuclear enlargement (arrowed in inset).
**Supplementary Table 1:** Fibroblasts used in this study.

<table>
<thead>
<tr>
<th>Coriell Code</th>
<th>Age/Sex</th>
<th>Passage No.</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
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<td>34F</td>
<td>P31</td>
<td>normal (mother of 06917)</td>
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<tr>
<td>AG11498</td>
<td>14M</td>
<td>P10</td>
<td>HGPS (G608G)</td>
</tr>
<tr>
<td>AG01972</td>
<td>14F</td>
<td>P16</td>
<td>HGPS (G608G)</td>
</tr>
<tr>
<td>AG06917</td>
<td>4M</td>
<td>P14</td>
<td>HGPS (G608G)</td>
</tr>
</tbody>
</table>

HGPS- Hutchinsom Gilford Progeria Syndrome, G608G indicates mutation. Passage No. indicates stage in tissue culture when used for Western Blot and cell counting.
Supplementary Table 2. VSMC isolates used in *in vitro* studies

<table>
<thead>
<tr>
<th>Sample</th>
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<th>Passage at senescence</th>
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<tr>
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<td>P14</td>
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<tr>
<td>78F</td>
<td>V</td>
<td>P4</td>
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</table>

Samples are indicated by age and sex of donor for VSMCs. The stage of atherosclerosis of the vessel (aorta) from which the VSMCs were isolated is indicated in column 2 using *AHA* lesion classification codes. The passage number at which each culture reached senescence after identical passaging conditions is shown in column 3.
**Supplementary Table 3**: Summary of human vessel IHC for Prelamin A and FACE1.

<table>
<thead>
<tr>
<th>Age/Sex/Vessel</th>
<th>Stage</th>
<th>Prelamin A</th>
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<td></td>
<td></td>
<td>M</td>
<td>In</td>
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<tr>
<td>14F.4A</td>
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<td>-</td>
<td>^</td>
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<td>VI</td>
<td>+++&lt;sup&gt;Ca&lt;/sup&gt;</td>
<td>++</td>
</tr>
</tbody>
</table>

First number indicates age in years, second number is sample identification; F, female; M, male; A, aorta; C, carotid. Stage indicates AHA lesion classification; M, media; In, intima. -, no positive VSMCs; +, <10% VSMCs positive, ++ < 50% VSMCs positive, +++; <80% VSMCs positive, ++++, 100% VSMCs positive. * not determined; ^intima not present. <sup>Ca</sup> indicates media was von Kossa positive at sites of prelamin A accumulation.
Prelamin A expression increases with age and disease: The expression of prelamin A in the arteries shown on Table 3 was converted to numerical values from 0-3 for each + indicated. The levels of expression in the intima were compared with the stage of atherosclerosis (A) and a significant increase in expression was observed with advanced disease. The level of expression in the media was analysed with respect to age and there was a significant increase in prelamin A expression with age. (B). Mean +/-SD, Mann-Whitney Test.
Data Analysis of Table 3: Prelamin A and FACE1 expression:

Using Fisher’s exact t-test it can be shown that expression of prelamin A and FACE1 correlate. The higher the expression of FACE1 the lower the expression of Prelamin A in human vessels.