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

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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.[1] Normally, prelamin A is posttranslationally modified by farnesylation to facilitate nuclear envelope (NE) targeting and is subsequently cleaved by the metalloproteinase FACE1/Zmpste24 to remove the farnesyl groups and produce mature lamin A, which can then insert into the nuclear lamina.[2] 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.[3,4] More rarely, HGPS is caused by mutations in *Zmpste24*, leading to the accumulation of permanently farnesylated prelamin A.[5,6] In vitro, progerin and prelamin A accumulation in fibroblasts causes nuclear morphology defects, and the cells undergo premature

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senescence associated with changes in gene expression, heterochromatin organization, and failure to effect DNA repair.[7–10]

Clinical Perspective on p 2210

An important feature of HGPS is that aging symptoms appear to be tissue specific.[11–13] 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.[14,15] 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.[4,11,12] Large arteries and small arterioles show characteristic VSMC changes including calcification, lipid accumulation, fibrosis, and VSMC attrition.[16,17] 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.[18,19] 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.[9] Accumulation of progerin in a very small fraction of skin fibroblasts in aged individuals has also been demonstrated.[20] 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.[19] Human dermal fibroblasts were obtained from NIA Aging Cell Repository, Coriell Institute (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.[21] Cells were treated for 48 hours with the farnesyl transferase inhibitor (FTI) α-hydroxyfarnesylphosphonic 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 H_2O_2 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) were coamplified in triplicate samples, and the fluorescence intensity of the polymerase chain reaction products was measured with the use of the Rotor 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-H_2AX (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.[21] 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-H_2AX 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.[22]

Electron Microscopy

VSMCs were fixed in 2% gluteraldehyde, 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 progerin (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

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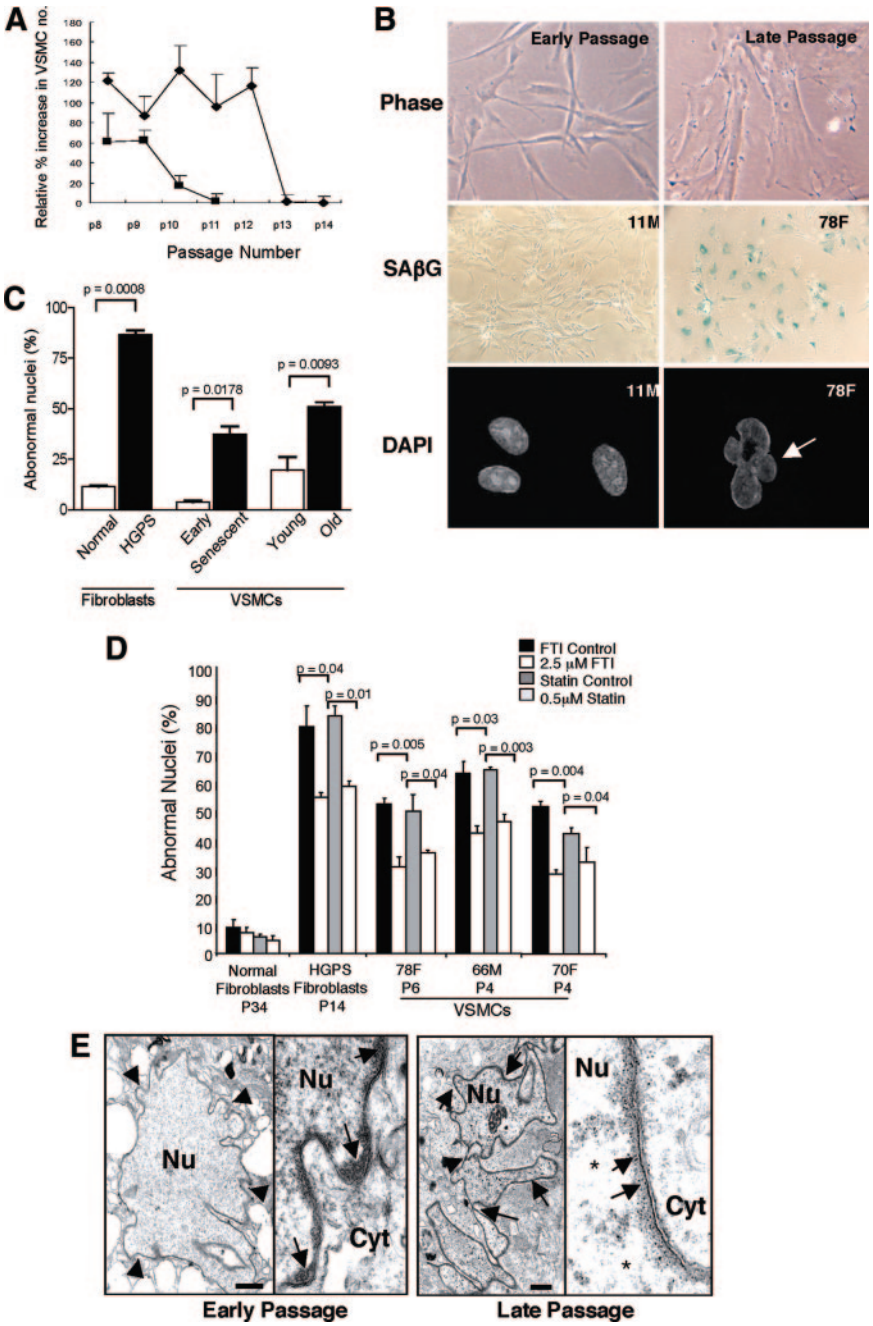

Figure 1. Aged VSMCs exhibit nuclear morphology defects. A, Proliferation of 2 VSMC isolates in vitro in a 54-year-old man (♦) and a 77-year-old woman (■). B, Nuclear morphology defects (arrow) and senescent cells (shown by SAβG staining and typical flattened appearance) were increased in aged VSMCs. 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.

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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 passaging, 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 A[23,24] (Figure 1D and Figure III in the online-only Data Supplement). This notion was supported by transmission electron microscopy ultrastructural analysis, which showed that VSMCs aged in vitro had severely convoluted and torturous NEs, thickening of the nuclear lamina, and loss of nuclear and NE-associated heterochromatin, features consistent with nuclear lamina defects (Figure 1E).

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

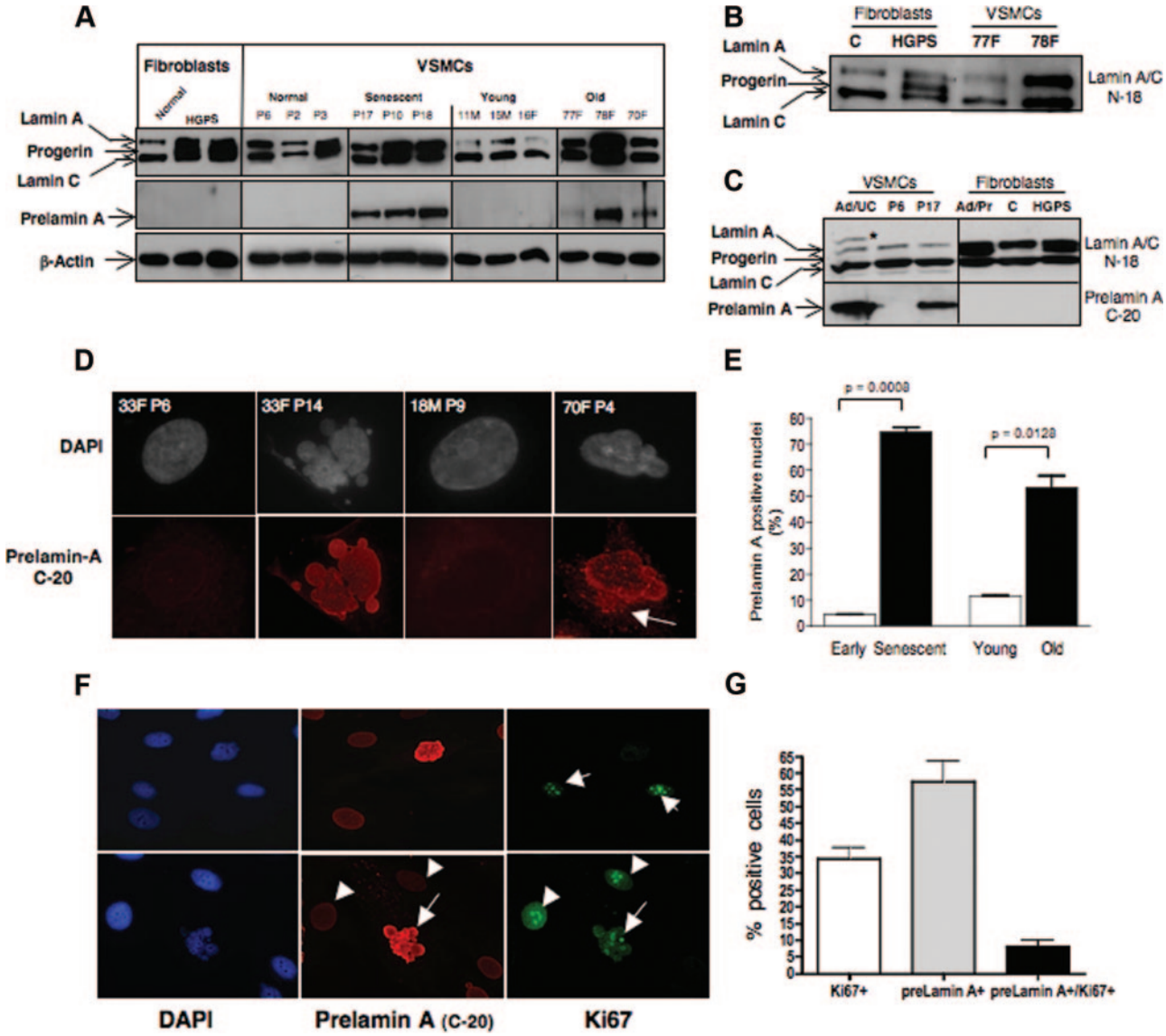

Figure 2. Aged VSMCs accumulate prelamin A. A, Western blotting using antibodies to lamin A/C (top, SC-6215, N-18) and prelamin A (middle, SC-6214, C-20) showing prelamin A accumulation in aged and senescent VSMCs. Normal and senescent VSMCs are paired (ie, from the same isolate). Abbreviations are as 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 that are also Ki67 positive (arrowheads) including a prelamin A–positive VSMC with a highly convoluted nucleus (arrow). G, Percentage of proliferating VSMCs that are prelamin A positive (54-year-old man, passage 8).

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 2D). Costaining with the proliferation marker Ki67 revealed that in presenescent cultures, ≈20% of proliferating VSMCs were also prelamin A positive, including VSMCs with highly convoluted nuclei (Figure 2F and 2G). This suggests that prelamin A accumulates before the onset of senescence and not as a consequence of it.

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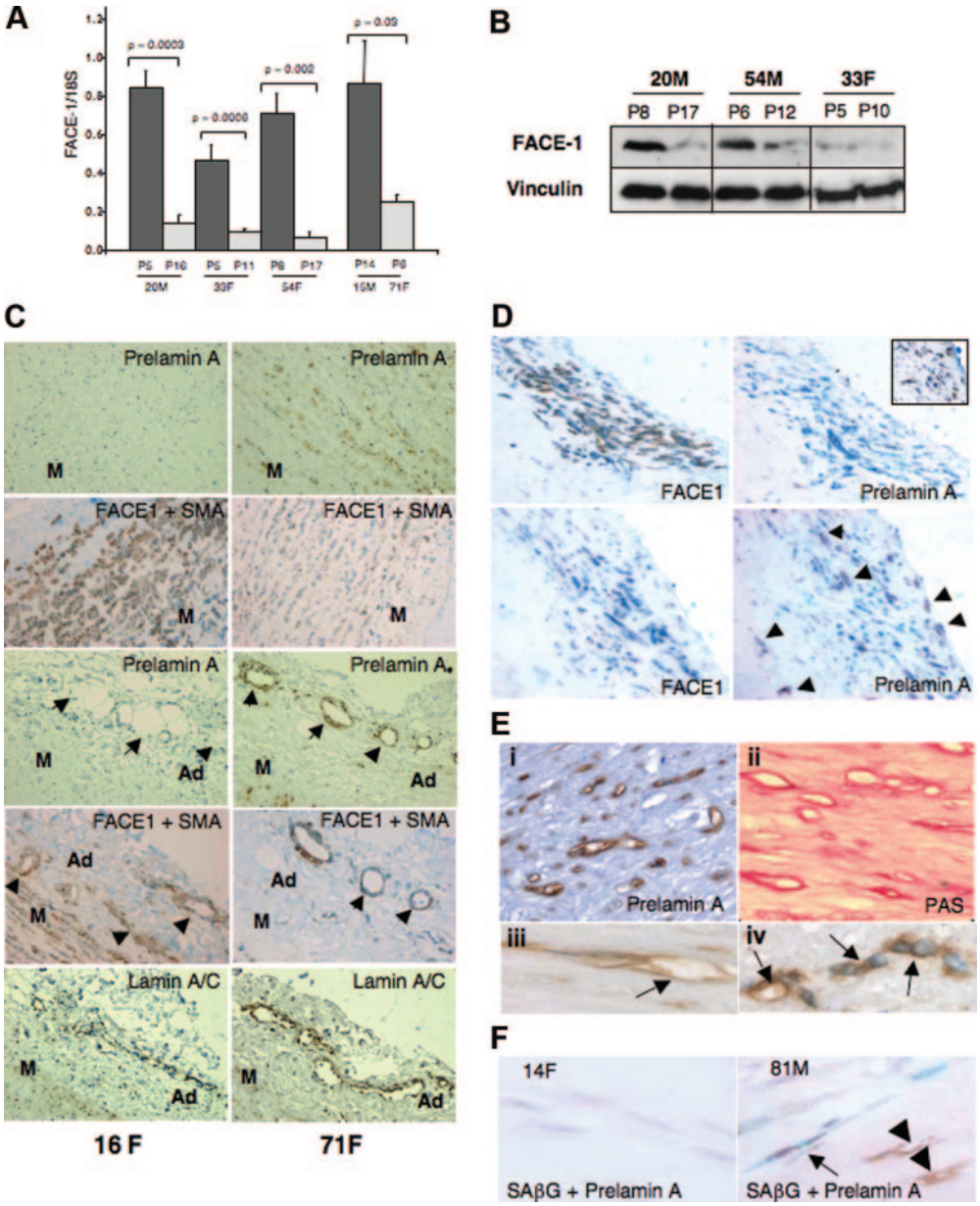

Figure 3. FACE1 downregulation correlates with prelamin A accumulation. A, Quantitative reverse transcription polymerase chain reaction showed a significant downregulation of FACE1 expression in late-passage and aged VSMCs. Each experiment was performed in triplicate, and results for 3 independent VSMC isolates are shown. Mean±SD values are shown. Unpaired *t* tests were used (early vs late passage). Abbreviations are as defined in Figure 1 legend. B, Western blotting showing consistent decrease in FACE1 protein levels in late-passage VSMCs from 3 different isolates. C, Prelamin A staining (brown) was absent in medial VSMCs from a young donor (16-year-old female subject) but deposited in the aortic media and VSMCs of the adventitial vasa vasorum of an aged donor (71-year-old woman). Lamin A/C (control) was present in all VSMCs. Costaining with α-smooth muscle actin (SMA) (blue) and FACE1 (brown) in adjacent sections showed that VSMC FACE1 levels were high in the young donor but decreased in the old donor coincident with prelamin A accumulation. M indicates media; Ad, adventitia. D, Adjacent sections of 2 regions of intima from an atherosclerotic plaque (84-year-old man). Top panels show that VSMCs with high FACE1 expression rarely showed prelamin A accumulation. Box shows that VSMCs are all positive for lamin A/C in the same region. Bottom panels show a different region where low FACE1 in VSMCs was coincident with prelamin A staining (arrow heads) in the adjacent section. E, High levels of prelamin A (i) were found in VSMCs surrounded by periodic acid–Schiff (PAS)–positive matrix (ii). These VSMCs often exhibited absent (iii) or dysmorphic/fragmented nuclei (iv) (arrows). In many of these VSMCs, prelamin A was observed in both the cytoplasm and the nucleus (arrows). Hematoxylin-eosin counterstain was used to show nuclei. F, Some prelamin A–positive VSMCs colocalized with SAβG-positive VSMCs in arteries from old individuals. Arrow shows a senescent (blue) VSMC that is prelamin A positive; arrowheads show prelamin A–positive cells that are not senescent. Prelamin A antibody, C-20; lamin A/C antibody, N-18.

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

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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 H_2O_2 treatment showed reduced FACE1 mRNA expression (Figure 4A and 4B). Short-term treatment with high-dose H_2O_2 also decreased FACE1 protein levels and upregulated p21, indicative of cell cycle arrest (Figure 4C). Longer-term treatment with low-dose H_2O_2 decreased FACE1 protein and increased prelamin A, and this was associated with activation of the cell stress marker phospho-p38 as well 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 H_2O_2 ex vivo. Nuclear staining for the oxidative damage marker 8-oxo-dG was detectable after exposure to H_2O_2. 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

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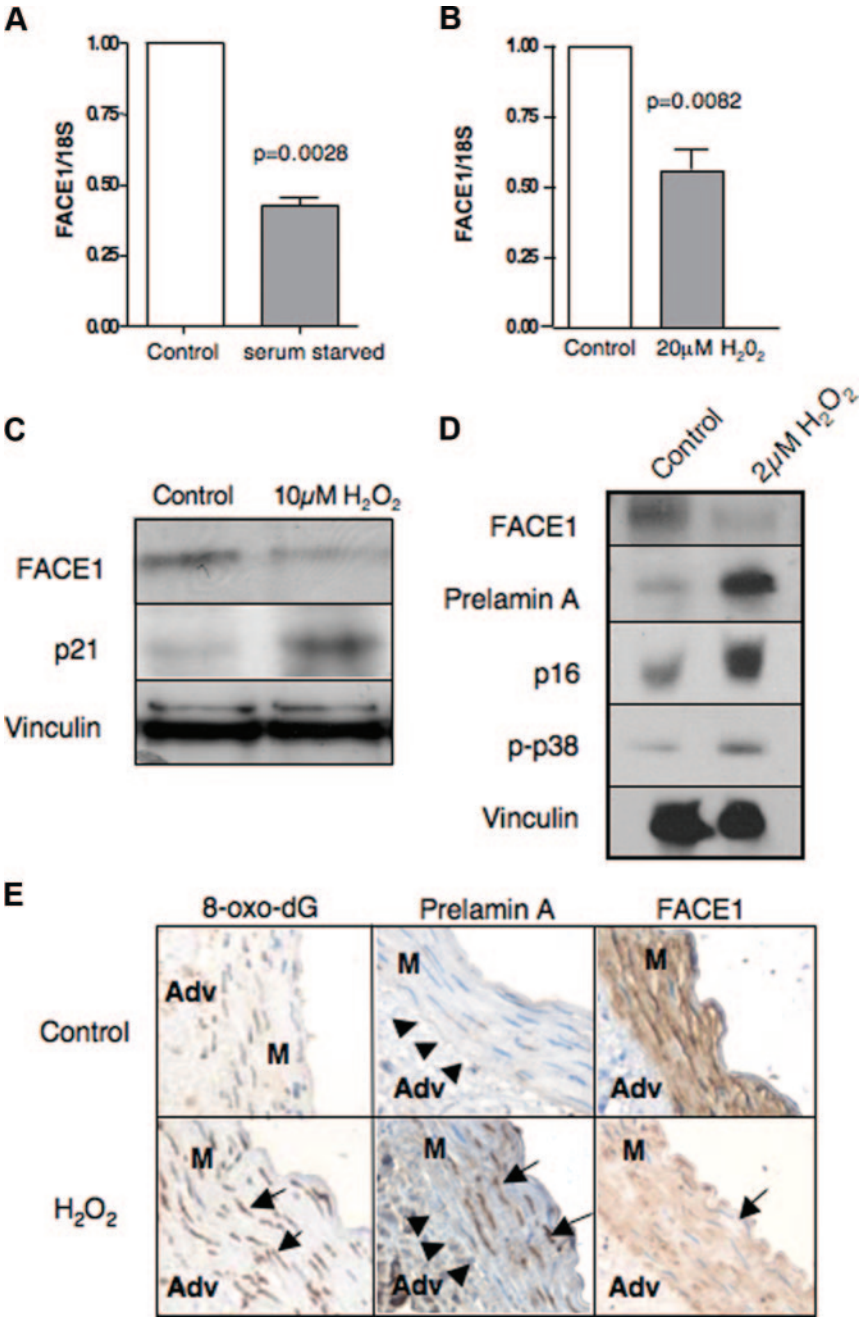

Figure 4. Oxidative stress downregulates FACE1 expression. Quantitative reverse transcription polymerase chain reaction showed decreased FACE1 mRNA levels in VSMCs serum starved for 24 hours (A) or treated with H_2O_2 (B). Data are mean±SD for experiments performed in triplicate on 3 independent VSMC isolates. Paired *t* test was used (control vs treated). C, Western blotting of VSMCs treated for 24 hours with 10 μmol/L H_2O_2 showing reduced FACE1 and increased p21. D, Treatment for 3 days with 2 μmol/L H_2O_2 decreased FACE1 and increased prelamin A, p16, and phospho-p38 in VSMCs. Vinculin loading control was used. E, Adjacent sections of renal artery from a normal 7-year-old female subject showing minimal staining for prelamin A, high levels of FACE1 protein, and no evidence of oxidative DNA damage by 8-oxo-dG staining. Treatment for 24 hours with 4 μmol/L H_2O_2 induced prelamin A accumulation (arrows) in most medial VSMCs, and FACE1 protein levels were diminished. Elevated oxidative DNA damage is shown by 8-oxo-dG staining in most VSMCs. M indicates media; Adv, adventitia. Boundary is shown by arrowheads.

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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.[14,15] 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.[13] VSMCs show mesenchymal stem cell–like properties and can undergo both osteogenic and adipocytic differentiation,[25,26] leading to calcification and lipid accumulation, pathologies also prevalent in the vasculature of children with HGPS and in transgenic mice overexpressing progerin.[15,16] We found that prelamin A accumulation was greatest in aged, calcified arteries, suggesting that it may promote osteogenic differentiation of VSMCs.[13] 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.[18,27–29]

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

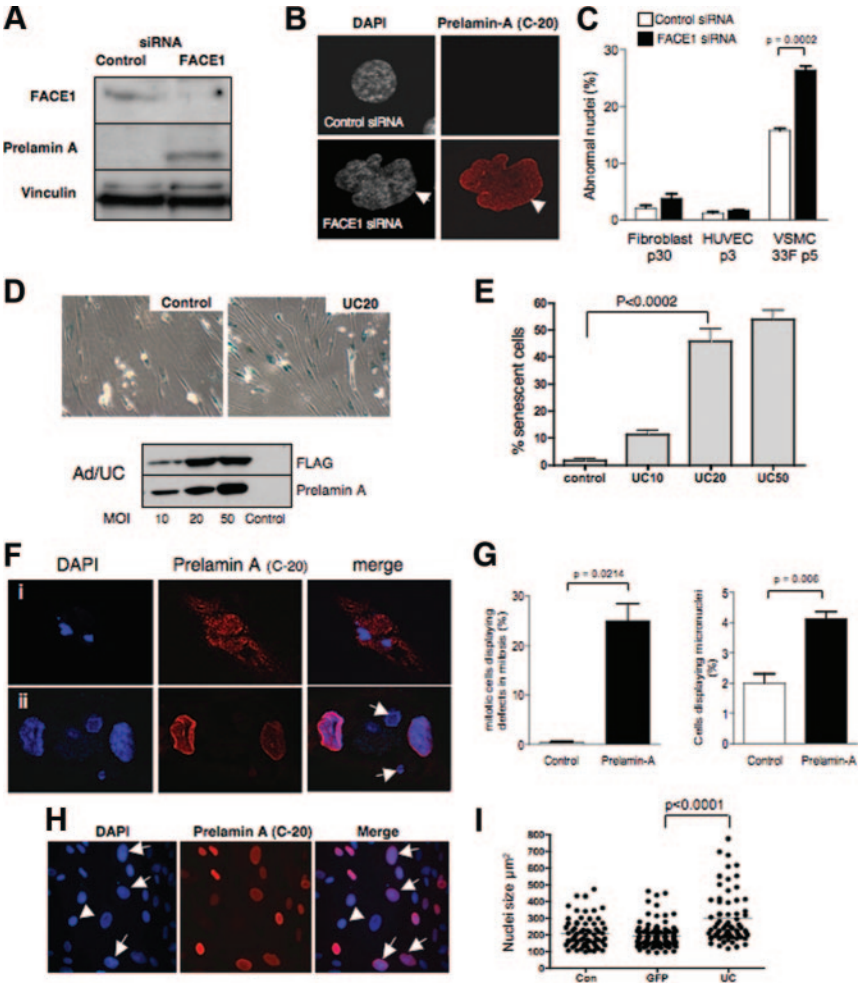

Figure 5. Prelamin A induces senescence and mitotic defects. A, Western blotting showing decreased FACE1 and increased prelamin A expression 72 hours after FACE1 siRNA treatment of early-passage VSMCs compared with control siRNA. B and C, Confocal immunofluorescence showing increased prelamin A accumulation and nuclear morphology defects after FACE1 depletion in VSMCs. FACE1 siRNA had no effect on nuclear morphology 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 SAβG 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.

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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.[30,31] Similarly, we observed aberrant prelamin A accumulation in the cytoplasm during mitosis in late-passage and FACE1-depleted VSMCs, and this was associated with uneven partitioning of DNA into daughter nuclei and giant nuclei. Second, we found that prelamin A induced DNA damage in VSMCs, and we also observed an increased frequency of VSMCs with DNA damage entering mitosis. Entry into mitosis before the completion of DNA damage repair is indicative of mitotic checkpoint failure, and this may also have been causal in the induction of mitotic defects and genomic instability. Importantly, our observations in VSMCs are consistent with previous studies in fibroblasts and transformed cells, showing that both progerin and prelamin A are dominant-negative toxins that disrupt nuclear lamina integrity, deregulating DNA damage signaling and mitosis.[32] Delayed recruitment of 53BP1 to sites of DNA damage has been demonstrated in FACE1$^{-/-}$ fibroblasts, leading to delayed DNA damage repair, accelerated DNA damage, and G2/M mitotic checkpoint failure and resultant aberrant mitosis and genomic instability.[10]

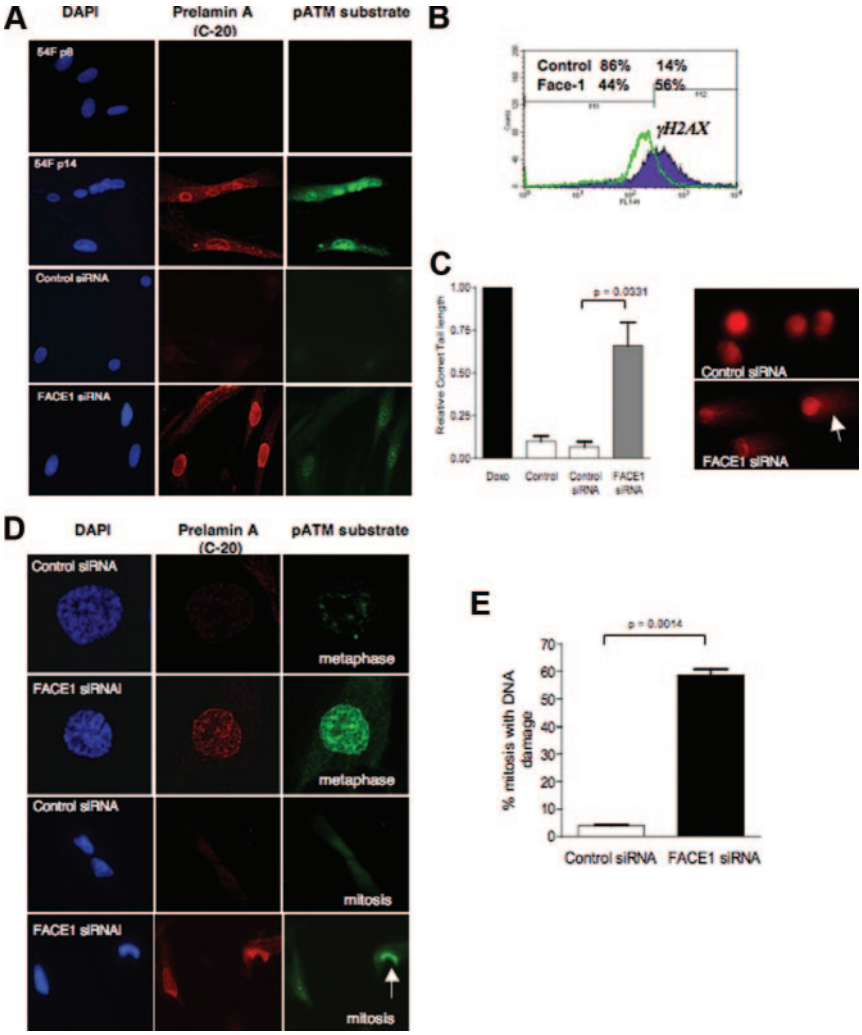

Figure 6. Prelamin A induces DNA damage. A, Increased DNA damage (shown by phosphor-ATM [pATM] substrate antibody) coincident with prelamin A accumulation in late-passage VSMCs and after FACE1 siRNA in early-passage VSMCs (54-year-old woman, passage 8). Abbreviations are as defined in Figure 1 legend. B, Fluorescence-activated cell sorting data showing that FACE1 siRNA treatment increased the percentage of γ-H2AX–positive VSMCs from 14% to 56% (a representative experiment shown of n=4). C, Comet assay showed increased tail length after FACE1 siRNA (arrow) indicative of increased DNA strand breakage. Graph shows quantification of tail length for n=200 VSMCs from 3 independent experiments. Unpaired *t* test was used (control siRNA vs FACE1 siRNA). Doxo indicates DNA damage positive control; control, no siRNA. D, After FACE1 siRNA, prelamin A–positive VSMCs entered mitosis with high levels of DNA damage. Control cells lacking prelamin A did not exhibit DNA damage at mitosis. Prelamin A–positive VSMCs with DNA damage showed mitotic defects such as abnormal DNA contents in daughter nuclei (arrow). E, Increased numbers of VSMCs with DNA damage at mitosis after FACE1 siRNA. Mean±SD values for n=100 mitotic cells from 3 independent experiments are shown. Unpaired *t* test was used (control siRNA vs FACE1 siRNA).

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.[21] 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.[33] 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.[8] Nuclear fragmentation is a hallmark of mitotic catastrophe, a form of cell death induced when cells carrying severe DNA damage enter mitosis.[34] 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.[35] 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.[36,37]

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.[38] 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

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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

1. Gruenbaum Y, Margalit A, Goldman RD, Shumaker DK, Wilson KL. The nuclear lamina comes of age. *Nat Rev Mol Cell Biol*. 2005;6:21–31.
2. Sinensky M, Fantle K, Trujillo M, McLain T, Kupfer A, Dalton M. The processing pathway of prelamin A. *J Cell Sci*. 1994;107(pt 1):61–67.
3. De Sandre-Giovannoli A, Bernard R, Cau P, Navarro C, Amiel J, Boccaccio I, Lyonnet S, Stewart CL, Munnich A, Le Merrer M, Levy N. Lamin A truncation in Hutchinson-Gilford progeria. *Science*. 2003; 300:2055.
4. Dechat T, Pfleghaar K, Sengupta K, Shimi T, Shumaker DK, Solimando L, Goldman RD. Nuclear lamins: major factors in the structural organization and function of the nucleus and chromatin. *Genes Dev*. 2008;22: 832–853.
5. Denecke J, Brune T, Feldhaus T, Robenek H, Kranz C, Auchus RJ, Agarwal AK, Marquardt T. A homozygous ZMPSTE24 null mutation in combination with a heterozygous mutation in the LMNA gene causes Hutchinson-Gilford progeria syndrome (HGPS): insights into the pathophysiology of HGPS. *Hum Mutat*. 2006;27:524–531.
6. Shackleton S, Smallwood DT, Clayton P, Wilson LC, Agarwal AK, Garg A, Trembath RC. Compound heterozygous ZMPSTE24 mutations reduce prelamin A processing and result in a severe progeroid phenotype. *J Med Genet*. 2005;42:e36.
7. Goldman RD, Shumaker DK, Erdos MR, Eriksson M, Goldman AE, Gordon LB, Gruenbaum Y, Khuon S, Mendez M, Varga R, Collins FS. Accumulation of mutant lamin A causes progressive changes in nuclear architecture in Hutchinson-Gilford progeria syndrome. *Proc Natl Acad Sci U S A*. 2004;101:8963–8968.
8. Bridger JM, Kill IR. Aging of Hutchinson-Gilford progeria syndrome fibroblasts is characterised by hyperproliferation and increased apoptosis. *Exp Gerontol*. 2004;39:717–724.
9. Scaffidi P, Misteli T. Lamin A-dependent nuclear defects in human aging. *Science*. 2006;312:1059–1063.
10. Liu B, Wang J, Chan KM, Tjia WM, Deng W, Guan X, Huang JD, Li KM, Chau PY, Chen DJ, Pei D, Pendas AM, Cadinanos J, Lopez-Otin C, Tse HF, Hutchison C, Chen J, Cao Y, Cheah KS, Tryggvason K, Zhou Z. Genomic instability in laminopathy-based premature aging. *Nat Med*. 2005;11:780–785.
11. Merideth MA, Gordon LB, Clauss S, Sachdev V, Smith AC, Perry MB, Brewer CC, Zalewski C, Kim HJ, Solomon B, Brooks BP, Gerber LH, Turner ML, Domingo DL, Hart TC, Graf J, Reynolds JC, Gropman A, Yanovski JA, Gerhard-Herman M, Collins FS, Nabel EG, Cannon RO III, Gahl WA, Introne WJ. Phenotype and course of Hutchinson-Gilford progeria syndrome. *N Engl J Med*. 2008;358:592–604.
12. Kudlow BA, Kennedy BK, Monnat RJ Jr. Werner and Hutchinson-Gilford progeria syndromes: mechanistic basis of human progeroid diseases. *Nat Rev Mol Cell Biol*. 2007;8:394–404.
13. Scaffidi P, Misteli T. Lamin A-dependent misregulation of adult stem cells associated with accelerated ageing. *Nat Cell Biol*. 2008;10:452–459.
14. McClintock D, Gordon LB, Djabali K. Hutchinson-Gilford progeria mutant lamin A primarily targets human vascular cells as detected by an anti-lamin A G608G antibody. *Proc Natl Acad Sci U S A*. 2006;103: 2154–2159.
15. Varga R, Eriksson M, Erdos MR, Olive M, Harten I, Kolodgie F, Capell BC, Cheng J, Faddah D, Perkins S, Avallone H, San H, Qu X, Ganesh S, Gordon LB, Virmani R, Wight TN, Nabel EG, Collins FS. Progressive vascular smooth muscle cell defects in a mouse model of Hutchinson-Gilford progeria syndrome. *Proc Natl Acad Sci U S A*. 2006;103: 3250–3255.
16. Stehbens WE, Delahunt B, Shozawa T, Gilbert-Barness E. Smooth muscle cell depletion and collagen types in progeric arteries. *Cardiovasc Pathol*. 2001;10:133–136.
17. Stehbens WE, Wakefield SJ, Gilbert-Barness E, Olson RE, Ackerman J. Histological and ultrastructural features of atherosclerosis in progeria. *Cardiovasc Pathol*. 1999;8:29–39.
18. Davies JD, Carpenter KL, Challis IR, Figg NL, McNair R, Proudfoot D, Weissberg PL, Shanahan CM. Adipocytic differentiation and liver x receptor pathways regulate the accumulation of triacylglycerols in human vascular smooth muscle cells. *J Biol Chem*. 2005;280:3911–3919.
19. Shanahan CM, Cary NR, Salisbury JR, Proudfoot D, Weissberg PL, Edmonds ME. Medial localization of mineralization-regulating proteins in association with Monckeberg's sclerosis: evidence for smooth muscle cell-mediated vascular calcification. *Circulation*. 1999;100:2168–2176.
20. McClintock D, Ratner D, Lokuge M, Owens DM, Gordon LB, Collins FS, Djabali K. The mutant form of lamin A that causes Hutchinson-Gilford progeria is a biomarker of cellular aging in human skin. *PLoS ONE*. 2007;2:e1269.
21. Matthews C, Gorenne I, Scott S, Figg N, Kirkpatrick P, Ritchie A, Goddard M, Bennett M. Vascular smooth muscle cells undergo telomere-based senescence in human atherosclerosis: effects of telomerase and oxidative stress. *Circ Res*. 2006;99:156–164.
22. Olive PL, Banath JP. Induction and rejoining of radiation-induced DNA single-strand breaks: "tail moment" as a function of position in the cell cycle. *Mutat Res*. 1993;294:275–283.
23. Toth JI, Yang SH, Qiao X, Beigneux AP, Gelb MH, Moulson CL, Miner JH, Young SG, Fong LG. Blocking protein farnesyltransferase improves nuclear shape in fibroblasts from humans with progeroid syndromes. *Proc Natl Acad Sci U S A*. 2005;102:12873–12878.
24. Capell BC, Erdos MR, Madigan JP, Fiordalisi JJ, Varga R, Conneely KN, Gordon LB, Der CJ, Cox AD, Collins FS. Inhibiting farnesylation of progerin prevents the characteristic nuclear blebbing of Hutchinson-Gilford progeria syndrome. *Proc Natl Acad Sci U S A*. 2005;102: 12879–12884.
25. Tintut Y, Alfonso Z, Saini T, Radcliff K, Watson K, Bostrom K, Demer LL. Multilineage potential of cells from the artery wall. *Circulation*. 2003;108:2505–2510.
26. Mounkes LC, Kozlov S, Hernandez L, Sullivan T, Stewart CL. A progeroid syndrome in mice is caused by defects in A-type lamins. *Nature*. 2003;423:298–301.
27. Tyson KL, Reynolds JL, McNair R, Zhang Q, Weissberg PL, Shanahan CM. Osteo/chondrocytic transcription factors and their target genes exhibit distinct patterns of expression in human arterial calcification. *Arterioscler Thromb Vasc Biol*. 2003;23:489–494.
28. Zaidi SK, Javed A, Pratap J, Schroeder TM, J JW, Lian JB, van Wijnen AJ, Stein GS, Stein JL. Alterations in intranuclear localization of Runx2 affect biological activity. *J Cell Physiol*. 2006;209:935–942.
29. Lloyd DJ, Trembath RC, Shackleton S. A novel interaction between lamin A and SREBP1: implications for partial lipodystrophy and other laminopathies. *Hum Mol Genet*. 2002;11:769–777.
30. Dechat T, Shimi T, Adam SA, Rusinol AE, Andres DA, Spielmann HP, Sinensky MS, Goldman RD. Alterations in mitosis and cell cycle progression caused by a mutant lamin A known to accelerate human aging. *Proc Natl Acad Sci U S A*. 2007;104:4955–4960.
31. Cao K, Capell BC, Erdos MR, Djabali K, Collins FS. A lamin A protein isoform overexpressed in Hutchinson-Gilford progeria syndrome interferes with mitosis in progeria and normal cells. *Proc Natl Acad Sci U S A*. 2007;104:4949–4954.
32. Fong LG, Ng JK, Meta M, Cote N, Yang SH, Stewart CL, Sullivan T, Burghardt A, Majumdar S, Reue K, Bergo MO, Young SG. Heterozygosity for LMNA deficiency eliminates the progeria-like phenotypes in Zmpste24-deficient mice. *Proc Natl Acad Sci U S A*. 2004;101:18111–18116.
33. Martinet W, De Bie M, Schrijvers DM, De Meyer GR, Herman AG, Kockx MM. 7-Ketocholesterol induces protein ubiquitination, myelin figure formation, and light chain 3 processing in vascular smooth muscle cells. *Arterioscler Thromb Vasc Biol*. 2004;24:2296–2301.
34. Vakifahmetoglu H, Olsson M, Zhivotovsky B. Death through a tragedy: mitotic catastrophe. *Cell Death Differ*. 2008;15:1153–1162.
35. Minamino T, Komuro I. Vascular cell senescence: contribution to atherosclerosis. *Circ Res*. 2007;100:15–26.
36. Matturri L, Cazzullo A, Turconi P, Lavezzi AM. Cytogenetic aspects of cell proliferation in atherosclerotic plaques. *Cardiologia*. 1997;42:833–836.

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37. Jones MR, Ravid K. Vascular smooth muscle polyploidization as a biomarker for aging and its impact on differential gene expression. *J Biol Chem*. 2004;279:5306–5313.
38. Martinet W, Knaapen MW, De Meyer GR, Herman AG, Kockx MM. Elevated levels of oxidative DNA damage and DNA repair enzymes in human atherosclerotic plaques. *Circulation*. 2002;106:927–932.
39. Yang SH, Qiao X, Fong LG, Young SG. Treatment with a farnesyltransferase inhibitor improves survival in mice with a Hutchinson-Gilford progeria syndrome mutation. *Biochim Biophys Acta*. 2008; 1781:36–39.
40. Yang SH, Meta M, Qiao X, Frost D, Bauch J, Coffinier C, Majumdar S, Bergo MO, Young SG, Fong LG. A farnesyltransferase inhibitor improves disease phenotypes in mice with a Hutchinson-Gilford progeria syndrome mutation. *J Clin Invest*. 2006;116:2115–2121.
41. Fong LG, Frost D, Meta M, Qiao X, Yang SH, Coffinier C, Young SG. A protein farnesyltransferase inhibitor ameliorates disease in a mouse model of progeria. *Science*. 2006;311:1621–1623.
42. Capell BC, Olive M, Erdos MR, Cao K, Faddah DA, Tavarez UL, Conneely KN, Qu X, San H, Ganesh SK, Chen X, Avallone H, Kolodgie FD, Virmani R, Nabel EG, Collins FS. A farnesyltransferase inhibitor prevents both the onset and late progression of cardiovascular disease in a progeria mouse model. *Proc Natl Acad Sci U S A*. 2008;105:15902–15907.
43. Varela I, Pereira S, Ugalde AP, Navarro CL, Suarez MF, Cau P, Cadinanos J, Osorio FG, Foray N, Cobo J, de Carlos F, Levy N, Freije JM, Lopez-Otin C. Combined treatment with statins and aminobisphosphonates extends longevity in a mouse model of human premature aging. *Nat Med*. 2008;14:767–772.

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 Hutchison-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). Hutchison-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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