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Progerin and telomere dysfunction collaborate to trigger cellular senescence in normal human fibroblasts

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Hutchinson-Gilford progeria syndrome (HGPS), a devastating premature aging disease, is caused by a point mutation in the lamin A gene (LMNA). This mutation constitutively activates a cryptic splice donor site, resulting in a mutant lamin A protein known as progerin. Recent studies have demonstrated that progerin is also produced at low levels in normal human cells and tissues. However, the cause-and-effect relationship between normal aging and progerin production in normal individuals has not yet been determined. In this study, we have shown in normal human fibroblasts that progressive telomere damage during cellular senescence plays a causative role in activating progerin production. Progressive telomere damage was also found to lead to extensive changes in alternative splicing in multiple other genes. Interestingly, elevated progerin production was not seen during cellular senescence that does not entail telomere shortening. Taken together, our results suggest a synergistic relationship between telomere dysfunction and progerin production during the induction of cell senescence, providing mechanistic insight into how progerin may participate in the normal aging process.

Introduction

Cellular senescence, the finite division capacity of normal somatic cells in vitro, has long been used as a cellular model for understanding mechanisms underlying normal aging (1, 2). A popular hypothesis of cellular senescence is that progressive attrition of telomeric DNA results in loss of telomere capping proteins, exposing DNA breaks that activate cell cycle arrest and senescence (3). Telomeres are the specialized structures at the chromosome ends, made up of many kilobases of a simple DNA repeat (TTAGGG), bound by a multiprotein complex known as shelterin (4). Telomeres cannot be copied to their extreme termini by DNA polymerase, and so undergo progressive shortening unless elongated by a ribonucleoprotein named telomerase (5, 6). Telomerase is composed of a reverse transcriptase (TERT) and an RNA component (TERC) that serves as a template for telomere elongation (6, 7). In somatic cells lacking telomerase, gradual telomere loss and ultimate senescence are inevitable (8). Consistent with this model, there is a strong association between cell immortalization and persistent telomerase expression (9). Besides telomere shortening, cellular senescence can be triggered by a variety of environmental and intracellular stimuli, including γ-irradiation, oxidative stress, and overexpression of certain oncogenes, such as H-rasV12 (10–12). Moreover, mutations in certain genes, such as lamin A (LMNA; accession no. NM_170707), have been linked to premature senescence (13, 14).

LMNA encodes 2 intermediate filament proteins, lamin A and lamin C. Together with lamin B, they form a dynamic meshwork located just inside the nuclear inner membrane, named the nuclear lamina. The lamina provides important mechanical support to the nuclear structure and also influences chromatin organization, gene expression, and DNA replication (15–17). To date, at least 13 human diseases (referred to as the laminopathies) have been associated with the mutations in lamin genes (13). Among them, Hutchinson-Gilford progeria syndrome (HGPS) has received the most attention because of its striking premature aging phenotype, including alopecia, diminished subcutaneous fat, premature atherosclerosis, and skeletal abnormalities. Children with HGPS die at an average age of 12 years, usually from heart attack or stroke (18). The vast majority of HGPS cases are associated with a de novo nucleotide substitution at position 1824 (C→T) in the LMNA gene (14, 19). This mutation does not affect the coded amino acid (and is thus generally referred to as G608G), but partially activates a cryptic splice donor site in exon 11 of LMNA, leading to the production of a prelamin A mRNA that contains an internal deletion of 150 base pairs (14). This transcript is then translated into a protein known as progerin, which lacks 50 amino acids near the C terminus (14). Indeed, progerin has been found to accumulate in multiple tissues in biopsies from HGPS patients, including skin, tongue, breast, heart, liver, kidney, stomach, bladder, diaphragm, pancreas, spleen, thyroid, adipose tissue, joint cartilage, bone, skeletal muscle, heart, and large and small arteries (20).

The cellular phenotypes in HGPS include blebbing (i.e., abnormal shape) of nuclei, thickening of the nuclear lamina, loss of peripheral heterochromatin, clustering of nuclear pores, and premature senescence (21). Interestingly, prior to developing these obvious nuclear morphological changes, fibroblasts from HGPS patients exhibit broad abnormalities in histone modification patterns (22). HGPS fibroblasts also show global changes in gene expression (23, 24) and a delayed response in DNA-damage repair (25).

Gene transfer experiments have left no doubt that progerin acts as a dominant negative. Progerin expression induces multiple defects during mitosis: cytokinesis delay, abnormal chromosome segregation, and binucleation (26, 27). Several Lmna mutant mouse models have been created. A transgenic model carrying the G608G mutated human LMNA in a bacterial artificial chromo-
some (BAC) shows progressive loss of vascular smooth muscle cells in the medial layer of large arteries, closely resembling the most lethal aspect of the human phenotype (28).

The molecular mechanism of progerin toxicity is at least partially understood. Normal lamin A is farnesylated at its C terminus, and that posttranslational modification is thought to play a role in targeting lamin A to the inner nuclear membrane. But subsequently, the ZMPSTE24 endoprotease cleaves off the last 18 amino acids at the C terminus of lamin A, including the farnesyl tail. This releases lamin A from its membrane anchor and allows it to take its place in the nuclear scaffold (29). The 50–amino acid internal deletion in progerin includes the ZMPSTE24 cleavage site, and thus progerin remains permanently farnesylated (27). Recently, treatment with farnesyl transferase inhibitors (FTIs) has been shown to improve progerin-induced cellular phenotypes in vitro and in several HGPS mouse models (13, 27, 30–34). A clinical trial with an FTI in children with HGPS was initiated in May 2007.

It has always been of great interest to determine the biological relevance of HGPS to normal aging. This interest was heightened by the detection of progerin mRNA and progerin protein in cells obtained from healthy individuals, which indicates that the cryptic splice site activated by the HGPS mutation is also capable of being used in the presence of the normal sequence of exon 11 (35). Recently, progerin transcripts and progerin were identified in vivo in skin biopsies from healthy individuals, and a recent analysis suggested that progerin transcript levels increase in late-passage cells from HGPS patients and parental controls (36, 37).

Using a reporter construct, Scaffidi et al. showed that the normal LMNA sequence at G608G could also be used as a weak splice donor to produce progerin in normal individuals (35). We have previously shown that a significant percentage of progerin-positive cells from normal individuals are morphologically abnormal, exhibiting phenotypes that resemble HGPS cells (26). This suggests that activation of progerin expression in normal cells, triggered by some unknown signal, may contribute to senescence.

However, the cause-and-effect relationship between normal aging and progerin production in normal individuals has not been determined. In the present study, we assayed the effects of cellular passage and donor age on the activation of progerin production and found that the cryptic splice donor site that produces progerin is activated in senescent cells. Screening various primary and transformed cell lines revealed an interesting inverse correlation between cell immortalization and progerin transcription, and ectopic expression of telomerase in normal fibroblast cells resulted in a significant decrease in progerin production. In addition, we found that progerin production was not induced in telomere-independent, oncogene-driven senescence, further supporting a potential causal relationship between telomere-induced senescence and progerin production. Consistent with this model, we found that elevated levels of progerin were induced in fibroblast cells whose telomeres had been uncapped. With splicing-sensitive exon microarrays, we further showed that extensive changes in alternative splicing of multiple genes, including LMNA, occurred as telomeres shortened in the nuclear scaffold (29).
and cells approached senescence. Taken together, our findings are suggestive of synergism between telomere damage and progerin production in induction of cellular aging and provide what we believe to be the first genome-wide analysis of the changes in alternative splicing during cell senescence.

Results

Cellular senescence activates progerin production. To elucidate the cause-and-effect relationship between aging and progerin production, we designed a splicing reporter assay that permits observation of the immediate effects of cell passage or donor age on the activation of the progerin cryptic splice donor site. The cryptic splicing reporter originally described by Scaffidi et al. (35) was PCR amplified and subcloned into a bicistronic vector (pIRES-DsRed-Expression 2) (Figure 1A). This 2-color progerin splicing reporter was then transiently transfected into normal fibroblast cells. In this assay, all transfected cells expressed red fluorescence protein, and cells using the cryptic splice site also expressed GFP (Supplemental Figure 1, A and B; supplemental material available online with this article; doi:10.1172/JCI43578DS1). At 96 hours after transfection, we sorted those fibroblasts with fluorescence-activated cell sorting (FACS) analysis and quantified 2 subpopulations (Figure 1B and Supplemental Figure 1A): the fraction positive for both GFP and DsRed, consisting of transfected cells that had activated the cryptic splice site in LMNA (referred to herein as double-positive), and the fraction positive for DsRed only, containing the transfected cells that did not use the cryptic splice (referred to herein as DsRed-only).

To examine the effect of cellular passage and donor age, we performed the reporter assay in 6 independent primary fibroblast lines, derived from healthy donors ranging 10–92 years in age, at increasing cellular passages. Percent double-positive cells relative to total DsRed cells was used as an indicator of the progerin splicing activity and plotted against cell passage and donor age. In all tested cell lines, we consistently observed that double-positive cell percentage increased with cell passage (Figure 1C and Supplemental Figure 2), which suggests that cellular senescence correlates with activation of progerin splicing. Furthermore, in agreement with previous reports (35, 36, 38, 39), we did not observe an obvious correlation of progerin production with donor age (Supplemental Figure 2).

Progerin-expressing cells from normal individuals show signs of senescence. To gain mechanistic insights into the activation of the progerin cryptic splice site in cellular aging, we next compared telomere length in the double-positive and DsRed-only subgroups after FACS analysis. The telomeres were labeled using FISH with a peptide nucleic acid probe against telomere repeat sequence (PNA-FISH). The telomere length of each cell was quantified as the total fluorescence intensity from PNA-FISH in each nucleus, which was defined by the DAPI staining of DNA (Figure 2A). We examined 2 normal fibroblast cell lines, AG06299 at passage 35 (p35) and HGFDFN168 at p18. Because both cell lines demonstrated similar results, we showed results only from HGFDFN168. Box plot analysis suggested that the telomeres in the double-positive subgroup were significantly shorter than those in the DsRed-only subgroup from the same cell line \( (P < 0.0001; \text{Figure } 2B) \). Taken together, these results indicate that the progerin-producing double-positive cells define a subpopulation of cells with short telomeres, which suggests a potential connection between telomere erosion and progerin splicing activation.

Immortalized cells suppress progerin production. To further test the connection between progressive telomere shortening and progerin production, we measured the amount of progerin mRNA in immortalized cancer cells with stable telomeres as a result of telomerase expression as well as in primary cells lacking telomerase expression. A RT-PCR assay with progerin- or lamin A–specific primers was designed to assess relative use of the endogenous lamin A cryptic splice site (Figure 3A). Paired with a forward primer at the exon 7–8 junction, these primers specifically amplified truncated progerin or lamin A products with the expected sizes. A high level of specificity and identity was demonstrated using in vitro–purified cDNAs (Supplemental Figure 3). DNA sequencing verified the identities of these PCR products (data not shown). We tested a total of 16 cell lines, including 12 primary cell lines derived from 3 cell types (fibroblasts, aortic SMCs, and B lymphocytes) and 4 immortalized cell lines from various telomerase-positive cancers (Figure 3B). Consistent with previous reports (40), quantitative RT-PCR (qRT-PCR) analysis revealed substantial variation in LMNA expression in different cell types, with the lowest expression found in B lymphocytes (15-fold less than fibroblasts; data not shown). To directly compare the level of progerin mRNA among these different types of cells, we normalized the amount of progerin mRNA to the LMNA mRNA in each cell line. Interestingly, we found that progerin mRNA was essentially absent in immortalized cells with stable telomeres (Figure 3B).

Next, we examined how changes in telomere length would influence progerin production. We studied 2 human TERT–immortalized normal fibroblast cell lines, hTERT-HGFDFN090 and...
hTERT-AG09838, and their parent cells. qRT-PCR analysis with human TERT primers confirmed the presence of TERT mRNA in the immortalized fibroblast cells, whereas no TERT mRNA was detected in their nonimmortalized counterparts because of its extremely low amount in differentiated primary fibroblasts (data not shown). In addition, quantitative telomere PNA-FISH analysis demonstrated that stable infection of human TERT resulted in elongated telomeres (Figure 3C and Supplemental Figure 4). Consistent with our finding in immortalized cancer cells, we found that the ectopic expression of telomerase in normal fibroblasts led to significant downregulation in progerin production: progerin mRNA decreased 3- and 10-fold in hTERT-AG08398 and hTERT-HGFDFN090 fibroblasts, respectively, compared with their nonimmortalized passage-matched counterparts (Figure 3D). Therefore, we concluded that forced elongation of telomeres in primary fibroblast cells leads to suppression of progerin production.

Together, our data demonstrated an inverse correlation between telomerase expression (cell immortalization) and progerin production and suggested that telomeres serve as the upstream signals in regulating progerin production.

Progerin production is not altered in telomere-independent premature cellular senescence. To examine whether progerin splicing is activated in cellular senescence that does not entail telomere shortening, we analyzed progerin production in premature senescence induced by oncogenic ras (12). We chose 2 well-characterized normal fibroblast lines, AG08470 and HGFDFN168, and introduced an activated ras allele (H-rasV12) to those cells using the pBABE retrovirus, as previously described (12). The vector coexpresses a puromycin-resistant gene that allows selection for transduced cells in 4–5 days. After selection, those cells were grown in media without puromycin for an additional 6 days. Expression of H-rasV12 induced morphological changes in both normal fibroblast cell

Figure 3
Immortalized cells suppress progerin transcription. (A) Schematic representation of the positions of progerin- or lamin A–specific primers used for RT-PCR analysis. (B) qRT-PCR. Primary lines included normal fibroblast cell lines HGFDFN168 and HGFDFN090 (Fb1 and Fb2, respectively); human aortic SMC line; and B lymphocyte lines AG09393 and AG11659 (BL1 and BL2, respectively). HGPS fibroblast lines were HGADFN167 and HGADFN003 (HGPS1 and HGPS2, respectively). 4 immortalized lines of indicated cell types are also shown. qRT-PCR showed more progerin mRNA than LMNA mRNA in HGPS cell lines, which was an artifact caused by the difference in priming efficiency of progerin- and lamin A–specific primers (see Supplemental Figure 4C). (C) Representative images of quantitative telomere PNA-FISH analysis of human TERT–immortalized (+TERT) and primary (–TERT) fibroblast cells (AG09838, p8). DNA was stained with DAPI in blue to show the boundary of the nucleus (outlines), and telomere-FISH signals are in green. (D) qRT-PCR analysis of the total progerin mRNA amount in normal and human TERT–immortalized cell lines with progerin-specific primers. The relative expression values for progerin were normalized to the mean values of endogenous LMNA. HeLa and 293T lines are shown as controls.
lines. A substantial portion of H-rasV12–transduced cells became flat and enlarged and exhibited increased SA-β-gal activity (Figure 4A). No changes in morphology were detected in cells transduced with vector control (Figure 4A). Consistent with previous studies (12), quantitative telomere PNA-FISH analysis demonstrated no obvious changes in telomere length in H-rasV12–induced cells compared with that in control vector–induced cells (Figure 4B). Importantly, although the morphological features of H-rasV12–induced cells closely resembled cells that have passed their proliferative capacity and become senescent, qRT-PCR analysis indicated no upregulation of progerin production in either cell line tested (Figure 4C). In addition, we found no significant upregulation of progerin mRNA production in the premature senescent cells that had been induced by treatment with 0.5 mM sodium butyrate, a histone deacetylase inhibitor (41), for 2 weeks (data not shown). Together, these data suggest that upregulation of progerin production is not a common feature for all types of senescence, but appears specific for telomere-driven senescence.

Fibroblast cells from dyskeratosis congenita patients carrying a heterozygous null mutation in human TERT produce more progerin. To further test the hypothesis that short telomeres activate progerin production in senescent cells, we used 2 fibroblast lines, JH-1 and JH-2, derived from patients with dyskeratosis congenita (DC) (42). These cells carry a heterozygous loss-of-function mutation (K902N) in exon 11 of TERT that results in haploinsufficiency of telomerase and accelerated telomere shortening (42). DNA sequencing verified the presence of K902N in both DC fibroblast lines (Supplemental Figure 5). Quantitative telomere PNA-FISH analysis confirmed accelerated telomere loss during cellular senescence in these DC cells compared with passage-matched normal fibroblasts (P < 0.0001; Figure 5A). Using qRT-PCR on cells at p12–p15, we found a more than 2-fold increase in progerin production in these DC fibroblasts compared with normal passage-matched control cells (P = 0.013; Figure 5B). Interestingly, both JH-1 and JH-2 cells exhibited a more than 2-fold increase in cell blebbing (Figure 5, C and D; see Supplemental Figure 6 for passage-matched normal fibroblast control), reminiscent of the hallmark cellular phenotype in HGPS. In addition, we noted a significant increase in JH-1 and JH-2 cells containing micronuclei (small unincorporated chromosome fragments; Figure 5, C and D), potentially caused by prior defects in cell division, which has also been reported in HGPS cells (26, 27). Immunostaining of the JH-1 and JH-2 cells with an anti-progerin antibody revealed positive progerin staining inside micronuclei in those abnormal cells (Figure 5E). The specificity of the anti-progerin antibody was verified using immunostaining in HeLa cells expressing either GFP-progerin or GFP-lamin A (Supplemental Figure 7). Interestingly, we observed positive cytoplasmic signals for progerin in a small percentage of DC cells (Figure 5E), which contrasts with the predominant nuclear lamina localization of WT lamin A and lamin C. Our data in DC cells provide further support for a model in which short telomeres act as an upstream signal to activate progerin production in primary fibroblast cells.

**Fibroblasts expressing TRF2ΔBΔM show increased progerin production.** To address further the effect of telomere-directed senescence on progerin production, we used TRF2 inhibition as a tool to induce immediate telomere dysfunction in normal cells. TRF2 is a major component of the telomere-capping complex, shelterin (4). When TRF2 is dislodged from telomeres by a dominant-negative allele (TRF2ΔBΔM), a fraction of chromosome ends lose their protection, leading to a growth arrest that is indistinguishable from replicative senescence (43). We infected normal human fibroblasts with retroviruses expressing TRF2ΔBΔM or WT TRF2. Cells were kept under puromycin-containing medium for 5 days before being used for analysis. qRT-PCR analysis with progerin- and lamin A–specific primer pairs indicated no significant changes of WT LMNA mRNA level, but more than 8-fold upregulation of progerin mRNA was observed in TRF2ΔBΔM-expressing cells compared with control cells (Figure 6, A and B). Western blotting analysis with the anti-progerin antibody revealed a distinct band corresponding to progerin in cells infected with TRF2ΔBΔM, but not in control cells infected with

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**Figure 4**

Progerin production is not increased on oncogene ras–induced premature cellular senescence. (A) Vector control and H-rasV12–infected AG08470 cells stained for SA-β-gal activity at day 6 after puromycin selection. (B) Box plot representation of the quantitative telomere PNA-FISH in the vector control or H-rasV12 transfected AG08470 cells (P = 0.138; n = 22 per group). Box denotes 25th and 75th percentiles; line within box denotes 50th percentile; whiskers denote 9th and 91st percentiles. (C) qRT-PCR analysis of total progerin mRNA in H-rasV12– or control-infected normal fibroblast cells with progerin-specific primers. Relative expression values for progerin were normalized to the mean values of endogenous LMNA. Fb1 and Fb2, replicates performed in AG08470 and HGFDFN168, respectively, at p14.
Figure 5
Progerin production is more abundant in passage-matched DC fibroblasts that carry a TERT mutation. (A) Box plot representation of the quantitative telomere PNA-FISH in JH-1 and JH-2. JH-1 and JH-2 had significantly shorter telomeres compared with passage-matched normal fibroblast controls ($P < 0.0001$). All cell lines were analyzed at p12–p15. NR1 and NR2, normal fibroblast controls HGDFN168 and HGDFN090, respectively, at p14. n is indicated for each group. Box denotes 25th and 75th percentiles; line within box denotes 50th percentile; whiskers denote 9th and 91st percentiles. (B) qRT-PCR analysis of progerin mRNA in JH-1 and JH-2 cells. The relative expression values for progerin were normalized to the mean values of LMNA. Significantly greater amounts of progerin were observed in p12 JH-1 and p15 JH-2 cells, which have accelerated telomere shortening ($P = 0.013$, p12 JH-1 and p15 JH-2 vs. p14 NR-1 and p14 NR-2). (C) Immunofluorescence of JH-1 and JH-2 fibroblast cells with anti-lamin A/C antibody (green) and anti-α-tubulin (MT; red) antibody. DNA is labeled with DAPI in blue. (D) Quantification of nuclear blebbing and micronuclei in p12 JH-1 and p15 JH-2 cells. A normal fibroblast (NR; AG08470 at p14) was used as a control. (E) Immunostaining with anti-progerin antibody in selected DC cells. DNA is stained with DAPI in blue. Scale bar: 10 μm.
WT TRF2 (Figure 6C). Similarly, immunofluorescence analysis revealed positive progerin staining in the TRF2ΔBΔM cells (Figure 6D). Interestingly, we found that progerin staining in TRF2ΔBΔM cells was quite diffuse in the nucleoplasm and was even seen in the cytoplasm in some cells. This differs with the predominant nuclear lamina localization of WT lamin A/C. We reasoned that the apparent lack of signal from the anti–lamin A/C antibody in the progerin-positive cytoplasm might be just a sensitivity issue, since there is such a large amount of lamin A/C in the nucleus. Indeed, simply by increasing the gain of the signal for lamin A/C, we observed colocalization of signals from anti-progerin and anti–lamin A/C antibodies (Supplemental Figure 8). In summary, these data support the conclusion that uncapped dysfunctional telomeres activate the cryptic splice site in normal fibroblast cells to produce progerin.

Extensive alternative splicing events occur during cell senescence. Next, we asked whether telomere dysfunction induces a broader set of alternative splicing changes, beyond the effects on LMNA. To monitor global effects on alternative splicing, we used the human Exon 10ST array, a splicing-sensitive microarray with probes targeted to individual exons or exon junctions, although it is not designed to detect use of the progerin cryptic splice site. This design allowed us to detect RNA isoforms expressed while simultaneously profiling gene expression. The expression level of each exon on each array was detected independently and analyzed using XRAY microarray analysis software.

The profiles of exon usage were compared between the same fibroblast lines with either long or short telomeres (group A) and between passage-matched fibroblasts where no significant variations in telomere length were present (group B), as controls (Figure 7A). Group A included 2 comparisons between early- and late-passage normal fibroblast cells (groups A1 and A2) and 2 comparisons between passage-matched human TERT–immortalized and nonimmortalized cells (groups A3 and A4). Group B comparisons included 4 comparisons between passage-matched normal and HGPS fibroblasts. Using a false discovery rate (FDR) threshold of 0.01, we identified numerous changes in gene expression in each comparison, even between the passage-matched fibroblasts from 2 normal individuals (data not shown). Surprisingly, changes in alternative splicing were much more prominent in group A, in which significant telomere shortening had occurred (Figure 7A and Supplemental Table 1). Between 10% and 50% of the genes showing changes in alternative splicing also exhibited differential regulation of gene expression (Figure 7A), which suggested distinct but also partially overlapping programs of transcriptional and posttranscriptional regulation. An overlap of 82 genes was observed in the 4 comparisons of group A (Figure 7B and Supplemental Table 2), which implies that 1 or more common regulatory pathways might be activated in these cells by shortening of telomeres. Statistical analysis (see Methods) indicated that the overlap of genes showing altered expression and altered splicing within all 4 group A sets was not significant \(P > 0.75\); however, the overlap of 82 genes with altered splicing between the 4 sets was highly significant \(P < 0.0001\). We were unable to identify any obvious pattern or sequence motifs in the set of genes that showed alternative splicing in the presence of telomere shortening.

In an attempt to address the functional relevance of these differentially spliced genes induced by changes in telomere length, gene
ontology (GO) enrichment analyses (see Methods) were carried out for these 82 common genes, using the complete set of genes covered by the exon microarray as a reference. Interestingly, among the top 10 most overrepresented categories, 5 were related to cytoskeleton organization and function, including the genes regulating intermediate filaments and microtubules, such as actin, fibronectin, vimentin, and tubulin (Figure 7C). These results indicate that cellular senescence induces extensive changes in alternative splicing in multiple genes and suggest a potential role of differential splicing in cytoskeleton reorganization during cellular aging.

Discussion

Considerable progress has been made in understanding the connection between HGPS and normal aging. In 2006, Scaffidi et al. first showed that the normal LMNA sequence could produce progerin at a low level in normal individuals, potentially implicating progerin in normal physiological aging (35). With a progerin-specific antibody, we previously demonstrated that the progerin-expressing cells from normal individuals mimic some aspects of HGPS cells, further supporting the idea that the premature aging disease HGPS and normal aging may share a common cellular and molecular basis (26). Furthermore, a recent analysis has shown that levels of progerin mRNA transcript increase in late-passage cells in vitro (37), and progerin protein accumulates with age in vivo in normal individuals (36). However, the cause-and-effect relationship between progerin production and aging has not been clear.

In this study, we applied a splice reporter assay that permits observation of the immediate effects of cell passage or donor age on the activation of the progerin cryptic splice site in LMNA. In normal fibroblasts, we showed that this cryptic site was increasingly activated as cells reached later cellular passage numbers. Thus, cellular senescence correlated with enhanced progerin production. No obvious correlation was found, however, between progerin production and donor age. One potential explanation is that the cells capable of growing in culture from a skin biopsy are relatively young in vivo (39).

We further showed that the fibroblast cells that used the LMNA cryptic splice site had shorter telomeres and exhibited high senescence-associated β-gal (SA-β-gal) activity. The finding that most of

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**Figure 7**

Extensive alterations in alternative splicing occur as cells senesce. (A) Number of genes that showed significant changes in alternative splicing in each indicated comparison. Gray, genes that exhibited changes only in alternative splicing; black, genes that exhibited changes both in alternative splicing and in gene expression. Group A included binary comparisons between normal fibroblasts before and after senescence: A1, p34 vs. p52 for normal fibroblast AG06299; A2, p7 vs. p22 for normal fibroblast HGDFDN168; A3, normal vs. human TERT-immortalized fibroblast HGDFDN090 at p6; A4, human TERT-immortalized vs. nonimmortalized normal fibroblast AG08398 at p8. Group B compared passage-matched fibroblasts where no significant variations in telomere length are present: B1, HGPS fibroblast HGADFN167 at p15 vs. HGPS fibroblast HGADFN003 at p16; B2, normal fibroblast HGDFDN168 at p14 vs. normal fibroblast HGDFDN090 at p14; B3, HGPS fibroblast HGADFN167 at p15 vs. age-matched normal fibroblast AG08470 at p14; B4, HGPS fibroblast HGADFN003 at p16 vs. age-matched normal fibroblast AG08470 at p14. (B) There were 82 overlapping genes among the 4 lists of genes in group A. (C) GO analysis (sorted by process networks) of the 82 overlapping genes in B. The top 10 enriched categories are shown; cytoskeleton-related categories are denoted with asterisks.
HGPS patients fail to immortalize despite the exogenous expression of telomerase, which suggests that the growth arrest in HGPS confers resistance to telomerization (44). Another report indicated that telomerase failed to protect progerin-induced DNA damage (45). However, Decker et al. reported shortened telomeres in HGPS fibroblasts, although their finding was limited to late-passage HGPS fibroblasts, where telomere attrition may well have been on the basis of cell passage number rather than being specific for HGPS (46). Most recently, a report by Benson et al. showed that exogenous expression of progerin in human diploid fibroblasts rapidly induces telomere aggregation, appearance of DNA-damage signals at telomeres, and chromosomal aberrations. Importantly, these effects were abolished by telomerase expression (47). Using quantitative telomere PNA-FISH in HGPS fibroblasts, we found no clear evidence of shortened telomeres compared with passage-matched normal fibroblasts (data not shown). Similarly, we found that the telomere length in vascular smooth muscle cells derived from a transgenic mouse model of HGPS was no different from that in nontransgenic control mice (data not shown). In support of the conclusion that progerin’s effect on telomeres is independent of shortening of the telomeric DNA, Benson et al. (47) indicated that progerin induces rapid telomere dysfunction well before telomere attrition would be detectable. Therefore, we suggest that progerin induces an acute DNA damage response at telomeres — perhaps on the basis of well-established effects of progerin on disrupting heterochromatin structure (21) — that leads to deprotection of the telomeric 3’ overhang.

Cellular senescence reflects a complex process of gradual deterioration of the molecular components, checkpoints, and cellular structures. Inspired by the progerin results, we hypothesized that alternative splicing, a key pre-mRNA processing step, might be more broadly influenced by senescence. Using splicing-sensitive exon microarrays, we showed that not only LMNA splicing was affected by short telomeres, but a significant number of other genes were also affected. Interestingly, GO analysis further demonstrated that the most overrepresented categories in this set of differentially spliced genes were related to cytoskeleton function. This finding may connect with the longstanding observation that senescent cells demonstrate characteristic changes in cytoskeleton organization, including cell shapes, cell adhesion, and cell mobility. Previous studies reported that fibronectin (48), a cell adhesion protein, and vimentin (49, 50), an intermediate filament protein, are alternatively spliced in senescent cells. Both were identified in our analysis, as were other cytoskeleton-related proteins, such as actin and tubulin. Our studies present what we believe to be the first systematic genome-wide analysis of alternative splicing during cellular senescence and suggest a significant potential role of differential splicing in cytoskeleton reorganization during cellular aging.

To summarize, we demonstrated that progressive telomere loss in normal cells acted as an upstream signal to activate the cryptic splice site in LMNA to produce progerin as well as to induce alternative splicing of a number of other genes. Telomere-induced senescence has previously been primarily attributed to the activation of the tumor suppressor p53 (11). Interestingly, in HGPS cells, progerin-induced senescence has also been partially linked to the activation of p53 (51). Taken together, our data suggest a more complex model: in addition to activation of p53, progressive telomere shortening triggers alternative splicing of a suite of genes, including production of progerin mRNA and progerin protein. These changes, in turn, contribute to cellular senescence.
(Figure 8). The recent report from Benson et al. (47) adds another potential feature to this mechanism—a positive feedback loop between telomeres and progerin.

The specific signaling pathway from dysfunctional telomeres to the spliceosome machinery remains undefined. An attractive hypothesis is that certain telomere-binding/capping proteins are released from telomere ends when telomeres are progressively shortened or damaged, and then influence spliceosome function in senescent cells. One potential candidate for carrying out this function is the shelterin complex, consisting of 6 telomere-binding proteins: TRF1, TRF2, POT1, TIN2, Rap1, and TPP1. Acting together with DNA repair factors, these proteins protect telomere ends by forming a specialized complex that masks the telomeres from being seen as sites of DNA damage (4). The released shelterin proteins could potentially act as signal transducers for alternative splicing in senescent cells. Other factors that may directly influence alternative splicing would include the heterogeneous nuclear ribonucleoproteins (hnRNPs), which bind to both RNA and single-stranded telomere DNA. In fact, hnRNPs have been suggested from being seen as sites of DNA damage (4). The released shelterin proteins are believed to be a novel signaling pathway for cellular senescence and further enhance the conclusion that the study of HGPS can provide critical clues to the normal aging process.

**Methods**

**Cell culture.** Primary human dermal fibroblasts were cultured under 5% CO₂ (balanced by ambient air) in MEM (Gibco; Invitrogen) supplemented with 15% FBS (Invitrogen) plus 2 mM l-glutamine. The primary fibroblast cell lines used in our studies included AG06299 (normal), AG08470 (normal), AG07306 (normal), AG08048 (normal), AG06277 (normal), AG09838 (normal), and AG09682 (normal) from Coriell Cell Repositories and HGADFN167 (HGPS), HGADFN003 (HGPS), HGFDFN168 (normal), and HGFDFN090 (normal) from the Progeria Research Foundation. See Supplemental Table 3 for detailed information for each cell line. The hTERT-HGFDFN090 and hTERT-AG09838 stable fibroblast cell lines were generated and provided by T. Glover (University of Michigan, Ann Arbor, Michigan, USA). To subclone the splicing reporter into the pIRES-DsRed-Expression 2 vector, the pPRO reporter previously described (35) was PCR amplified using the following primers: forward, CCGCTCGAGGTACGGCTCTCATCAACTCC; reverse, CCGCTCGAGTTACTTGTAGCTCGTC. The PCR product was subsequently cloned into the XhoI site of pIRES-DsRed-Expression 2 vector, and the correct orientation of the insert was determined by sequencing.

**Transfection.** All transient transfections with the progerin splicing reporter in the primary fibroblasts were performed using the Amaxa nucleofection device and nucleofector kits for adult human–dermal fibroblasts (NHDF; Lonza) according to the manufacturer’s instructions.

**Antibodies.** The antibodies used in the study included a rabbit polyclonal antibody against progerin (provided by K. Djabali, Columbia University, New York, New York, USA), mouse anti-lamin A/C (MAB3211; Chemicon), mouse anti-tubulin (DM1α; Sigma-Aldrich), rabbit anti-GFP (Abcam), and mouse anti-GAPDH (Abcam).

**FACS.** Primary fibroblasts transfected with the progerin splicing reporter were excised at 488 nm and sorted with a FACSaria machine (BD) according to the manufacturer’s instructions.

**RNA isolation, reverse transcription, qRT-PCR, and data analysis.** Cells were harvested at approximately 75% confluence. RNA for each sample was isolated with the Qiagen RNAasy kit according to the manufacturer’s instructions. RNA was reverse transcribed using the superscript III kit (Invitrogen) according to the manufacturer’s instructions. qRT-PCR was performed to measure expression of progerin, LMNA, TERT, and ACTB. All reactions were carried out at least in triplicate on an Applied Biosystems 7900HT Fast Real-Time PCR System using SYBR Green mix (Qiagen) according to the manufacturer’s instructions. Reaction conditions were as follows: 1 cycle of 2 minutes at 50°C; 1 cycle of 10 minutes at 95°C; and 40 cycles of 15 seconds at 95°C, 15 seconds at 58°C, and 30 seconds at 72°C. Primers for TERT and β-actin were obtained from R&D and Ambion, respectively. The sequence of the forward primer for amplifying progerin/lamin A is CCAACAAATCGTCAAGGACCA. The progerin- and lamin A-specific reverse primers were designed according to amplification-refractory mutation system strategy, by introducing a mutation at the penultimate base to increase specificity. The progerin-specific primer sequence is CATGAT-GCTGCGATTTGCGGCTTGAC, and that for lamin A is CATGAT-GCTGCGATTTGCGGCTTGAC.

**Quantitative telomere PNA-FISH analysis.** FISH was performed with the Telomere PNA Kit (Dako) according to the manufacturer’s protocol. Confocal images were acquired at room temperature using a Zeiss LSM 510 NLO Meta system mounted on a Zeiss Axiovert 200M microscope with an oil immersion Plan-Apochromat ×63/1.4 differential image contrast objective lens. Excitation wavelengths of 488 nm (4%), 561 nm (7%), and 740 nm (3%) were used for detection of the FITC-tagged telomeric probe, the Cy3-tagged telomeric probe, and DAPI, respectively. Fluorescent emissions were collected in a BP 500- to 550-nm IR blocked filter, LP 575-nm filter, and BP 390- to 465-nm IR blocked filter, respectively. All pinholes were set with a range from 1.21 to 1.39 Airy units, which correspond to an optical slice of 1.0 μm (excluding the DAPI channel, for which a multichannel laser was used). All confocal images were of frame size 512 pixels × 512 pixels, scan zoom 2, and were line averaged 8 times. Confocal images were postprocessed using MediaCybernetics Image-Pro Plus software (version 6.3). Every image was processed using a custom macro designed to calibrate, filter (Gaussian), segment (touching nuclei or nuclei touching the image border were not included), outline the nuclei, count, and record measurements. Measurements included nuclear area (μm²) and fluorescent intensity...
resulting cRNA was purified. A first-strand cDNA was generated from the
antibody solution containing 0.5 mg/ml biotinylated anti-streptavidin
PBS for 20 minutes at room temperature followed by a 5-minute treatment
with 0.5% Triton-X 100 in PBS, or with methanol/Acetone (1:1) at −20°C as
described previously (26). The fixed cells were rinsed with PBS and blocked
with 10% horse serum and 4% BSA in PBS for 30 minutes. Cells were then
incubated for 1 hour with the primary antibodies diluted in blocking solution.
The secondary antibodies were Alexa 488- or Alexa 594-conjugated
donkey anti-rabbit or donkey anti-mouse IgG antibodies (Invitrogen). All
samples were also counterstained with DAPI (Vector Laboratories). Cells
were observed with a LSM510 or LSM710 confocal microscope (Zeiss) or an
Axioplan fluorescence microscope (Zeiss).

SA--β-gal activity assay. Subconfluent primary fibroblasts grown on
chamber slides (Nunc) were fixed in 3% formaldehyde and stained over-
night at 37°C with X-gal buffered with sodium phosphate at pH 6.0 in the
presence of potassium ferrocyanide and potassium ferricyanide as
described previously (55).

Exon microarray processing and analysis. Total RNAs were prepared according
to Affymetrix protocols (Affymetrix). RNA quality and quantity was
ensured using the Bioanalyzer (Agilent) and NanoDrop (Thermo Scientif-
ic), respectively. For RNA labeling, 1 μg total RNA was used in conjunction
with the Affymetrix-recommended protocol for GeneChip WT Sense Target
Labeling. The total RNA was first subjected to a ribosomal RNA reduction
using the Invitrogen RiboMinus Transcriptome Isolation Kit. The remaining
RNA was then reverse transcribed in the presence of dT7N6 primers
to generate cDNA. An in vitro transcription was then performed, and the
resulting cRNA was purified. A first-strand cDNA was generated from the
cRNA in the presence of dUTPs. After RNA hydrolysis, the single-stranded
cDNA was fragmented and end-labeled with biotin. The hybridization cock-
tail containing the fragmented and biotin-labeled cDNAs were hybridized to the
Affymetrix GeneChip Human Exon 1.0 ST Array. The chips were washed
with 0.5% Triton-X 100 in PBS, or with methanol/Acetone (1:1) at −20°C as
shown in the FITC and Spectrum Orange channels. At least 20–30 randomly
chosen nuclei were processed per cell line.

Expression score for a probe set was calculated as the median of all probes
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shown in the FITC and Spectrum Orange channels. At least 20–30 randomly
chosen nuclei were processed per cell line.

Immunofluorescence analysis. Immortalized cells or primary fibroblasts
grown on chamber slides (Nunc) were fixed with 4% formaldehyde in
PBS for 20 minutes at room temperature followed by a 5-minute treatment
with 0.5% Triton-X 100 in PBS, or with methanol/Acetone (1:1) at −20°C as
described previously (26). The fixed cells were rinsed with PBS and blocked
with 10% horse serum and 4% BSA in PBS for 30 minutes. Cells were then
incubated for 1 hour with the primary antibodies diluted in blocking solution.
The secondary antibodies were Alexa 488- or Alexa 594-conjugated
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