Telomere Length Is a Determinant of Emphysema Susceptibility

Jonathan K. Alder¹, Nini Guo¹, Frant Kembou¹, Erin M. Parry¹, Collin J. Anderson¹, Amany I. Gorgy¹, Michael F. Walsh², Thomas Sussan³, Shyam Biswal^{3,4}, Wayne Mitzner³, Rubin M. Tuder⁵, and Mary Armanios^{1,2,4}

¹Department of Oncology, ²McKusick-Nathans Institute of Genetic Medicine, and the ⁴Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins University School of Medicine, Baltimore, Maryland ³Department of Environmental Health Sciences, Johns Hopkins Bloomberg School of Public Health, Baltimore, Maryland; and ⁵Division of Pulmonary Sciences and Critical Care Medicine, University of Colorado Denver, Aurora, Colorado

Rationale: Germline mutations in the enzyme telomerase cause telomere shortening, and have their most common clinical manifestation in age-related lung disease that manifests as idiopathic pulmonary fibrosis. Short telomeres are also a unique heritable trait that is acquired with age.

Objectives: We sought to understand the mechanisms by which telomerase deficiency contributes to lung disease.

Methods: We studied telomerase null mice with short telomeres.

Measurements and Main Results: Although they have no baseline histologic defects, when mice with short telomeres are exposed to chronic cigarette smoke, in contrast with controls, they develop emphysematous air space enlargement. The emphysema susceptibility did not depend on circulating cell genotype, because mice with short telomeres developed emphysema even when transplanted with wild-type bone marrow. In lung epithelium, cigarette smoke exposure caused additive DNA damage to telomere dysfunction, which limited their proliferative recovery, and coincided with a failure to down-regulate p21, a mediator of cellular senescence, and we show here, a determinant of alveolar epithelial cell cycle progression. We also report early onset of emphysema, in addition to pulmonary fibrosis, in a family with a germline deletion in the Box H domain of the RNA component of telomerase.

Conclusions: Our data indicate that short telomeres lower the threshold of cigarette smoke-induced damage, and implicate telomere length as a genetic susceptibility factor in emphysema, potentially contributing to its age-related onset in humans.

Keywords: telomerase; chronic obstructive pulmonary disease; dyskeratosis congenita; interstitial lung disease

Lung function declines with age (1). This decline frequently manifests as progressive, irreversible organ failure notably in two recognized clinical settings: emphysema and idiopathic pulmonary fibrosis (IPF). These disorders represent major burdens

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

The inherited factors that underlie emphysema susceptibility are not known.

What This Study Adds to the Field

This study identifies telomere length, which is known to shorten with age, as a genetic determinant of cigarette smoke-induced emphysema susceptibility.

of disability and mortality world-wide, and currently no therapies short of lung transplant are known to significantly change their natural history. Although sometimes considered distinct, emphysema and IPF frequently coexist (2, 3), suggesting they may have a shared etiology and pathobiology. In addition to age, cigarette smoke (CS) exposure is known to accelerate the onset of both emphysema and IPF (4, 5). In some patients, even long after CS cessation, there is often a progressive decline in lung function (6), suggesting that age-related factors may cooperate with sustained CS-induced lung damage to cause these disorders. Understanding the biology underlying the susceptibility to these fatal disorders holds promise for rational prevention and therapy strategies that can improve outcome.

Telomeres are DNA-protein structures that protect chromosome ends from degradation. Telomeres shorten progressively with cell division and critically short telomeres signal a DNA damage response that can lead to apoptosis (7–9). Short telomeres are also a potent inducer of senescence, a permanent state of impaired cell cycle progression associated with accumulation of cyclin-dependent kinase inhibitors (7, 10). Telomerase is a specialized polymerase that synthesizes telomere repeats (11, 12). Telomerase has two essential components: a catalytic reverse transcriptase, TERT, which copies from a template within the RNA component, TR, to add new telomere sequence onto chromosome ends (13–16). In humans and mice, telomerase deficiency and short telomeres cause a stem cell failure syndrome, which manifests as a loss of regenerative capacity in tissues of rapid turnover: the skin, mucosa, and bone marrow (reviewed in Reference 17). Recent studies in families with IPF have indicated that telomerase mutations play a critical role in the genetics of lung disease (18, 19). In fact, inherited mutations in the essential telomerase components *hTERT* and *hTR* are the most commonly identifiable defect in families with pulmonary fibrosis, accounting for 10–15% of all cases (17). Short telomeres are also a risk factor for IPF (20). A recent study additionally noted shorter telomeres in the lungs of individuals with emphysema when compared with unaffected individuals

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Correspondence and requests for reprints should be addressed to Mary Armanios, M.D., Department of Oncology, Johns Hopkins University School of Medicine, 1650 Orleans Street, CRB Room 186, Baltimore, MD 21287. E-mail: marmani1@jhmi.edu

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with comparable CS exposure (21). However, the role of telomere length as a determinant of CS-induced lung disease in a genetically defined animal model has not been examined.

To approach this question, we studied telomerase-deficient mice. In telomerase-null mice, degenerative phenotypes are seen only when telomeres are short, which has established telomere length, and not telomerase loss itself, as the relevant genetic defect (8, 22–25). Studies in two mouse strains have shed light on the role of telomere length in disease. On the C57BL/6 strain of *Mus musculus*, mice have long heterogeneous telomere lengths (~50 kb) and short telomeres can only be generated after several generations of breeding of telomerase RNA-null, mTR^{-/-}, mice (23). The CAST/EiJ strain has shorter telomere lengths comparable with humans (~15 kb), and telomerase-null mice on this strain develop more severe phenotypes (24, 26). In both genetic backgrounds, short telomeres cause epithelial defects manifesting clinically as impaired wound healing in the skin, and mucosal atrophy in the intestinal tract (22, 24). However, it is not known whether short telomeres affect the homeostasis of alveolar epithelium, a putative site of injury in emphysema and IPF. Here we show that, although mice with short telomeres have no baseline histologic defects, they are more susceptible to developing emphysema after CS exposure. The emphysema susceptibility defect is intrinsic to the lung parenchyma, and CS causes additive DNA damage to telomere dysfunction thus impairing epithelial recovery. We also report young-onset emphysema cases in telomerase mutation carriers with CS exposure history. Our data identify short telomere length as a genetic determinant of emphysema susceptibility and of CS-induced lung disease.

METHODS

Mice

Mice were housed at the Johns Hopkins University School of Medicine campus, Baltimore, Maryland, and all procedures were approved by its Institutional Animal Care and Use Committee. CAST/EiJ mTR^{+/-} mice with short telomeres were generated by interbreeding heterozygous mice for 8–10 generations as described (24). C57BL/6J mTR^{-/-} G4 mice were generated by successive breeding of mTR^{-/-} mice for four generations (referred to as G4) (23), and generation 4 was the terminal, infertile generation in our colony. p21^{-/-} mice were purchased from the Jackson Laboratory (B6129S2-Cdkn1a^{-/-} and B6129SF2/J controls; Bar Harbor, ME). For proliferation studies, EdU was purchased from Invitrogen (Carlsbad, CA) and a mini-osmotic pump was placed subcutaneously for 14 days (Alzet, Cupertino, CA). Pumps were loaded with EdU dissolved in sterile saline at 10 mg/ml.

CS Exposure

Mice were exposed for 6 hours each day, 5 days per week, with 3R4F cigarettes (University of Kentucky, Tobacco Research Institute, Lexington, KY) at a total suspended particle count of 150 mg/m³ using a TE-10 smoking apparatus (Teague Enterprises, Woodland, CA) (27). The smoking apparatus was adjusted to produce a mixture of sidestream smoke (89%) and mainstream smoke (11%). On the final experiment day, lungs were harvested within 2 hours of exposure or left in filtered air to recover. For the transplant and short-term experiments, mice were exposed for 2.5 hours each day, 5 days per week at a total suspended particle count of 250 mg/m³, to deliver an intensive exposure over a shorter daily time frame.

Fixation and Morphometry

After pulmonary function testing, lungs were perfused with 10 ml of phosphate-buffered saline. The right lung was ligated and the left lung was then inflated with warm (50°C) 1% low-melt agarose at 25 cm H₂O. The inflation pressure was measured continuously until the agarose started to gel. The trachea was then clamped and the lungs excised

and placed on ice. The right lung was snap frozen and stored at -80°C. Lung tissues were then placed in 10% phosphate-buffered formalin for at least 48 hours. Before fixation, the left lung was dissected and sliced. For morphometry studies, 5-μm sections were cut and stained with hematoxylin and eosin (H&E), and Masson's trichrome stains were performed in a clinical laboratory. Fifteen images were acquired using a Nikon Eclipse 50i (Nikon, Tokyo, Japan) at ×100 magnification. Mean linear intercept (MLI) was determined by computer-assisted morphometry using a macro designed with MetaMorph software (Molecular Devices, Sunnyvale, CA) (27).

Pulmonary Function Studies and Quasistatic Pressure–Volume Curves

Mice were sedated with ketamine and xylazine, and a tracheostomy was performed with an 18-gauge cannula. The tracheal cannula was occluded for 5 minutes, which led to complete degassing of the lungs by absorption atelectasis. Air was infused with a syringe pump, and airway pressure and volume were recorded on a PowerLab digital data acquisition system running Chart v5.3 software (ADInstruments, Castle Hill, Australia). Once a pressure of 35 cm H₂O was reached, lungs were deflated to -10 cm H₂O at a rate of 3 ml/min. Two sequential pressure–volume loops between 0 and 35 cm H₂O were then acquired. Residual volume was measured at a pressure of -10 cm H₂O during the first deflation. TLC was defined as the volume at 35 cm H₂O from the first inflation loop. V₁₀ was defined as the percentage of the TLC at 10 cm H₂O. Specific compliance of the quasistatic respiratory system was computed from the pressure–volume relationships as the slope of the deflation limb from 3–8 cm H₂O divided by the lung volume at 35 cm H₂O.

Bone Marrow Transplant

Female recipient mice were lethally irradiated (9.5 Gy; MSD Nordion Gammacell 40 Exactor, Nordion, Ottawa, ON, Canada) and 5 × 10⁶ whole bone marrow cells from a male donor were injected intravenously. After smoke exposure, marrow was harvested and genomic DNA was purified using a Puregene kit (Qiagen, Valencia, CA). We quantified engraftment by measuring the levels of *Sry* relative to *β-actin* by quantitative polymerase chain reaction (PCR) (28).

Immunofluorescence and Immunohistochemistry

Tissue sections were deparaffinized and hydrated through sequential xylene incubations and decreasing ethanol concentrations. Antigen retrieval was performed in unmasking solution (Vector Laboratories, Burlingame, CA). Slides were blocked and prepared using standard procedures and incubated with primary antibodies from the following manufacturers: SPC (Chemicon, Billerica, MA); 53BP1 (Novus Biologicals, Littleton, CO); Mac-3 (BD Biosciences, Franklin Lakes, NJ); and CC10 and p21 (SantaCruz Biotechnology, Santa Cruz, CA). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining was performed using an *in situ* cell death detection kit (Roche, Indianapolis, IN). Telomere length was measured in paraffin-embedded tissues in alveolar type 2 cells using quantitative fluorescence *in situ* hybridization (FISH) (20). Images were obtained on a Zeiss Axioscope (Zeiss, Jena, Germany). Immunohistochemistry was performed using a Vectastain Elite ABC kit (Vector Laboratories). All histology and immunofluorescence analyses were performed masked to genotype.

Quantitative Real-time Reverse Transcription PCR

Total RNA was extracted from approximately 100 mg of frozen lung tissue. The tissue was placed in Trizol (Invitrogen) and homogenized in a bullet blender (Next Advance Inc., Cambridge, MA). RNA was DNase treated and column purified (RNAeasy, Qiagen). cDNA was prepared using superscript III (Invitrogen). A total of 50 ng of cDNA was used for each PCR reaction. A standard curve was generated for each target by cloning the PCR product into a plasmid and preparing serial dilutions. Primers were designed to span introns, and all products were sequence-verified. All PCR efficiencies were greater than 80% and reactions were performed in triplicate. Quantitative reverse transcription PCR was performed on a CFX96 thermocycler using iQ SYBR

Green Supermix (BioRad, Hercules, CA). The expression of each gene was normalized to hypoxanthine phosphoribosyltransferase 1 and β_2 -microglobulin using the Bio-Rad software. Primer sequences are listed in the online supplement.

Subjects

Subjects were evaluated at Johns Hopkins Hospital. The study was approved by the Johns Hopkins Medicine Institutional Review Board and participants gave written, informed consent. *hTR* was sequenced from genomic DNA, and lymphocyte telomere length was measured using flow-FISH (18). *hTR* levels were measured in early passage lymphoblastoid cells from mutation carriers and noncarriers using quantitative reverse transcription PCR (29).

Statistics

We used GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego CA). Means were compared using Student *t* test, and all *P* values are two-sided.

RESULTS

Mice with Short Telomeres Do Not Have Obvious *De Novo* Fibrosis or Emphysema

To examine whether mice with short telomeres develop *de novo* disease, we first examined lung histology in adult CAST/EiJ *mTR*^{+/-} late-generation and C57BL/6J *mTR*^{-/-} G4 mice. We did not detect any fibrosis as quantified by Masson's trichrome staining (see Figures E1 and E2 in the online supplement). There was also no obvious baseline air space disease in short telomere mice from either strain (Figures 1A and 1B; see Figures E1 and E2). Specifically, morphometry studies revealed no differences in the MLI (Figures 2A and 2B; see Figure E1A). These data indicated that adult mice with short telomeres, at least on the CAST/EiJ and C57BL/6J strains, do not develop spontaneous fibrosis or air space enlargement in the age groups we examined.

Mice with Short Telomeres Are More Susceptible to CS-induced Lung Disease

CS exposure is a risk factor in age-related lung disease. We therefore tested whether genetically determined short telomere length predisposes mice to develop lung disease after a chronic exposure. We randomized age- and sex-matched CAST/EiJ wild-type and short telomere mice to either filtered air or CS in an automated chamber for 6 months; however, neither group

developed weight loss or morphometry defects indicating this is a resistant strain (30), even when telomeres are short (data not shown). We similarly randomized age- and sex-matched C57BL/6J wild-type and G4 mice. C57BL/6J mice are known to be modestly susceptible to CS (30), and indeed both wild-type and G4 mice had decreases in body weight confirming their susceptibility (Figure 1C; see Figure E3A).

We then examined whether short telomeres determined the severity of CS-induced injury assessed by lung morphometry and lung function studies. Wild-type C57BL/6J mice developed no significant air space disease compared with air-exposed controls (Figures 1A and 1B), as previously shown (27). In contrast, G4 mice had a significantly larger MLI than air-exposed controls, indicating emphysematous air space changes (Figures 1A and 1B). The emphysematous changes were regional in G4 mice with focal areas more prominently affected (Figure 1A). The increased MLI was paralleled by alterations in pulmonary function with G4 mice having the largest ratio of residual volume to TLC, a physiologic measure of emphysema (Figure 1C; see Figure E3B). Lung mechanics further showed that, in contrast to air-exposed controls, G4 mice had significant decreases in the percentage of TLC at a fixed pressure (V_{10}), indicative of altered functional residual capacity (Figure 1C; see Figure E3C). G4 mice exposed to CS also had decreased lung volume-adjusted compliance (Figure 1C; see Figure E3D). Similar compliance defects have been reported in CS-exposed murine models (31, 32). Because humans develop increased compliance in the setting of emphysema, the trends we report, along with others' previously, likely represent differences in the consequences of emphysema on lung mechanics in rodents especially because we found no evidence of increased collagen deposition or synthesis in G4 mice after CS exposure by both Masson's trichrome staining and active collagen expression (see Figure E2). These morphometric and lung function studies indicated that short telomeres are a determinant of CS susceptibility in murine emphysema.

The Telomere-mediated CS Susceptibility Is Independent of Circulating Cell Genotype

CS induces an exuberant inflammatory response in the lung, and inflammation is considered a critical determinant of emphysema pathogenesis (6). Compared with air-exposed controls, CS-exposed mice had significantly higher levels of alveolar macrophages (Figures 2A and 2B), as has been seen in other

Figure 1. C57BL/6J mice with short telomeres develop emphysematous changes after cigarette smoke (CS) exposure. (A) Representative images show air space enlargement in G4 mice compared with controls after 6 months exposure (hematoxylin and eosin, original magnification $\times 100$). RA = room air. The emphysematous changes shown in the lower right panel represent regional areas of emphysema that were seen in short telomeres CS-exposed mice. (B) Mean linear intercept (MLI) quantification in a blinded analysis from 12–15 mice per group. Mice were 9–11 months of age, and were sex-matched across groups. (C) Table shows mean values of body weight and pulmonary function studies. RV/TLC = ratio of the residual volume relative to the total lung capacity ($n = 7$ –9 mice per group). V_{10} = percentage of TLC at 10 cm H_2O . Specific compliance = compliance corrected for TLC. Error bars = SEM. * $P < 0.05$; ** $P < 0.01$. *P* values in C refer to comparisons with mean in respective RA-exposed control group. WT = wild-type.

Figure 2. The telomere-mediated emphysema susceptibility is independent of circulating cells. (A) Representative images of alveolar macrophages detected by Mac-3 immunohistochemistry indicated by arrows. (B) Quantitation of alveolar macrophages in room air (RA) and cigarette smoke (CS) exposed groups after 6 months exposure. The mean number of macrophages was calculated based on counts per high power field (HPF) ($n = 8-9$ mice per group, eight images per lung). WT = wild-type. (C) WT mice transplanted with WT bone marrow (hematoxylin and eosin, original magnification 100 $\times$). (D) Representative image of air space enlargement in a G4 mouse that received a bone marrow transplant from a WT donor. (E) Mean linear intercept (MLI) after 6 months CS exposure ($n = 3-5$ mice per group). (F) Macrophage quantitation of transplanted mice shown in E. For E and F, mice were 11–14 months at the time of analysis. Error bars = SEM. * $P < 0.05$; ** $P < 0.01$.

studies (27). However, when we compared the two groups of CS-exposed mice, we did not identify statistically significant differences in the number of alveolar macrophages even after correcting for the MLI (Figure 2B; see Figure E4A).

The observation that G4 mice were more susceptible to CS suggested that the emphysema observed in G4 mice may be caused by a telomere defect in either the inflammatory cells themselves or resident lung cells. To distinguish these possibilities, we performed an adoptive transfer anticipating that if the susceptibility were derived from circulating cells, wild-type bone marrow would rescue the CS susceptibility in G4 mice. Similarly, wild-type mice that receive bone marrow from G4 donors would acquire the susceptibility. To control for the effects of radiation, we transplanted wild-type mice with wild-type bone marrow, and G4 mice with G4 bone marrow. By Day 28 after lethal irradiation, in contrast to saline-injected mice, all the transplanted mice survived indicating successful donor engraftment. Mice were then exposed to CS for 6 months, and at the end of the exposure, we confirmed successful donor engraftment (see Figure E4B). In wild-type recipients that received bone marrow from either a wild-type or G4 donor, there was no evidence of CS-induced emphysema as evidenced by the unchanged MLI, similar to data in untransplanted mice (Figures 1A, 1B, and 2E). In contrast, G4 mice showed significant increases in MLI regardless of whether they received wild-type or G4 marrow (Figure 2E). Importantly, when we quantified the inflammatory response, we found that CS-exposed recipient mice, both wild-type and G4, had similar alveolar macrophage counts independent of donor genotype (Figure 2F), suggesting that macrophage recruitment in our model was independent of telomere length. These data indicated that the telomere-mediated CS susceptibility did not depend on the genotype of circulating cells, but was likely intrinsic to resident lung cells.

CS Causes Additive DNA Damage to Short Telomeres in Lung Epithelium

Because our data indicated that short telomeres in resident lung cells likely determine the emphysema susceptibility, we

focused on defining how telomere length affects epithelial homeostasis after CS, a key event in the pathogenesis of the air space enlargement that characterizes this disease (6). We first quantified DNA damage by examining DNA double-strand breaks detected by 53BP1 foci specifically in lung epithelial cells. The 53BP1 protein binds to DNA at the site of double-strand breaks, and we found that at baseline the percentage of damaged terminal bronchiole epithelial cells was fivefold higher in G4 mice (Figures 3A and 3B), consistent with their known dysfunctional telomeres that bind 53BP1 (9, 23, 33). In mice that were exposed to CS, there was additive damage with G4 mice accumulating the greatest burden of cells with double-strand DNA breaks (Figure 3B). We could not specifically stain for 53BP1 foci in type 2 alveolar epithelial cells (AECs) because of technical difficulties, but adjacent terminal bronchiole Clara epithelia likely reflect damage patterns in these adjacent AECs similarly exposed to CS. We next examined whether CS may have caused an acquired state of telomere shortening by measuring telomere length by quantitative FISH in AECs (20). We detected fewer telomere signals in G4 mice compared with wild-types as predicted; however, we could not detect additional telomere shortening in CS-exposed groups (Figures 3C and 3D). Because C57BL/6 mice have long heterogeneous telomeres making it difficult to detect subtle differences in mean telomere length (23), this highly sensitive FISH-based assay does not entirely exclude the possibility of minor telomere shortening after a 6-month exposure to CS. To assess whether other forms of damage, such as oxidative stress, may be increased in the susceptible group, we examined nitrotyrosine and 8-hydroxy-2-deoxyguanosine immunohistochemistry, but did not detect differences between wild-type and G4 mice exposed to CS (not shown). Together, these data indicate that the short telomeres, as genetically determined in G4 mice, and the environmentally acquired CS-induced DNA damage are additive.

Short Telomeres Limit Epithelial Recovery after CS

To determine the cellular consequences of CS-induced damage and short telomeres, we examined evidence of apoptosis. The

Figure 3. Cigarette smoke (CS) causes additive DNA damage to telomere dysfunction. (A) Representative images of terminal bronchioles identifies damage foci detected by 53BP1 nuclear foci (green) in Clara cells identified by cytoplasmic CC10 (red). (B) Percentage of cells containing DNA damage foci after 6 months CS exposure ($n = 7$ –10 mice per group). WT = wild-type. (C) Merged triple color immunofluorescence images of telomere signals (red) in type 2 alveolar cells marked by SPC staining (green) and nuclei (blue). The number of nuclear signals per DAPI area is fewer in G4 cells. (D) The number of telomere signals relative to DAPI area is shown in the bar graph ($n = 12$ –15 mice per group, 20–30 nuclei were imaged and quantified). Mice were exposed to CS for 6 months. AEC = alveolar epithelial cells; DAPI = 4',6-diamidino-2-phenylindole; error bars = SEM. * $P < 0.05$; *** $P < 0.001$.

baseline apoptosis rate was very low and G4 mice did not exhibit a significant increase in TUNEL-positive cells relative to wild-type controls (see Figure E4C). Although there was an increase in both groups with CS, this difference was not statistically significant (see Figure E4C). Thus, short telomeres do not seem to contribute significantly to elevated apoptosis after 6 months of CS at the single time point we examined.

Because short telomeres are a potent inducer of cellular senescence, we next examined epithelial proliferation *in vivo* during and after CS. Because the proliferative rate of AECs is slow at any given time, we studied the dynamics of epithelial proliferation during a 14-day experiment by implanting mini-osmotic pumps that continuously delivered EdU, a thymidine analog, and measured proliferation at baseline, during CS exposure, and in the recovery period after CS exposure, 14 days later. At baseline, control and G4 mice had similar fractions of proliferating AECs (Figure 4A). During a 14-day CS exposure, the proliferation of type 2 AECs dropped similarly in wild-type and G4 mice (Figure 4A). However, when we quantified the proliferative AEC fraction after a 14-day recovery period, it significantly lagged in G4 mice (Figure 4A). We also found similar defective proliferation of terminal bronchiole Clara cells during the recovery period (Figure 4B). These data indicate that epithelial proliferation is dynamic during and after CS exposure, and that short telomeres limit the proliferative recovery of epithelial cells after CS injury.

Failure to Down-Regulate p21 in Short Telomere Lungs after CS

The fact that short telomeres impair the recovery of epithelial cells after CS suggested that some cells may show hallmarks of senescence. Because senescence caused by telomere shortening has been associated with accumulation of specific cyclin-dependent kinase inhibitors (34–36), we measured their expression in whole-lung lysates by real-time PCR. We compared levels in two different experiments. First, in a long-term experiment, we examined mice exposed to air and CS at baseline, at the end of a 6-month CS exposure, and during the recovery period, 1 week later. In a short-term experiment, we measured levels at baseline, immediately after a 14-day CS exposure, and during recovery 14 days later. The latter time points are identical

to the *in vivo* labeling experiments shown in Figures 4A and 4B. p15^{INK4b} and p27 had similar baseline levels in wild-type and G4 lungs, whereas p16^{INK4a} levels were higher in G4 lungs from older mice (Figure 5D), as seen previously (35). Nonetheless, levels of all three of these cyclin-dependent kinase inhibitors were not affected by CS immediately or during the recovery periods in both the short- and long-term experiments (Figures 4C, 4D, 4F–4H, and 4J). However, in both experiments, p21 expression levels were dynamic, increasing modestly after CS as seen previously (37). Remarkably, p21 levels fell precipitously during the recovery levels dropping up to 15-fold in wild-type lungs during the proliferative recovery (Figures 4E and 4I). Importantly, p21 levels failed to down-regulate in G4 lungs at the recovery time point in both experiments with up to sixfold higher levels relative to control lungs (Figures 4E and 4I). These data, which paralleled the defects in epithelial proliferation in G4 mice, indicated that higher levels of p21 may contribute to the telomere-mediated proliferative lag we observed in the recovery period (Figures 4A and 4B).

To investigate whether p21 plays a role in epithelial cell cycle progression, we examined alveolar cells and found that the p21 protein was detectable in wild-type luminal alveolar cells by immunofluorescence (Figure E4D). We next examined the cell cycle progression of pulmonary epithelium in p21 knockout mice by examining EdU incorporation. We found that p21^{-/-} animals had strikingly a twofold increase in the fraction of proliferating AECs and Clara cells lining terminal bronchioles (Figures 5A–5F). Importantly, there was no concurrent increase in the basal proliferation rate of intestinal villous epithelium indicating that p21 may specifically regulate the cell cycle progression of pulmonary epithelium (Figures 5G–5I). These data indicated that p21 is a determinant of distal pulmonary epithelial-cell cycle progression.

Young-onset Emphysema in Telomerase Mutation Carriers

Telomerase mutations are a risk factor for IPF, but heterogeneous pulmonary disease phenotypes have been seen in telomerase mutation carriers (18, 38, 39). We sought to determine whether telomerase mutations and short telomeres are susceptibility factors in human CS-induced emphysema. We identified a family with a combined emphysema-fibrosis spectrum of lung disease. The proband was a 55-year-old female who was

Figure 4. Short telomeres limit epithelial recovery after cigarette smoke (CS). (A) Type 2 alveolar epithelial proliferation was measured at three time points: at baseline, immediately after 14 days CS exposure, and 14 days after CS ended. For each time point, a mini-osmotic pump delivered EdU subcutaneously for 14 days, and proliferation in type 2 alveolar cells was identified by costaining with cytoplasmic SPC ($n = 5$ mice per group, 6–8 high power fields per lung). AEC = alveolar epithelial cells. (B) Proliferation of terminal bronchiolar Clara cells was measured similar to A, but with EdU colocalization with CC10 ($n = 5$ mice per group, 5–10 terminal bronchioles per lung). WT = wild-type. (C–F) Relative expression levels of cyclin-dependent kinase inhibitors from three groups of mice are shown from a long-term experiment: room air (RA)-exposed mice represent baseline time point, mice exposed to 6 months of CS, and mice that were exposed to 6 months of CS that were then allowed to recover in RA for 7 days ($n = 5$ –8 mice for each data point, 9–11 mo old). (G–J) Relative expression levels

of cyclin-dependent kinase inhibitors from three groups of mice are shown from a short-term experiment: RA-exposed mice represent baseline time point, mice exposed to 14 days CS exposure, and mice that were exposed to 14 days CS that were then allowed to recover in RA for 14 days ($n = 5$ mice for each data point, 6 mo old). Expression in whole lung lysates was measured by real-time polymerase chain reaction and normalized to *Hprt* and β_2m expression. Wild-type RA control group transcript levels were assigned a value of 1 for relative comparison. Error bars = SEM. * $P < 0.05$; ** $P < 0.01$.

diagnosed with emphysema at the age of 44 after a 29 pack-year smoking history (Figures 6A–6C). The family history was notable for her father who died from IPF at the age of 70, and a niece with bone marrow failure (pedigree in Figure 6A). Her sister was diagnosed at the age of 34 with a combined emphysema and pulmonary fibrosis syndrome after a 15 pack-year smoking history and subsequently died at age 46 from end-stage lung disease (Figures 6A, 6D, and 6E). Both the proband and her sister had normal α_1 -antitrypsin levels. In light of the family history suggestive of a telomerase defect (40), we sequenced the telomerase genes and identified a novel heterozygous mutation *hTR* del375–377 within the essential Box H motif, which is critical for the biogenesis and stability of hTR (41–43). The *hTR* mutation segregated with the pulmonary disease in affected family members, and quantification of hTR levels in cells from mutation carriers showed they had approximately 50% of the levels of their first-degree noncarrier relatives, consistent with a haploinsufficiency mechanism of telomerase deficiency (Figure 6H). Mutation carriers also had significantly short lymphocyte telomere length relative to age-matched controls (less than the first percentile; Figure 6I). The observations in this family indicated that inherited mutations in telomerase might be risk factors for young-onset emphysema, alone or in combination with pulmonary fibrosis, in some individuals with a smoking history.

DISCUSSION

We sought to understand the mechanisms by which short telomeres might contribute to lung disease in humans by studying telomerase-null mice. We identified telomere length and telomerase deficiency as a susceptibility factor in emphysema. Late-generation telomerase-null mice developed emphysematous air space enlargement after a chronic CS exposure as evidenced by morphometric differences that affected lung function. Telomere length is heterogeneous and heritable across populations, and short telomeres accumulate with age (7, 25, 44). Our data indicate that short telomere length is a genetic determinant of emphysema in mice, and may contribute to the susceptibility to CS-induced lung disease with age in humans.

Several pieces of evidence in our study point to epithelial injury being a primary mechanism of the telomere-associated CS-induced susceptibility. We show that the telomere-associated emphysema susceptibility does not depend on a telomere defect in bone marrow-derived cells. In adoptive transfer, short telomeres caused emphysema susceptibility independent of inflammatory cell genotype indicating that although the inflammatory response after CS is striking, the recruitment of macrophages *per se* is not sufficient to induce emphysema in our model. In addition, we show that in epithelial cells, DNA damage caused by CS is additive to telomere dysfunction with short telomere mice carrying the greatest burden. Because of the slow turnover rate

Figure 5. p21 is a determinant of basal pulmonary epithelial proliferation. (A) Representative immunofluorescence showing EdU staining (red) in type 2 alveolar epithelial cells (AECs) marked by cytoplasmic SPC (green) in wild-type (WT) mice and in (B) p21^{-/-} mice. (C) The percentage of proliferating AECs in p21^{-/-} mice was higher as shown in the bar graph. (D and E) Representative images showing EdU staining (red) in terminal bronchiole Clara cells (green) in WT and p21^{-/-} lungs, respectively. (F) Increased proliferative fraction of EdU-CC10-positive cells in bar graph. (G and H) Intestinal tract epithelium shows EdU incorporation (red) in WT and p21^{-/-} mice, respectively. (I) Bar graph shows quantitation after twice-daily injections for 2 days. n = 5 mice per group, age 3–4 months, 10 HPF were analyzed per mouse. Error bars = SEM. * P < 0.05.

of lung epithelium, we developed an assay to track epithelial-specific proliferation *in vivo* and show that it is dynamic during and after CS exposure. In parallel to the dynamics of epithelial proliferation, p21 levels are significantly down-regulated during

the recovery phase (increased proliferation), and in short telomere mice there is a failure of this down-regulation. *In situ* studies have also found p21 up-regulation in human lungs with emphysema (21). These data suggest that p21 is a candidate

Figure 6. Early onset emphysema as a manifestation of inherited telomerase mutation. (A) Pedigree with features of telomere syndrome including idiopathic pulmonary fibrosis (IPF), bone marrow failure, and premature hair graying shows autosomal-dominant inheritance. The proband is indicated by an arrow and the shaded squares and circles indicate mutation carrier. I1 is a probable carrier, and I13 is an obligate carrier. The summary of the clinical history is listed below with CS referring to the cigarette smoke exposure history. (B and C) Apical and mid-lung computed tomography (CT) images from the proband show evidence of emphysema with apical bullae and hyperinflation. (D and E) CT images of I13 show subcutaneous emphysema caused by a spontaneous pneumothorax, apical bullae, and mid-lung ground glass infiltrates with septal thickening consistent with a combined emphysema and interstitial lung disease phenotype. (F) Chromatogram showing heterozygous deletion in hTR, the RNA component of telomerase.

(G) Secondary structure of hTR shows site of the three nucleotide deletion within the essential Box H domain in red. (H) Quantification of hTR levels in cells from unaffected relatives (n = 3), affected mutation carriers (n = 3), and DKC1 mutation carriers (n = 2). Data were generated by quantitative real-time polymerase chain reaction and hTR levels were normalized to ARF3 levels. ** P < 0.01. (I) Lymphocyte telomere length in proband and mutation carriers shows significant shortening relative to age-matched controls. Identifiers refer to pedigree in A.

effector of the senescence-like phenotype we observe and the associated emphysema susceptibility. Choudhury and coworkers (45) have shown that p21 loss rescues telomere degenerative defects in the bone marrow. Future studies in compound p21 knockout mice with short telomeres can definitively establish whether rescue of the telomere-mediated emphysema susceptibility by p21 deletion is feasible and are ongoing. p21 is a known tumor suppressor in lung cancer and p21^{-/-} mice are prone to develop spontaneous lung adenocarcinoma with age (46). These data together highlight the role of p21 as a regulator of epithelial cell cycle.

How might epithelial proliferative defects in short telomere mice lead to alveolar breakdown? Our data suggest at least two possibilities: First, it is possible that the defective type 2 AEC proliferation directly leads to regenerative failure and remodeling of the lung. We identified proliferative defects up to 14 days after CS exposure, so in the setting of repetitive injury cycles as occurs with CS, it may be that defects become eventually irreversible and directly lead to regional alveolar loss. Chronic low-grade apoptosis caused by the combined effect of CS-induced and telomere damage may similarly contribute to alveolar loss. Another possibility is that the combined DNA damage induces a senescence phenotype and this indirectly contributes to alveolar destruction. Senescence is a complex process associated with gene expression changes and a cytokine and protease secretory phenotype (47). A recent study showed that senescent alveolar cells are associated with a higher proinflammatory cytokine profile *in vitro* (48); however, the relevance of this phenotype *in vivo* has not been examined. Although our data do not entirely exclude other lung parenchymal cells or extrapulmonary factors, such as nutritional deficiency, as contributing to the telomere-mediated emphysema, our studies support a model where epithelial injury is the primary determinant of the emphysema susceptibility.

We did not identify *de novo* pulmonary fibrosis or emphysema in the mice with short telomeres in the age groups we examined. Our findings are in contrast to a prior study that identified *de novo* airspace changes in late-generation mTR^{-/-} mice, a difference that may reflect differences in phenotype severity caused by telomere length heterogeneity, or other strain-specific factors (49). The absence of a phenotype in our mice within the relatively short mouse lifespan is consistent with the finding that in individuals who inherit telomerase mutations, disease onset is rare before the age of 40 (18, 19, 29, 39, 50). Emphysema onset is also age-dependent and is rare before the sixth decade (3, 6). Thus, although short telomere length alone does not cause disease phenotypes, the combined telomere and CS-induced damage together overcomes a threshold and manifests as emphysema. The clinical observations, along with findings in our model, indicate that even though the short telomere defect alone is not sufficient to induce lung disease in mice, it serves as a first genetic hit in a likely multistep process that is cumulative with age, and accelerated with CS.

Previous work has established a causal role for short telomeres in IPF, and our studies here extend the role of short telomeres in age-related lung disease susceptibility to include emphysema. Emphysema and pulmonary fibrosis have traditionally been considered distinct clinical entities; however, in recent years it has become clear that as many as 20% of patients with emphysema have concurrent interstitial lung disease (2, 3). Here we show that within a single family, the pulmonary manifestations of telomerase insufficiency are heterogeneous and can include emphysema, IPF, and the combined syndrome. In a cohort of telomerase mutation carriers, 5% of cases were reported to have a history of spontaneous pneumothorax or had the diagnosis of chronic obstructive pulmonary disease (39). It may therefore be that

emphysema alone or combined with pulmonary fibrosis are rare manifestations of inherited telomerase mutations. Identifying the factors that determine whether the first presentation in telomerase mutation carriers is primarily emphysema, IPF, or both is important to examine in larger studies. Given the early onset of disease in the patients we describe, consideration of telomerase genetic testing may be indicated in young-onset patients with emphysema who have a personal or family history suspicious for telomere-mediated disease (17, 40).

In summary, we report that short telomere length is a susceptibility factor for CS-induced emphysema in mice. Our data indicate that short telomeres may contribute to the differential susceptibility to CS across populations, and with aging.

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References

1. Fowler RW. Ageing and lung function. *Age Ageing* 1985;14:209–215.
2. Cottin V, Nunes H, Brillet PY, Delaval P, Devouassoux G, Tillie-Leblond I, Israel-Biet D, Court-Fortune I, Valeyre D, Cordier JF. Combined pulmonary fibrosis and emphysema: a distinct underrecognised entity. *Eur Respir J* 2005;26:586–593.
3. Washko GR, Hunninghake GM, Fernandez IE, Nishino M, Okajima Y, Yamashiro T, Ross JC, Estepar RS, Lynch DA, Brehm JM, *et al.* Lung volumes and emphysema in smokers with interstitial lung abnormalities. *N Engl J Med* 2011;364:897–906.
4. Mason RJ, Buist AS, Fisher EB, Merchant JA, Samet JM, Welsh CH. Cigarette smoking and health. *Am Rev Respir Dis* 1985;132:1133–1136.
5. Steele MP, Speer MC, Loyd JE, Brown KK, Herron A, Slifer SH, Burch LH, Wahidi MM, Phillips JA III, Sporn TA, *et al.* Clinical and pathologic features of familial interstitial pneumonia. *Am J Respir Crit Care Med* 2005;172:1146–1152.
6. Tudor RM, Yoshida T, Arap W, Pasqualini R, Petrache I. State of the art. Cellular and molecular mechanisms of alveolar destruction in emphysema: an evolutionary perspective. *Proc Am Thorac Soc* 2006; 3:503–510.
7. Harley CB, Futcher AB, Greider CW. Telomeres shorten during ageing of human fibroblasts. *Nature* 1990;345:458–460.
8. Lee HW, Blasco MA, Gottlieb GJ, Horner JW II, Greider CW, DePinho RA. Essential role of mouse telomerase in highly proliferative organs. *Nature* 1998;392:569–574.
9. d'Adda di Fagagna F, Reaper PM, Clay-Farrace L, Fiegler H, Carr P, Von Zglinicki T, Saretzki G, Carter NP, Jackson SP. A DNA damage checkpoint response in telomere-initiated senescence. *Nature* 2003; 426:194–198.
10. Campisi J, d'Adda di Fagagna F. Cellular senescence: when bad things happen to good cells. *Nat Rev Mol Cell Biol* 2007;8:729–740.
11. Greider CW, Blackburn EH. Identification of a specific telomere terminal transferase activity in tetrahymena extracts. *Cell* 1985;43: 405–413.
12. Greider CW, Blackburn EH. The telomere terminal transferase of tetrahymena is a ribonucleoprotein enzyme with two kinds of primer specificity. *Cell* 1987;51:887–898.
13. Greider CW, Blackburn EH. A telomeric sequence in the MA of tetrahymena telomerase required for telomere repeat synthesis. *Nature* 1989;337:331–337.
14. Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, *et al.* The RNA component of human telomerase. *Science* 1995;269:1236–1241.

15. Lingner J, Hughes TR, Shevchenko A, Mann M, Lundblad V, Cech TR. Reverse transcriptase motifs in the catalytic subunit of telomerase. *Science* 1997;276:561–567.
16. Singer MS, Gottschling DE. Tlc1: template MA component of *Saccharomyces cerevisiae* telomerase. *Science* 1994;266:404–409.
17. Armanios M. Syndromes of telomere shortening. *Annu Rev Genomics Hum Genet* 2009;10:45–61.
18. Armanios MY, Chen JJ, Cogan JD, Alder JK, Ingersoll RG, Markin C, Lawson WE, Xie M, Vulto I, Phillips JA III, et al. Telomerase mutations in families with idiopathic pulmonary fibrosis. *N Engl J Med* 2007;356:1317–1326.
19. Tsakiri KD, Cronkhite JT, Kuan PJ, Xing C, Raghu G, Weissler JC, Rosenblatt RL, Shay JW, Garcia CK. Adult-onset pulmonary fibrosis caused by mutations in telomerase. *Proc Natl Acad Sci USA* 2007;104:7552–7557.
20. Alder JK, Chen JJ, Lancaster L, Danoff S, Su SC, Cogan JD, Vulto I, Xie M, Qi X, Tudor RM, et al. Short telomeres are a risk factor for idiopathic pulmonary fibrosis. *Proc Natl Acad Sci USA* 2008;105:13051–13056.
21. Tsujii T, Aoshiba K, Nagai A. Alveolar cell senescence in patients with pulmonary emphysema. *Am J Respir Crit Care Med* 2006;174:886–893.
22. Rudolph KL, Chang S, Lee HW, Blasco M, Gottlieb GJ, Greider C, DePinho RA. Longevity, stress response, and cancer in aging telomerase-deficient mice. *Cell* 1999;96:701–712.
23. Blasco MA, Lee HW, Hande MP, Samper E, Lansdorp PM, DePinho RA, Greider CW. Telomere shortening and tumor formation by mouse cells lacking telomerase MA. *Cell* 1997;91:25–34.
24. Hao LY, Armanios M, Strong MA, Karim B, Feldser DM, Huso D, Greider CW. Short telomeres, even in the presence of telomerase, limit tissue renewal capacity. *Cell* 2005;123:1121–1131.
25. Armanios M, Alder JK, Parry EM, Karim B, Strong MA, Greider CW. Short telomeres are sufficient to cause the degenerative defects associated with aging. *Am J Hum Genet* 2009;85:823–832.
26. Hemann MT, Greider CW. Wild-derived inbred mouse strains have short telomeres. *Nucleic Acids Res* 2000;28:4474–4478.
27. Rangasamy T, Cho CY, Thimmulappa RK, Zhen L, Srisuma SS, Kensler TW, Yamamoto M, Petrache I, Tudor RM, Biswal S. Genetic ablation of *nrf2* enhances susceptibility to cigarette smoke-induced emphysema in mice. *J Clin Invest* 2004;114:1248–1259.
28. Kiel MJ, Yilmaz OH, Iwashita T, Terhorst C, Morrison SJ. Slam family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. *Cell* 2005;121:1109–1121.
29. Parry EM, Alder JK, Lee SS, Phillips JA III, Loyd JE, Duggal P, Armanios M. Decreased dyskerin levels as a mechanism of telomere shortening in x-linked dyskeratosis congenita. *J Med Genet* 2011;48:327–333.
30. Guerassimov A, Hoshino Y, Takubo Y, Turcotte A, Yamamoto M, Ghezzi H, Triantafyllou A, Whittaker K, Hoidal JR, Cosio MG. The development of emphysema in cigarette smoke-exposed mice is strain dependent. *Am J Respir Crit Care Med* 2004;170:974–980.
31. Foronjy RF, Mercer BA, Maxfield MW, Powell CA, D'Armiento J, Okada Y. Structural emphysema does not correlate with lung compliance: lessons from the mouse smoking model. *Exp Lung Res* 2005;31:547–562.
32. Wu ZX, Hunter DD, Kish VL, Benders KM, Batchelor TP, Dey RD. Prenatal and early, but not late, postnatal exposure of mice to side-stream tobacco smoke increases airway hyperresponsiveness later in life. *Environ Health Perspect* 2009;117:1434–1440.
33. Hemann MT, Strong MA, Hao LY, Greider CW. The shortest telomere, not average telomere length, is critical for cell viability and chromosome stability. *Cell* 2001;107:67–77.
34. Collado M, Serrano M. The power and the promise of oncogene-induced senescence markers. *Nat Rev Cancer* 2006;6:472–476.
35. Guo N, Parry EM, Li LS, Kembou F, Lauder N, Hussain MA, Berggren PO, Armanios M. Short telomeres compromise beta-cell signaling and survival. *PLoS ONE* 2011;6:e17858.
36. Jacobs JJ, de Lange T. Significant role for p16ink4a in p53-independent telomere-directed senescence. *Curr Biol* 2004;14:2302–2308.
37. Rangasamy T, Misra V, Zhen L, Tankersley CG, Tudor RM, Biswal S. Cigarette smoke-induced emphysema in a/j mice is associated with pulmonary oxidative stress, apoptosis of lung cells, and global alterations in gene expression. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L888–L900.
38. Cronkhite JT, Xing C, Raghu G, Chin KM, Torres F, Rosenblatt RL, Garcia CK. Telomere shortening in familial and sporadic pulmonary fibrosis. *Am J Respir Crit Care Med* 2008;178:729–737.
39. Diaz de Leon A, Cronkhite JT, Katzenstein AL, Godwin JD, Raghu G, Glazer CS, Rosenblatt RL, Girod CE, Garrity ER, Xing C, et al. Telomere lengths, pulmonary fibrosis and telomerase (TERT) mutations. *PLoS ONE* 2010;5:e10680.
40. Parry EM, Alder JK, Qi X, Chen JJ, Armanios M. Syndrome complex of bone marrow failure and pulmonary fibrosis predicts germline defects in telomerase. *Blood* 2011;117:5607–5611.
41. Chen JL, Blasco MA, Greider CW. Secondary structure of vertebrate telomerase MA. *Cell* 2000;100:503–514.
42. Mitchell JR, Cheng J, Collins K. A box h/aca small nucleolar MA-like domain at the human telomerase MA 3' end. *Mol Cell Biol* 1999;19:567–576.
43. Mitchell JR, Wood E, Collins K. A telomerase component is defective in the human disease dyskeratosis congenita. *Nature* 1999;402:551–555.
44. Njajou OT, Cawthon RM, Damcott CM, Wu SH, Ott S, Garant MJ, Blackburn EH, Mitchell BD, Shuldiner AR, Hsueh WC. Telomere length is paternally inherited and is associated with parental lifespan. *Proc Natl Acad Sci USA* 2007;104:12135–12139.
45. Choudhury AR, Ju Z, Djojicubroto MW, Schienke A, Lechel A, Schaetzlein S, Jiang H, Stepczynska A, Wang C, Buer J, et al. Cdkn1a deletion improves stem cell function and lifespan of mice with dysfunctional telomeres without accelerating cancer formation. *Nat Genet* 2007;39:99–105.
46. Martin-Caballero J, Flores JM, Garcia-Palencia P, Serrano M. Tumor susceptibility of p21(waf1/cip1)-deficient mice. *Cancer Res* 2001;61:6234–6238.
47. Coppe JP, Desprez PY, Krtolica A, Campisi J. The senescence-associated secretory phenotype: the dark side of tumor suppression. *Annu Rev Pathol* 2010;5:99–118.
48. Tsujii T, Aoshiba K, Nagai A. Alveolar cell senescence exacerbates pulmonary inflammation in patients with chronic obstructive pulmonary disease. *Respiration* 2010;80:59–70.
49. Lee J, Reddy R, Barsky L, Scholes J, Chen H, Shi W, Driscoll B. Lung alveolar integrity is compromised by telomere shortening in telomerase-null mice. *Am J Physiol Lung Cell Mol Physiol* 2009;296:L57–L70.
50. Alder JK, Cogan JD, Brown AF, Anderson CJ, Lawson WE, Lansdorp PM, Phillips JA III, Loyd JE, Chen JJ, Armanios M. Ancestral mutation in telomerase causes defects in repeat addition processivity and manifests as familial pulmonary fibrosis. *PLoS Genet* 2011;7:e1001352.