Telomerase reverse transcriptase ameliorates lung fibrosis by protecting alveolar epithelial cells against senescence

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Running title: Telomerase and senescence in fibrosis

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Key words: telomerase, telomerase reverse transcriptase (TERT), epithelial cell, senescence, fibrosis, idiopathic pulmonary fibrosis (IPF), lung disease, inflammation, pulmonary dysfunction

ABSTRACT
Mutations in the genes encoding telomerase reverse transcriptase (TERT) and telomerase’s RNA components, as well as shortened telomeres, are risk factors for idiopathic pulmonary fibrosis (IPF), wherein repetitive injury to the alveolar epithelium is considered to be a key factor in pathogenesis. Given the importance of TERT in stem cells, we hypothesized that TERT plays an important role in epithelial repair and that its deficiency results in exacerbation of fibrosis by impairing this repair/regenerative process. To evaluate the role of TERT in epithelial cells, we generated type II alveolar epithelial cell (AECII) specific TERT conditional knockout mice (SPC-Tert cKO) by crossing floxed TERT mice with inducible SPC-driven Cre mice. SPC-Tert cKO mice did not develop pulmonary fibrosis spontaneously up to 9 months of TERT deficiency. However, upon bleomycin treatment they exhibited enhanced lung injury, inflammation, and fibrosis compared with control mice, accompanied by increased pro-fibrogenic cytokine expression, but without a significant impact on AECII telomere length. Moreover, selective TERT deficiency in AECIIs diminished their proliferation and induced cellular senescence. These findings suggest that AECII specific TERT deficiency enhances pulmonary fibrosis by heightening susceptibility to bleomycin-induced epithelial injury and diminishing epithelial regenerative capacity because of increased cellular senescence. We confirmed evidence for increased AECII senescence in IPF lungs, suggesting potential clinical relevance of the findings from our animal model. Our results suggest that TERT has a protective role in AECIIs, unlike its pro-fibrotic activity previously observed in fibroblasts, indicating that TERT’s role in pulmonary fibrosis is cell type specific.

Telomere length shortening with or without telomerase gene mutations or polymorphisms have been reported in many disorders but their precise pathogenetic significance remains unclear (1-5). Telomerase is a ribonucleoprotein complex and consists of a catalytic component, telomerase reverse transcriptase (TERT) and an RNA template (TR). Telomerase adds a telomere repeat sequence to the 3’ end of telomeres on chromosomal ends, which are protective structures for chromosome
stabilization (6-8). Additionally telomeres are protected by a shelterin complex composed of six subunit proteins, namely TRF1, TRF2, TIN2, RAP1, TPP1, and POT1 (9). In addition to participation in telomere length regulation this complex prevents recognition of the telomere ends as double-strand breaks, which can incite an inappropriate DNA damage response. (10). Deficiency or loss of one of these subunits causes different aspects of telomere dysfunction via non-identical mechanisms (11). For example both TRF1 and TRF2 negatively regulate telomere length and prevent chromosome end-to-end fusions in vivo, but only TRF1 appears to be associated with mitotic spindle assembly while TRF2 plays key roles in t-loop formation and suppression of ATM activation (9,12-14). In addition to its telomere maintenance role extratelomeric roles for TERT in regulation of cellular proliferation, apoptosis, differentiation, and senescence have been reported (15-18). Moreover TERT is present in mitochondria, where it is implicated in reduction of reactive oxygen species and mitochondrial DNA (19). Thus the role of TERT likely involves both telomeric and/or extratelomeric mechanisms impacting on a multiplicity of cell functions.

Mutant TERT, TR and/or shortened telomeres in peripheral blood leukocytes are amply documented as risk factors for IPF (3,4,20,21). Induced telomerase and TERT expression is noted in lung fibroblasts from patients with fibrotic interstitial lung disease, including IPF, and in models of lung injury and fibrosis without impact on telomere length (22). Given the different and sometimes opposing roles of the cell types involved in fibrosis, the pathogenic significance of TERT in these different cell populations might reflect these differences. TERT in fibroblasts is essential for pulmonary fibrosis since fibroblast-specific TERT deficiency reduces bleomycin (BLM)-induced pulmonary fibrosis (23,24). However selective deletion of shelterin component TRF1 or TRF2 in type II alveolar epithelial cells (AECII) causes cellular senescence, regeneration defects and increased susceptibility to injury or fibrosis with or without telomere shortening (25-27). Moreover in TERT null mice intravenous administration of a TERT expressing viral vector reconstitutes its expression predominantly, but not exclusively in AECII, and reduces BLM-induced pulmonary inflammation and fibrosis (28). This reduction in fibrosis is associated with some lengthening of telomeres with reduction in the proportion of shortened telomeres.

Idiopathic pulmonary fibrosis (IPF) is an age-associated chronic progressive lung fibrosis with a fatal outcome (29-31). It is currently proposed to be an epithelial-fibroblast disease (32,33), in which repetitive epithelial cell injury and defective/deficient regeneration cause release of mediators that initiate interstitial fibroblast recruitment, propagation and activation to constitute fibrotic foci (32,34-36). However the precise mechanisms, especially the role of TERT in AECII have not been fully elucidated, although cell proliferation/regeneration, senescence and apoptosis have been implicated in the pathogenesis of IPF (37-39).

In this study the role for TERT in AECII was investigated using AECII specific TERT conditional knockout mice (23,40,41). The results showed that AECII specific TERT deficient mice did not spontaneously develop pulmonary fibrosis, inflammation, or induce cellular senescence in AECII. However this selective TERT deficiency in AECII caused significant enhancement of BLM-induced pulmonary injury, inflammation and fibrosis accompanied by inhibition of cellular proliferation and induction of senescence in AECII. Elevated level of AECII cellular senescence was also observed in human IPF lung tissue.

Results
Generation of AECII specific TERT deficient mice
The triple transgenic AECII specific TERT deficient mice were generated by breeding floxed TERT mice (23) with doxycycline induced Cre mice under control of the SPC promoter as described in the Methods (Figure 1A). The genotype of TERTfl/fl/Spc+,Cre+/- mice was confirmed by PCR, which showed presence (Figure 1B) of the PCR fragments for TERTfl/fl (343 bp) using primers P3 and P4, Spc (200 bp), Cre (500 bp), and TERT null allele (215 bp) using primers P1 and P4 (primer locations diagrammed in Figure 1A). To induce selective TERT deficiency in AECII, the triple transgenic mice were treated with doxycycline for 10 consecutive days and referred to as SPC-Tert cKO mice. The controls (“WT”) were the double transgenic (Spc+,Cre+/-) mice, which were similarly treated with doxycycline. Treatment
with doxycycline up to 9 months had no significant effects on body weight gain/growth, food intake and lung histology in both SPC-Tert cKO and WT mice (Data not shown). In the SPC-Tert cKO mice, TERT mRNA was significantly reduced by $\sim$50% in isolated AECII after 4 days of doxycycline treatment (Data not shown), which was further reduced to $\sim$30% (or $\sim$70% reduction) of control (WT AECII) levels at day 10 of treatment (Figure 1C). In lung tissue, TERT mRNA was reduced by 46.8% in the cKO mice (Figure 1C). Significant reduction of 39.1% was also observed for telomerase activity in TERT cKO AECII (Figure 1D), which was less than that observed for TERT mRNA. The lower decrease in protein vs mRNA levels could be due to some combination of contaminating non-AECII cells (routinely <10%), which would have intact TERT expression, and/or residual TERT protein and enzyme activity remaining after 7 days of doxycycline treatment, i.e. the half-life for protein could be longer or more stable than the mRNA. In contrast TERT mRNA was not significantly altered in mouse lung fibroblasts (MLF), macrophages, T cells and B cells from the cKO mice (Figure 1E). These results confirmed the selective ablation of TERT in AECII.

**TERT deficiency enhanced susceptibility to BLM-induced lung injury**

Telomerase deficiency is suggested to cause alveolar stem cell replicative senescence and impaired epithelial regeneration (42,43), which could diminish the ability for epithelial repair and exacerbate injury. To evaluate the effect of TERT deficiency on BLM-induced lung alveolar epithelial injury, bronchoalveolar lavage (BAL) was performed with PBS 7 days after BLM treatment to evaluate protein leak and inflammation. BLM treatment in WT mice caused the expected significant injury as reflected by a 19-fold increase in protein recovery in the BAL fluid, which was significantly increased to 28-fold in SPC-Tert cKO mice (Figure 2A). This indication of increased BLM-induced injury in the cKO mice was also reflected in an increased inflammatory response. Elevated total number of BAL cells was observed as expected in BLM-injured WT lungs (~ 6-fold over PBS controls), which was further increased to 10-fold over PBS controls in SPC-Tert cKO lungs after BLM treatment (Figure 2B). Further analysis on the differential counts revealed a significantly greater increase in the number of inflammatory/immune cells, including alveolar F4/80+ macrophages, B220+ B cells and Gr-1+ cells in cKO BAL fluid than that seen in WT controls, while a slight increase of CD3+ T cells from BLM-treated cKO mice was not statistically significant. BAL protein level or cell number in PBS treated SPC-Tert cKO mice were not significantly different from those in the PBS treated WT mice. These findings suggested that AECII TERT deficiency rendered alveolar epithelium more susceptible to the BLM induced injury, but had no significant effects without BLM treatment.

**SPC-Tert cKO mice developed enhanced pulmonary fibrosis induced by BLM**

BLM-induced lung fibrosis is significantly reduced in mice with TERT deficiency, globally or selectively in bone marrow (24), or in collagen I-expressing (mesenchymal) cells (23,24). To further examine if the in vivo role of TERT in lung injury and fibrosis is cell type specific, lung injury/fibrosis was induced by BLM in WT control and SPC-Tert cKO mice, and evaluated at day 7 or 21 after BLM injection. The results showed that selective TERT deficiency in AECII in PBS-treated group had no significant effects on lung histology and did not cause significant change in extracellular matrix and cytokine gene expression compared to WT controls (Figures 2C-G). However, in contrast to the diminished fibrosis in mice with TERT deficiency in bone marrow or mesenchymal cells (23,24), upon BLM injury TERT deficiency in AECII resulted in significant enhancement of pulmonary fibrosis as shown by more extensive and diffuse fibrosis with cystic changes resembling honeycomb lung (Figure 2C). Consistently, whole lung collagen content as measured by lung hydroxyproline content was increased $\times 2.4$-fold in BLM-treated SPC-Tert cKO mice, which was significantly higher than the 1.7-fold increase in BLM-treated WT mice (Figure 2D). Thus TERT deficiency in AECII caused a significant increase for 41.2% in responsiveness to BLM treatment. In addition, the enhanced lung fibrosis induced by BLM in SPC-Tert cKO mice was evidenced by significantly higher BLM-induced expression of type I collagen and $\alpha$-smooth muscle actin ($\alpha$-SMA) than that in WT mice at mRNA level (Figure 2E). These enhanced BLM-induced fibrotic changes in SPC-Tert cKO mice were accompanied by...
augmented BLM-induced expression of multiple fibrogenic associated cytokines, including TGFβ1, FIZZ1, FIZZ2 and amphiregulin (Figure 2F), and as well as inflammatory cytokines TNFα and MCP1 (Figure 2G). These findings together indicated that while AECII specific TERT deficient mice failed to exhibit spontaneous development of lung inflammation or fibrosis, they did show significantly enhanced lung injury, inflammation and fibrosis in response to BLM treatment, relative to that seen in WT mice.

**TERT deficiency inhibited AECII proliferation in response to BLM-induced injury**

Since epithelial cell proliferation is essential for epithelial regeneration, which is critical for successful lung repair, so the impact of TERT deficiency on AECII proliferation was evaluated to see if its impairment was associated with the enhanced fibrotic response to BLM. Thus potential impairment of AECII proliferation by TERT deficiency may represent a mechanism underlying the observed enhanced BLM-induced lung injury and fibrosis in SPC-Tert cKO lung. To assess proliferation in AECII, single cell suspensions from lung tissue samples were analyzed by flow cytometry for the expression of cell proliferation marker Ki67 and AECII marker SPC. The results showed that the number of proliferating AECII (double positive for Ki67 and SPC) was increased by 5-fold in WT lung cells in response to BLM-induced injury (0.25 vs. 1.25), but which was only increased <1.2-fold in AECII (0.32 vs. 0.38) from SPC-Tert cKO lungs (Figure 3A). Compared with WT controls, SPC+ AECII in cKO lungs was decreased from 22.5 to 18.8% after BLM treatment (Figure 3A). The proportion of proliferating AECII (Ki67/SPC double positive cells out of total SPC* cells) in BLM-treated WT mouse lungs was also significantly higher than that observed in BLM-treated cKO mice (11.8 vs. 3.27%, respectively) (Figure 3B). This negative impact of TERT deficiency on AECII proliferation was confirmed in lung tissue sections immunostained for Ki67 and SPC (Figure 3C). Direct cell counting showed that BLM treatment caused increased number of SPC+ AECII in WT, which was significantly diminished in cKO lungs (Figure 3D, upper panel). While abundant SPC/Ki67 double positive cells were observed in BLM treated WT lung tissue sections, they were less frequent in SPC-Tert cKO lung (Figure 3D, bottom panel) despite the presence of abundant Ki67 single positive cells (Figure 3D, upper panel) representing non-AECII cells, such as α-SMA-expressing fibroblasts (data not shown). These findings indicated that TERT deficiency impaired AECII proliferation and thus regeneration in response to BLM injury, which might contribute to the observed enhanced injury and fibrosis in SPC-Tert cKO mice. AECII telomere length was not significantly affected by TERT deficiency, and the proportion of short telomeres (<15 kb) was not significantly different between WT and Tert deficient AECII (Figure 3E and 3F). Thus the diminished proliferative response in AECII TERT deficient cells was not associated with detectable telomere shortening.

**Effect of AECII TERT deficiency on cellular senescence**

Proliferation arrest is a hallmark of cellular senescence, which may represent the basis for the noted proliferative impairment in TERT deficient AECII. Moreover Sirt1 suppression of senescence is associated with and depends on induction of TERT expression (18,25) and de-repression of TERT can reverse senescence (44). To evaluate this possibility expression of the cellular senescence marker genes, p16 and p21 were evaluated in AECII isolated from WT and SPC-Tert cKO mice. The results revealed no significant effect of AECII TERT deficiency on p16 or p21 expression in the absence of lung injury (Figure 4A and 4B). BLM treatment caused significant elevation in the AECII expression of both p16 and p21 in WT mice, but the BLM-induced increase was significantly greater in AECII of SPC-Tert cKO mice (Figure 4A and 4B). Induction of p16 in AECII occurred as early as day 3 after BLM treatment, and was >2-fold higher in the cKO mouse lungs compared to WT lungs at this time point. Induction of p16 diminished by day 7 (Figure 4A), while p21 induction was greater at this time point with similar higher level of expression in lungs of cKO mice (Figure 4B). The level of p21 mRNA was not significantly different between MLF isolated from WT and SPC-Tert cKO mice, although BLM induced slight (~30%) and comparable induction of p21 mRNA in both WT and cKO lungs (Figure 4B). Associated with this enhanced expression of senescence markers in TERT-deficient AECII in BLM-injured lungs were parallel alterations in both AECII and lung tissue
expression of IL-6, a characteristic component of the senescence-associated secretory phenotype (SASP). Thus the BLM induced IL-6 expression was significantly greater in lungs of cKO mice with selective AECII TERT deficiency than in WT mice (Figure 4C). These findings suggested that selective enhancement of AECII senescence due to TERT deficiency might represent the basis for the noted impaired AECII proliferation in SPC-Tert cKO mice. Interestingly analysis of human lung tissue sections from IPF patients also revealed selective increase in p16 expression in AECII. This was undertaken using double immunofluorescence staining for SPC and p16 on the lung tissue sections from IPF patients and control subjects to assess AECII specific senescence in situ. The results revealed that there were increased number of cells displaying double positive SPC and p16 staining in IPF lung compared to the rare double positive cell in control lung (Figure 4D), indicating that more AECII cells were senescent in IPF lung.

**Discussion**

Given that alveolar epithelial injury is associated with pulmonary fibrosis (32), we generated conditional (doxycycline-inducible) AECII specific TERT knockout mice (SPC-Tert cKO mice) to assess the role of TERT in this lung cell type. AECII specific TERT deficiency per se had no noticeable impact on lung histology or senescent marker expression. SPC-Tert cKO mice did not develop pulmonary inflammation or fibrosis, and exhibited no respiratory distress with normal body weight gain up to 9 months of AECII TERT deficiency. However selective AECII TERT deficiency enhanced susceptibility to BLM-induced lung injury and fibrosis. The enhanced fibrosis is consistent with the association of TERT mutations with IPF and other fibrotic interstitial lung diseases (3-5). TERT deficiency in AECII enhanced BLM-induced senescence and impaired proliferation resulting in diminished epithelial repair and regeneration, given the known property of AECII as progenitor cells (45). This is consistent with the observation that AEC injury and failure of regeneration increases susceptibility to pulmonary fibrosis (26,37). Interestingly, genetic ablation of TERT in hepatocyte progenitors causes a marked increase in stellate cell activation and liver fibrosis (46). In contrast, selective TERT deficiency in collagen-expressing mesenchymal cells protects the mice from lung fibrosis probably by limiting collagen-expressing lung fibroblast proliferation and increasing susceptibility to apoptosis (23). However global TERT deficiency is reported to either reduce or does not alter fibrosis (24,47). Thus TERT in epithelial and mesenchymal cells may have opposing effects on the pathogenesis of pulmonary fibrosis, but the net effect of deficiency in all cell types might depend on the variable impact of TERT deficiency in the different cell types involved in fibrosis. This further signifies that somatic TERT gene mutation(s) can have either a pro- or anti-fibrotic effects depending on which cell type(s) is/are affected. Interestingly, a recent study suggests telomere shortening primarily affects AECII in pulmonary fibrosis since it is observed in alveolar type 2 cells, but not in surrounding cells in the patient with familial or sporadic interstitial fibrosis (48), while telomere shortening is not detectable in fibrotic lung fibroblasts isolated from human IPF or TR KO mice (22). However telomere shortening in familial IPF with TERT mutation is not tissue specific, since the mutation takes place in germ line cells; and indeed telomere length in blood is found to correlate with that in fibrotic AECII in familial interstitial pneumonias with TERT mutation but not in sporadic IPF (48) The lack of significant telomere shortening in AECII from TERT cKO is in part due to the short time frame of TERT deficiency and that mice have much longer telomeres than human (2,7). Later generations of telomerase deficiency are required to detect significant telomere shortening (2,22), which could account for the lack of detectable shortening within the short time frame of the animal model experiments in this study. It may be that a critically short telomere length such as in IPF with TERT mutation could be a factor in pathogenesis, but this does not exclude non-telomere mediated or extratelomeric/non-canonical mechanisms. However this biological difference of telomere length between species should be considered when interpreting findings from mouse models vis-a-vis potential relevance to human telomere-related lung diseases.

The role of TERT in telomere maintenance and DNA replication accounts for its intimate involvement in cell proliferation, ageing and senescence (49-51). However its extratelomeric roles in cell proliferation, differentiation, and
mitochondrial function (17,19,46) should also be considered especially since the noted effects of TERT deficiency in AECII was unaccompanied by telomere shortening. Furthermore induction of telomerase and TERT in BLM-induced pulmonary fibrosis and in human lung fibroblasts isolated from patients with IPF is not associated with significant changes in telomere length (22,52). In the current study, the enhanced susceptibility of SPC-Tert cKO mice to BLM-induced lung injury and fibrosis is also not correlated with telomere shortening in the AECII during 3 weeks of fibrotic period, and telomere shortening is not observed in the lungs of mice after BLM treatment (22,25,28). Thus the noted enhancement of lung injury and fibrosis due to AECII TERT deficiency does not appear to depend on loss of the telomeric function of TERT, unlike the noted telomere dysfunction in TRF1 or TRF2 deficiency. The data in mouse cells from another study also suggest that telomere length is not a primary determinant of senescence/crisis in mouse cells since embryonic fibroblasts from late generation (G1-G6) TR knockout mice exhibit similar efficiency in colony forming ability as cells derived from WT mice (2). Although the quantitative analysis of the proportion of short telomeres (<15kb) from Southern blots showed that Tert deficiency did not affect significantly the distribution of short telomeres in AECII, it does not rule out potential shortening in select individual chromosomes. Hence the findings cannot completely exclude potential telomere dysfunction from loss of TERT expression, which might induce a DNA damage response.

While TRF1/2 deficiency promptly causes cellular senescence associated with uncapping of telomere ends and inflammation, TERT deficiency caused increased susceptibility to develop cellular senescence, inflammation and fibrosis, which was manifested only upon exposure to stressful or injurious stimuli, e.g. BLM treatment. This manifestation of susceptibility upon stress resembles the 2-hit model of human IPF, which postulates the requirement for a ‘second hit’ to expose an underlying susceptibility due to impaired AECII function or regenerative capacity (27,53). TERT deficiency negatively impacts AECII proliferation resulting in impaired epithelial regeneration, thus contributing to the enhanced pulmonary fibrosis. These findings are consistent with a recent report showing that in G2 TERT KO mice, reconstitution of TERT expression by IV injection of a TERT expressing viral vector preferentially rescues AECII from cellular senescence and increases proliferation. This effect on AECII results in higher regeneration potential, which is associated with diminished pulmonary fibrosis induced by low dose (0.5 mg/kg) BLM (28). Thus these studies using complementary approaches on TERT expression in AECII result in a similar conclusion on the protective role of TERT in lung injury and fibrosis.

Aging is considered a risk factor for IPF and recent evidence suggests the importance of cellular senescence, a characteristic of organismal aging, as a potential contributor to the susceptibility for development of IPF although the basis for this relationship is unclear (29,54-56). Lung cells from IPF patients exhibit reduction in Sirt1 expression (37,57), plus evidence of cellular senescence as manifested by induction of p53, p21 and/or p16, whose level of expression correlates with severity of fibrotic lung disease (58). Selective senescence in AECII due to TERT deficiency diminishes epithelial cell proliferation, which could negatively impact regeneration resulting in enhancement of fibrosis. Interestingly decreased AECII senescence due to miR-34a deficiency in aged mice affords protection from BLM-induced pulmonary fibrosis, but had an opposite effect in young mice probably due to a greater influence on reduced senescence in fibroblasts (relative to AECII) resulting in increased fibroproliferation (57). In IPF accumulation of senescent cells is seen primarily in the alveolar epithelium and less frequently in fibroblasts, and usually observed in areas of active fibrosis concomitant to the aberrant secretory pattern of the lung epithelium (54). This dichotomy between degree of senescence in AECII vs. fibroblasts was also observed in the current study in the BLM model (Figure 5). Moreover TERT deficiency in fibroblasts makes them more susceptible to apoptosis in BLM-induced pulmonary fibrosis (23), but failed to induce AECII apoptosis in SPC-Tert cKO (data not shown). This difference in responsiveness to TERT deficiency could account for the lesser degree of senescence in lung fibroblasts than AECII in BLM-induced lung fibrosis. Taken together role of TERT during pulmonary fibrosis may be cell type specific, reflecting the net effect on the lung of the
predominant active cell cycle impacted by TERT deficiency. Thus AECII vs. fibroblast at early vs. late stage of fibrosis in young or old animals might be differentially impacted by TERT deficiency, resulting in the differential effects on fibrosis. The precise molecular mechanisms by which TERT protects BLM-treated AECII in a telomere-independent manner requires further elucidation.

Experimental procedures

Mice and BLM model of pulmonary fibrosis

Floxed TERT mice were generated as before (23). To generate AECII specific TERT conditional knockout mice, the floxed TERT mice (TERT fl/fl) were crossed with SPC-rTATet-O-CMV-Cre mice bearing a doxycline inducible Cre-recombinase where (tetO)-CMV-driven Cre expression is driven by the doxycycline-induced reverse tetracycline transactivator (rtTA), under control of the human surfactant protein C (SPC) promoter (40,41). The genotype of TERT fl/fl/Spc-Cre/+ mice were confirmed by PCR, and the resulting triple transgenic AECII specific conditional knockout mice on a C57BL/6J background were used as described (Figure 1A and 1B). The Spc-Cre/+ mice (SPC-rTATet-O-CMV-Cre) were used as relevant controls, and referred to as wild type (WT) controls henceforth for simplicity.

To induce selective TERT deficiency in AECII the triple transgenic mice (8 to 10 weeks old) were treated with doxycycline in both diet (1.6-2.7 mg/ 3-5g diet) and drinking water (2 mg/L) for 10 consecutive days with 3 replacements/week. WT controls were also treated with doxycycline. Mice with selective AECII TERT deficiency (SPC-Tert cKO) and WT mice were treated with BLM (Blenoxane, Mead Johnson, NJ) dissolved in sterile PBS by endotracheal instillation on day 0 at a dose of 1.9 U/kg body weight as before (59). Control mice received PBS injection alone. Doxycycline only in drinking water was continued for another 14 days after BLM injection. Where indicated, BAL was performed using 1 ml of PBS and repeated 5 times for collection of BAL fluid and cells. Total BAL cell number was counted using a hemocytometer and the differential cell counts were analyzed by flow cytometry. Total protein was measured with the Pierce BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL).

Lung cell isolation and separation

Primary mouse lung fibroblasts (MLF) were isolated and maintained as before (60), and used at passages 2–5 in the indicated experiments. Primary AEC II were isolated as previously described (61). Briefly, lungs were instilled with dispase II (Roche Diagnostics, Indianapolis, IN) followed by low-melt agarose (Sigma, St. Louis, MO), and digested for 45 minutes. Lungs were then dissected and treated with DNase. The cell suspensions were negatively selected for CD16/32 and CD45 expressing cells by MACS separation system (Miltenyi Biotec. Inc., San Diego, CA) followed by further negative selection for non-adherent cells by incubation on the petri dishes. The AEC II were then plated and cultured for 2 days on 200 µg/ml Matrigel (BD Biosciences, San Jose, CA) coated plates before use. Lung macrophages, T and B lymphocytes were separated using MACS separation system with F4/80, pan-CD90.2 and B220 microbeads (Miltenyi Biotec. Inc.), respectively.

Telomerase activity assay and telomere length measurement

Telomerase activity was assayed using Telomerase PCR ELISA kit (Roche, Indianapolis, IN) in accordance with the manufacturer’s protocol. Cell lysates heated at 85°C for 15 minutes were used as negative controls. Telomere length was analyzed in the AEC II isolated from doxycycline-treated WT or TERT cKO mice as described above. Analysis was performed using terminal restriction fragment (TRF) Southern blot with the “Telo TAGGG telomere length assay” kit (Roche), as previously described (22). Enzyme digested AEC II DNA plug kit (CHEF genomic DNA plug kit, Bio-Rad Laboratories, Hercules, CA) was separated on agarose gel with pulsed field electrophoresis, and followed by Southern blotting. Carestream MI SE (Carestream Health, Inc., Rochester, NY) was used for the analysis of the proportion of the shortest telomere signals.

Real time qRT-PCR

For real time qRT-PCR analysis, Taqman primers for type I collagen (Colla2), α-SMA (Acta2), TERT (Tert), TGFβ1 (Tgfβ1), FIZZ1 (Retnlα), FIZZ2 (Retnbl), amphiregulin (Areg), TNFa (Tnfa), MCP1 (Ccl2), p16 (p16), p21 (p21), IL-6 (Il6), and 18s RNA were purchased from
ThermoFisher Scientific. The 18s RNA was used as internal control or reference for normalization. One-step RT-PCR was performed as before (60) using a GeneAmp 7500 sequence detection system (Applied Biosystems, Rockford, IL). Results were expressed as $2^{-\Delta\Delta CT}$ using the indicated control group as calibrator (62).

**Hydroxyproline assay**

Lung collagen content was determined by measuring the hydroxyproline (HYP) content from lung homogenates as previously described (60,63). The results were expressed as µg HYP per lung.

**Histology and immunofluorescence staining**

The paraffin embedded lung sections were stained with H & E and Masson’s trichrome for routine evaluation of histopathology. For immunofluorescence staining, the paraffin-embedded mouse or human lung tissue sections (human sections were obtained from Lung Tissue Research Consortium, National Heart, Lung, and Blood Institute, National Institute of Health (LTRC, NIH NHLBI) were antigen retrieved in 10 mmol/L sodium citrate (pH 6.0) using the microwave for 20 minutes. Anti-mouse Ki67 (ab16667, Abcam, Cambridge, MA, 1:500), anti-mouse SPC (sc-7706, Santa Cruz, Santa Cruz Biotechnology, Inc., Santa Cruz, CA, 1:300), mouse anti-human p16 (ab117443, Abcam, 1:200), and rabbit anti-human SPC (ab90716, Abcam, 1:200) were used as primary antibodies for mouse and human, respectively. Isotype IgGs were used as the controls for the primary antibodies. Anti-mouse/rabbit IgG-NL493/NL557 (NL004, NL-006, NL007, R&D Systems), or anti-goat IgG-Alexa Fluor 488 (ab150129, Abcam) were used as secondary antibodies for Ki67 and SPC, respectively. Mounting medium containing DAPI (Vector, Burlingame, CA) was applied, and the sections were examined with a Nikon Eclipse E600 fluorescence microscope (Nikon, Tokyo, Japan) using the Nuance 3.0.2 multispectral imaging system (PerkinElmer, Inc., Hopkinton, MA). For quantitation of cell counting, cells positive for Spc$^*$ or Ki67$^*$ or both were counted from at least 5 high power fields, and ≥1000 total cells (DAPI$^*$) were counted for each group.

**Flow cytometry**

This was undertaken as before (60). The lung single cell suspension was first stained with viability dye eFluor 506 (#65-0866-14, eBioscience, San Diego, CA, 1:100 dilution), and then fixed/permeabilized (BD Cytofix/Cytoperm, BD Biosciences) prior to the staining with anti-mouse Ki67 (ab15580, Abcam, 1:100) and anti-SPC-Alexa Fluor 647(bs-10067R-A647, Bioss Antibodies, Woburn, MA, 1:50). BAL cells were stained with the combination of anti-mouse F4/80-Alexa Fluor 647 (#122609, 1:200), CD3-PE-Cy7 (#100200, 1:100), CD45R/B220-APC-Cy7 (#103223, BioLegend, San Diego, CA, 1:100), and Gr1-PE (# 553128, BD Biosciences, 1:50). The cells were also stained with isotype controls conjugated with the same fluorochrome as their respective antibodies, plus each single color antibody that was included in the antibody mixes. The data was acquired with a NovoCyte flow cytometer, and analyzed by NovoExpress software (Acea Biosciences, Inc., San Diego, CA). The gating for each antibody was based on the isotype controls and only live cells were analyzed.

**Statistical analysis**

All data were expressed as mean ± SD unless otherwise indicated. Differences between means of various treatment and control groups were assessed for statistical significance by ANOVA followed by post hoc analysis using Scheffé’s test. A P value < 0.05 was considered to indicate statistical significance.

**Animal and human studies**

All animal studies were reviewed and approved by the Institutional Animal Care & Use Committee (IACUC) at the University of Michigan. All human studies were reviewed and approved by the Institutional Review Board at the University of Michigan, and abide by the Declaration of Helsinki principles.
Acknowledgements: We thank Lisa Riggs for the excellent technical assistance in the lung tissue section preparation and the H & E and Masson’s trichrome staining.

Conflict of interest: The authors declare that they have no conflicts of interest with the contents of this article.
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FOOTNOTES
Funding was provided by the National Institutes of Health grants to S.H.P (HL 052285, HL 112880 and HL 138417).

The abbreviations used are: TERT, telomerase reverse transcriptase; BLM, bleomycin; IPF, idiopathic pulmonary fibrosis; AECII, type II alveolar epithelial cells; α-SMA, α-smooth muscle actin; SASP, senescence-associated secretory phenotype.
Figure 1. Generation of doxycycline-induced SPC-Tert cKO mouse. (A) The strategy for generation of SPC-Tert cKO. Double transgenic mice bearing the SPC-rtTA and (tetO) 7-CMV-Cre transgenes were bred to mice bearing the floxed TERT to generate triple transgenic progeny. In the presence of doxycycline (“DOX”), the activated rtTA bound to (tetO) 7-CMV led to activation of Cre recombinase, which then excised the floxed TERT sequence (part of promoter and exons 1-2) in AECII. (B) PCR genotyping using mouse tail DNA. The PCR fragments for TERTfl/fl (343 bp) using primers P1 and P2, for Spc (200 bp), for Cre (500 bp), and Cre excised SPC-Tert cKO (215 bp) using primers P1 and P5 are shown. (C) TERT ablation in AECII and lung tissue. AECII and lung tissue total RNA were isolated from WT or SPC-Tert cKO mice (“cKO”), and analyzed for TERT (Tert) mRNA expression by qPCR. TERT mRNA levels were calculated as $2^{-\Delta\Delta CT}$ and shown as fold change over WT controls. (D) Cell lysates were collected from AECII isolated from mice 7 days after DOX treatment, and analyzed for telomerase activity by telomerase PCR ELISA, and shown as fold change over WT controls. (E) Tert level in the indicated cell types were analyzed as described in (C). Asterisks indicated P<0.05 between the two indicated groups with N=3 for AECII, fibroblasts, T and B cells, macrophages, and N=5 mice for lung tissue analyses.
Figure 2. TERT deficiency enhanced alveolar epithelial damage and pulmonary fibrosis in SPC-Tert cKO mice. (A) The WT and SPC-Tert cKO mouse lungs were lavaged at day 7 after PBS or BLM treatment. Total protein amount was measured by BCA protein assay kit. (B) Total BAL cell number was counted in the BAL fluids, and the numbers of F4/80⁺, CD3⁺, B220⁺ and Gr1⁺ BAL cells were determined using flow cytometry. (C) Representative H & E stained lung tissue sections are shown. Original magnification: × 20. All scale bars: 500μm. (D) The lung tissues from WT or SPC-Tert cKO mice were homogenized and the lung collagen contents were measured by hydroxyproline (HYP) assay in whole lung homogenates. (E) Lung tissue RNA was analyzed for fibrotic marker type I collagen (Colla2) and α-SMA (Acta2) expression at mRNA level by qPCR analysis. Lung tissue RNA was also analyzed for profibrotic cytokines FIZZ1 (Retnla), FIZZ2 (Retnlb), TGFβ1 (Tgfb1) and amphiregulin (Areg). (F) and inflammatory cytokines MCP1 (Ccl2) and TNFα (Tnfa) (G). Asterisks indicated P<0.05 between the two indicated groups with N=3 in (A).
and (B), N=5 mice in (C, E-G), in (D) N=11 for WT-PBS, N=13 for WT-BLM, N=12 for cKO-PBS and N=13 for cKO-BLM.
Figure 3. TERT deficiency suppressed AECII proliferation in BLM-induced pulmonary fibrosis. (A) Whole lung single cell suspensions were analyzed for SPC and Ki67 positive cells by flow cytometry. The total SPC positive population was further analyzed to show the percentage of Ki67 positive cells in the SPC positive population, which is shown in (B). A representative analysis from 3 separate flow cytometry runs showing similar results, is shown in (A). Analysis from the combination of all 3 runs showing the average percentage of proliferated AECII that were double SPC+ Ki67+ in total SPC+ AECII, is shown as a bar graph in (B). (C) SPC and Ki67 double immunofluorescence staining were performed on paraffin-embedded lung tissue sections. SPC signals are shown in green color, Ki67 in red, and nuclei in purple-blue (DAPI). Original magnification: × 400. All scale bars: 20µm. (D) Results of direct cell counting from the relevant micrographs are expressed as percentages of all lung cells (upper panel) or SPC+ cells (lower panel), with the latter indicating the percentage of proliferating AECII. Asterisks indicated p<0.05 between the two indicated groups with N=5 for WT-PBS, WT-BLM, cKO-PBS, and N=6 for cKO-PBS group. (E) Telomere length assay in AECII. TERT cKO and their control mice received doxycycline for 30 days, and AECII were isolated from mice without BLM treatment, and then embedded with agarose gel. Telomere length was performed using TRF-Southern blotting. (F) The proportion of the shortest telomere signals are
shown as the intensity percentage of shortest telomere (<15kb) of the total telomere signal. N=3 mice each group.
Figure 4. TERT deficiency enhanced BLM-induced senescence in lung cells. (A) AECII were isolated from WT or SPC-Tert cKO lung at day 3 and day 7 after PBS or BLM treatment, and analyzed for senescence marker p16 (Ink4a) mRNA expression by qPCR. (B) The p21 (Cdkn1a) mRNA levels in isolated AECII and MLF at day 7 after PBS or BLM treatment were analyzed. (C) AECII and lung tissue RNA samples were analyzed for IL6 (Il6) mRNA. Asterisks indicated P<0.05 between the two indicated groups with N=3 for PBS, and N=4 for BLM group in (A-C). (D) Cellular senescence in IPF. The SPC and p16 double immunofluorescence microscopy was performed on paraffin-embedded human lung tissue sections from control or IPF patients. SPC signals are shown in green color, p16 in red, and nuclei in purple-blue with DAPI. The representative single and merged images from control and IPF lung sections are shown. Original magnification: × 400. All scale bars: 20µm.
Figure 5. Schematic illustration of cell type specific role of TERT in pulmonary fibrosis. TERT is shown as a suppressor of senescence, which in turn interrupts the cell cycle in AECII and fibroblasts by de-repression of p53/p21/p16. This in turn will suppress epithelial cell repair/regeneration, a critical factor in limiting development and progression of pulmonary fibrosis. Thus the effect of TERT deficiency in AECII is to enhance fibrosis as demonstrated in the current study. In contrast, the suppression of fibroproliferation in the case of senescence in fibroblasts should limit fibrosis, as previously noted (57).
Telomerase reverse transcriptase ameliorates lung fibrosis by protecting alveolar epithelial cells against senescence
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J. Biol. Chem. published online April 18, 2019

Access the most updated version of this article at doi: 10.1074/jbc.RA118.006615

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