Telomerase and Telomere Length in Pulmonary Fibrosis

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In addition to its expression in stem cells and many cancers, telomerase activity is transiently induced in murine bleomycin (BLM)–induced pulmonary fibrosis with increased levels of telomerase transcriptase (TERT) expression, which is essential for fibrosis. To extend these observations to human chronic fibrotic lung disease, we investigated the expression of telomerase activity in lung fibroblasts from patients with interstitial lung diseases (ILDs), including idiopathic pulmonary fibrosis (IPF). The results showed that telomerase activity was induced in more than 66% of IPF lung fibroblast samples, in comparison with less than 29% from control samples, some of which were obtained from lung cancer resections. Less than 4% of the human IPF lung fibroblast samples exhibited shortened telomeres, whereas less than 6% of peripheral blood leukocyte samples from patients with IPF or hypersensitivity pneumonitis demonstrated shortened telomeres. Moreover, shortened telomeres in late-generation telomerase RNA component knockout mice did not exert a significant effect on BLM-induced pulmonary fibrosis. In contrast, TERT knockout mice exhibited deficient fibrosis that was independent of telomere length. Finally, TERT expression was up-regulated by a histone deacetylase inhibitor, while the induction of TERT in lung fibroblasts was associated with the binding of acetylated histone H3K9 to the TERT promoter region. These findings indicate that significant telomerase induction was evident in fibroblasts from fibrotic murine lungs and a majority of IPF lung samples, whereas telomere shortening was not a common finding in the human blood and lung fibroblast samples. Notably, the animal studies indicated that the pathogenesis of pulmonary fibrosis was independent of telomere length.

Keywords: fibrosis; telomere; telomerase

Telomerase participates in the maintenance of telomere length by adding TTAGGG telomeric DNA repeats onto the ends of chromosomes *de novo*, while telomeres are shortened with each round of cell division (1–3). Telomerase consists of a catalytic reverse transcriptase (TERT) and an RNA component (TR) that serves as a template for telomere elongation. Cells lacking telomerase activity do not express TERT, whereas they still express TR constitutively (4). This suggests that the limiting factor for cellular expression of telomerase activity is TERT gene expression.

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Telomerase activity is widely expressed in cancerous cells and is undetectable in adult somatic cells, but it is transiently induced in various tissues undergoing injury, repair, and fibrosis. Bleomycin (BLM), hypoxia, and silica-induced lung injury and fibrosis in rodents are characterized by the induction of telomerase in epithelial cells and fibroblasts (5–7). Our previous animal studies showed that telomerase activity is transiently induced in BLM-treated fibrotic lung tissue. This is accompanied by increased TERT expression, which is obligatory for the full expression of fibrosis, because TERT deficiency impairs fibrosis along with a decreased expression level of α–smooth muscle actin (α-SMA), a myofibroblast differentiation marker. Reduced fibrosis in TERT knockout (KO) mice can be restored by transplantation with wild-type (WT) bone marrow (BM), thus implicating the importance of a TERT-inducible, BM-derived cell during fibrosis (5, 8–10). A recent report revealed that fibroblast telomerase expression is higher in areas of early remodeling in lung tissue of idiopathic pulmonary fibrosis (IPF), which precedes the α-SMA expression that dominates in areas of late remodeling (11).

Recent studies suggested a potential role for telomerase in a subset of patients with a familial form of IPF that is associated with telomere length shortening (12–16). Mutations in the TERT and/or TR genes have been identified in some familial cases of IPF accompanied with shortened telomeres. These shortened telomeres are thought to constitute a predisposing factor in the development of fibrosis upon exposure to injurious stimulus, perhaps by an impairment of tissue repair. Some patients with sporadic IPF exhibit telomere shortening independent of any mutations in the TERT or TR genes, and the mechanism of this telomere shortening is unknown. Moreover, the precise roles, as well as the mechanism of the genesis, of telomerase/telomere alterations in IPF remain unclear.

The objective of the present study was to investigate whether telomerase was also induced in human lung fibroblast (HLF) cultures from patients with interstitial lung disease (ILD), and to evaluate how this could relate to telomere length in pulmonary fibrosis.

MATERIALS AND METHODS

Human Subjects

Human leukocyte DNA was extracted from 70 patients with IPF, 86 patients with hypersensitivity pneumonitis (HP), and 117 normal control subjects. The mean ages of these three groups were not statistically significantly different from each other ($P = 0.33$). HLFs were isolated from 36 patients with IPF, five patients with systemic sclerosis (SSc), six patients with HP, three patients with idiopathic nonspecific interstitial pneumonia (iNSIP), and 21 control subjects, and were maintained in Dulbecco's Modified Eagle's Medium (DMEM; Life Technologies, Carlsbad, CA) supplemented with 10% FBS (more information on human demographics, sample sources, disease diagnoses, and human study approval is available in the online supplement).

Mice and Induction by BLM of Pulmonary Fibrosis

C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME). TERT and TR heterozygous KO mice on a C57BL/6

background were the kind gifts of Dr. Fuyuki Ishikawa (Kyoto University, Kyoto, Japan) and Dr. Carol Greider (Johns Hopkins University School of Medicine, Baltimore, MD), respectively (animal study approval and more breeding details are available in the online supplement). For the induction of BLM-induced pulmonary fibrosis, BLM (Mead Johnson, Yonkers, NY) was instilled endotracheally at a dose of 2 U/kg body weight (n = 3–5 mice per group). Mouse lung fibroblasts (MLFs) isolated from lung tissue were maintained in DMEM supplemented with 10% plasma-derived fetal bovine serum (PDS; Animal Technologies, Tyler, TX), 10 ng/ml epithelial growth factor, and 5 ng/ml platelet derived growth factor (R&D Systems, Inc., Minneapolis, MN; more details are available in the online supplement).

Quantitative RT-PCR and Western Blotting Analysis

One-step real-time TaqMan or SYBR green–based RT-PCR was performed using a GeneAmp 7500 Sequence Detection System (Applied-Biosystems, Foster City, CA). The primer sequences are listed in the online supplement. Western blotting to detect mouse or human TERT (hTERT) and acetyl-H3K9 (H3K9Ac) protein expression was performed with anti-mouse TERT (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-hTERT (Abcam, Cambridge, MA), anti-H3K9Ac, and horseradish peroxidase–conjugated glyceraldehyde 3–phosphate dehydrogenase antibodies (Sigma, St. Louis, MO).

Telomerase Activity Assay and Telomere Length Measurement

Telomerase activity was assayed using a telomerase PCR ELISA kit (Roche, Indianapolis, IN) in accordance with the manufacturer's protocol. Cell lysates heated to 80°C for 15 minutes were used as negative controls. Telomere length was measured by terminal restriction fragment (TRF) Southern blotting, using the Telo TAGGG Telomere Length Assay kit (Roche). An enzyme-digested human cell DNA or MLF DNA plug was separated on an agarose gel with regular electrophoresis for human cell DNA, or pulsed-field electrophoresis for MLF DNA, and this was followed by Southern blotting (more details are available in the online supplement). The mean TRF was calculated as previously described (17, 18).

Chromatin Immunoprecipitation Assay

A chromatin immunoprecipitation (ChIP) assay was performed using a ChIP assay kit (Millipore Corporation, Temecula, CA), following the manufacturer's protocol. Anti–acetyl-H3K9 (Sigma) was used to immunoprecipitate the histone–DNA complex. The PCR primers specific for the human or mouse TERT promoter are listed in the online supplement.

Statistical Analysis

ANOVA followed by *post hoc* analysis using the Scheffé test was applied for comparisons between the means of various groups. A P value of less than 0.05 was considered significant. Data are presented as means ± SE, unless otherwise indicated.

RESULTS

Telomerase Activity in Lung Fibroblast Cultures from Patients with ILD

To determine whether telomerase was also induced in cells from patients with IPF, HLFs from these and other patients with ILD were analyzed for telomerase activity using telomerase repeat amplification protocol (TRAP)–ELISA. The results showed that fewer than 30% of control samples exhibited telomerase activity (Figure 1A). In cases where control cells were isolated from cancer resections and the distance from the tumor margins was available, telomerase activity was found only in cells closest to the margins. Other control samples with telomerase activity were obtained from resections for pneumothorax or unused lung transplant specimens. In contrast, the majority (> 60%) of IPF and two thirds of iNSIP patient samples exhibited significant telomerase activity. Only 1 of 6 HP patient samples was positive for telomerase activity (Figure 1A). Forty percent of the SSc samples were also positive. The relative telomerase activities in each disease category are shown in Figure E1 in the online supplement. These findings suggested some association between the induction of telomerase in HLF and fibrotic ILD.

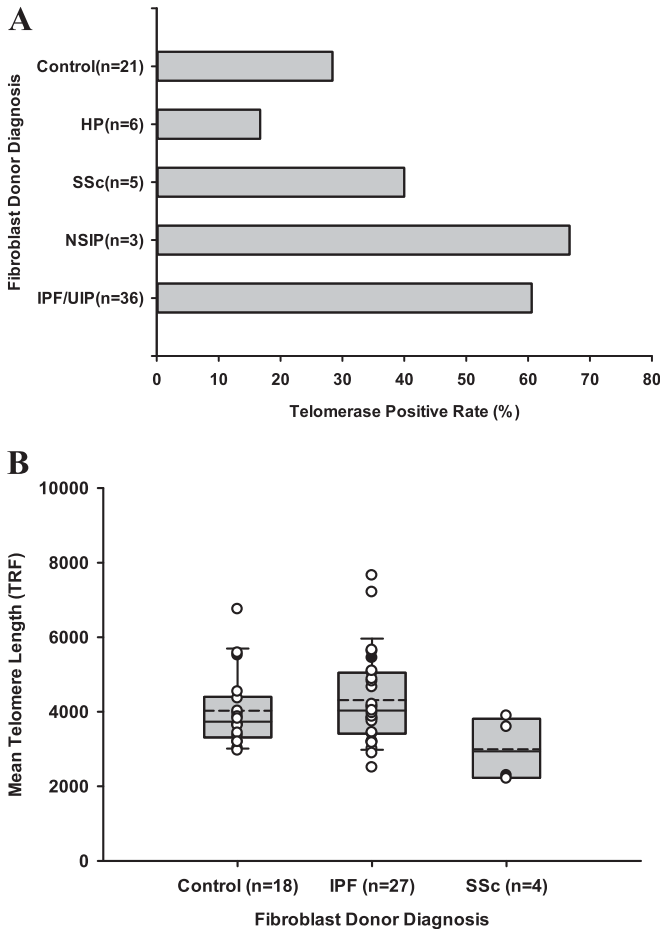


Figure 1. Telomerase activity and telomere length analysis in lung fibroblasts from patients with interstitial lung disease (ILD). Human lung fibroblasts were isolated from control subjects or patients with the indicated diagnoses. Fibroblast lysates were harvested from passage 6–10 cells. (*A*) Five micrograms of cell lysates were used for a telomerase activity assay with telomerase repeat amplification protocol (TRAP)-ELISA. The samples with absorbance at greater than or equal to 0.25, after subtracting the absorbance reading of the negative control, were considered positive for telomerase activity. The results are shown as percentages of samples that were telomerase-positive in each disease category, with the total number of samples indicated. (*B*) Genomic DNA from these fibroblasts was analyzed for telomere lengths by terminal restriction fragment (TRF) analysis and Southern blotting. The results are plotted as mean telomere length. The median is indicated with a *solid line* inside the *shaded box*, whereas the mean is indicated with a *dashed line*. The *lower error bar* indicates the 10th percentile, whereas the *upper error bar* indicates the 90th percentile. The *bottom boundary* of the *shaded box* indicates the 25th percentile, and the *upper boundary* indicates the 75th percentile. Each data point represents results from cells from a single donor. HP, hypersensitivity pneumonitis; IPF, idiopathic pulmonary fibrosis; NSIP, nonspecific interstitial pneumonia; UIP, usual interstitial pneumonia; SSc, systemic sclerosis.

Telomere Length Measurements in IPF and SSc Fibroblasts

Given the evidence of telomerase induction in HLF cultures from patients with IPF, and the role of telomerase in telomere

maintenance, telomere lengths in these cultures were measured by TRF Southern blotting. The results showed that telomere lengths were not significantly different between control, IPF, and SSc cell samples (Figure 1B). The distribution of telomere lengths was not significantly different between these three groups. Only one out of the 27 (3.7%) IPF samples had a mean telomere length below 10% of predicted, based on the control samples. Thus, despite the induction of telomerase activity in the cells from patients with IPF, no significant effect on telomere length was evident.

Recent studies indicated shortened telomeres in some peripheral blood leukocyte samples from patients with sporadic and familial IPF, involving variable associations with mutations in TERT and/or TR genes (12–15). For comparison, genomic DNA isolated from peripheral blood leukocytes from the Mexican cohort group (70 patients with IPF, 86 patients with HP, and 117 normal subjects) was similarly analyzed for telomere length, and the results are shown in Figures 2A and 2B. Telomere length shortening (<10% of predicted) was detected in 4 out of 70 patients with IPF (5.71%), with one other patient manifesting borderline shortening, whereas 5.81% of patients with HP exhibited telomere shortening, with two other patients displaying borderline shortening. One of 117 (0.86%) control samples displayed shortening, with an additional four exhibiting borderline shortening. The age dependence of telomere length was defined by the linear regression line constructed from the control data, and the few samples from the control, IPF, and HP groups with shortened telomeres were defined as those with a telomere length below the 10th percentile boundary of this regression. Thus the few cases of shortened telomeres were identified after controlling for age on the basis of this regression line. Smoking history did not exert a significant effect on telomere length for the control ($P = 0.142$), IPF ($P = 0.327$), or HP ($P = 0.598$) groups. The proportions of shortest telomere lengths (< 2.5 kb) were also compared between the three groups, and the results revealed no significant differences (Figure 2C). Thus the incidence of telomere shortening in samples from patients with IPF and HP was comparable (5.71% and 5.81%, respectively), indicating that this low frequency of telomere shortening was not specific for IPF or chronic progressive fibrotic lung disease.

Telomere Length and BLM-Induced Pulmonary Fibrosis

To assess whether telomere length was a significant factor in the pathogenesis of pulmonary fibrosis, BLM-treated TR KO mice were examined first for telomere shortening as a function of the number of generations after establishing the homozygous KO of the respective gene. TR KO MLF cultures revealed a gradual shortening of telomeres up to the fourth generation, as reflected by the higher amounts of shorter telomeres in blots of later generations (Figure 3A). However, the mean TRF values were not statistically significant between the TR KO samples, but were all significantly different from the WT samples. Notably, telomere length was not affected by BLM treatment. The impact of TR deficiency (second generation) on BLM-induced pulmonary fibrosis was then analyzed by estimating lung collagen content, using a biochemical assay for hydroxyproline (HYP), as previously described (18) in whole-lung homogenates on Day 21 after BLM or saline administration. The results showed that BLM injection caused the expected increase in lung HYP content in WT mice (Figure 3B). The response to BLM in TR KO mice was not significantly different from that in WT mice, and this lack of effect for TR deficiency on fibrosis was also demonstrated by lung collagen I mRNA concentrations that showed responses comparable to those of BLM treatment in both WT

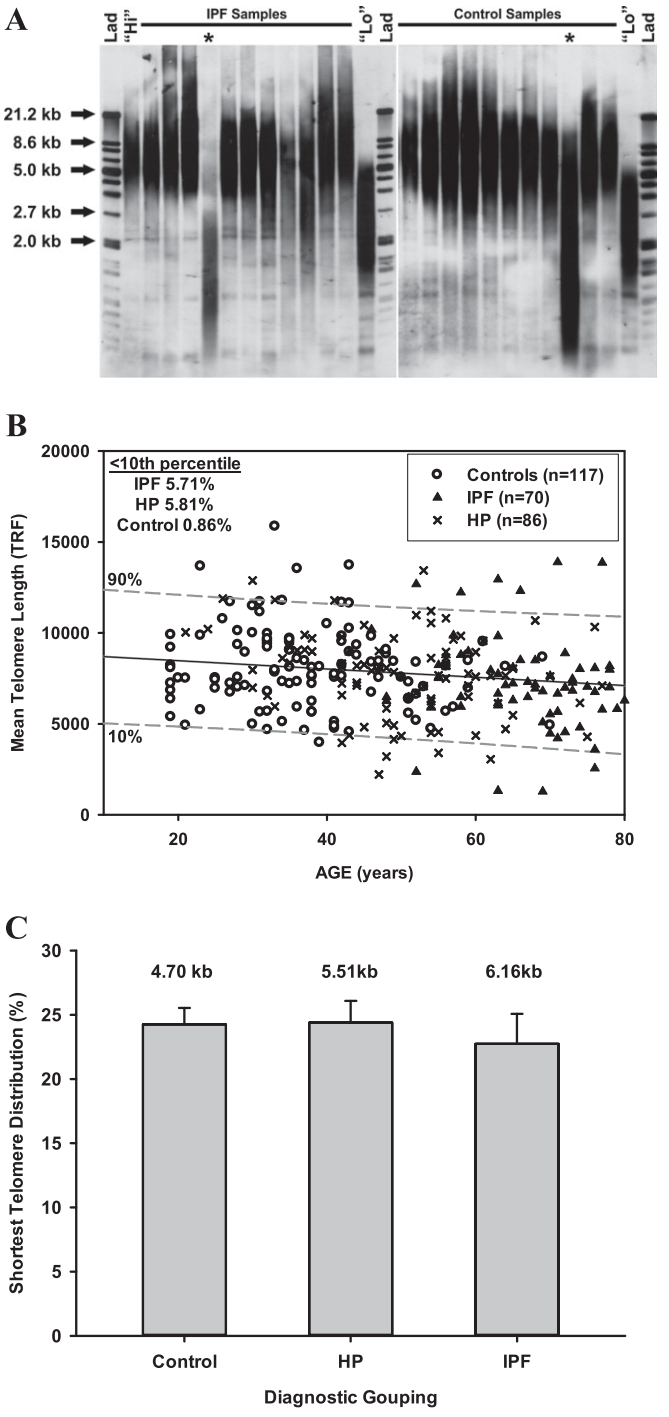
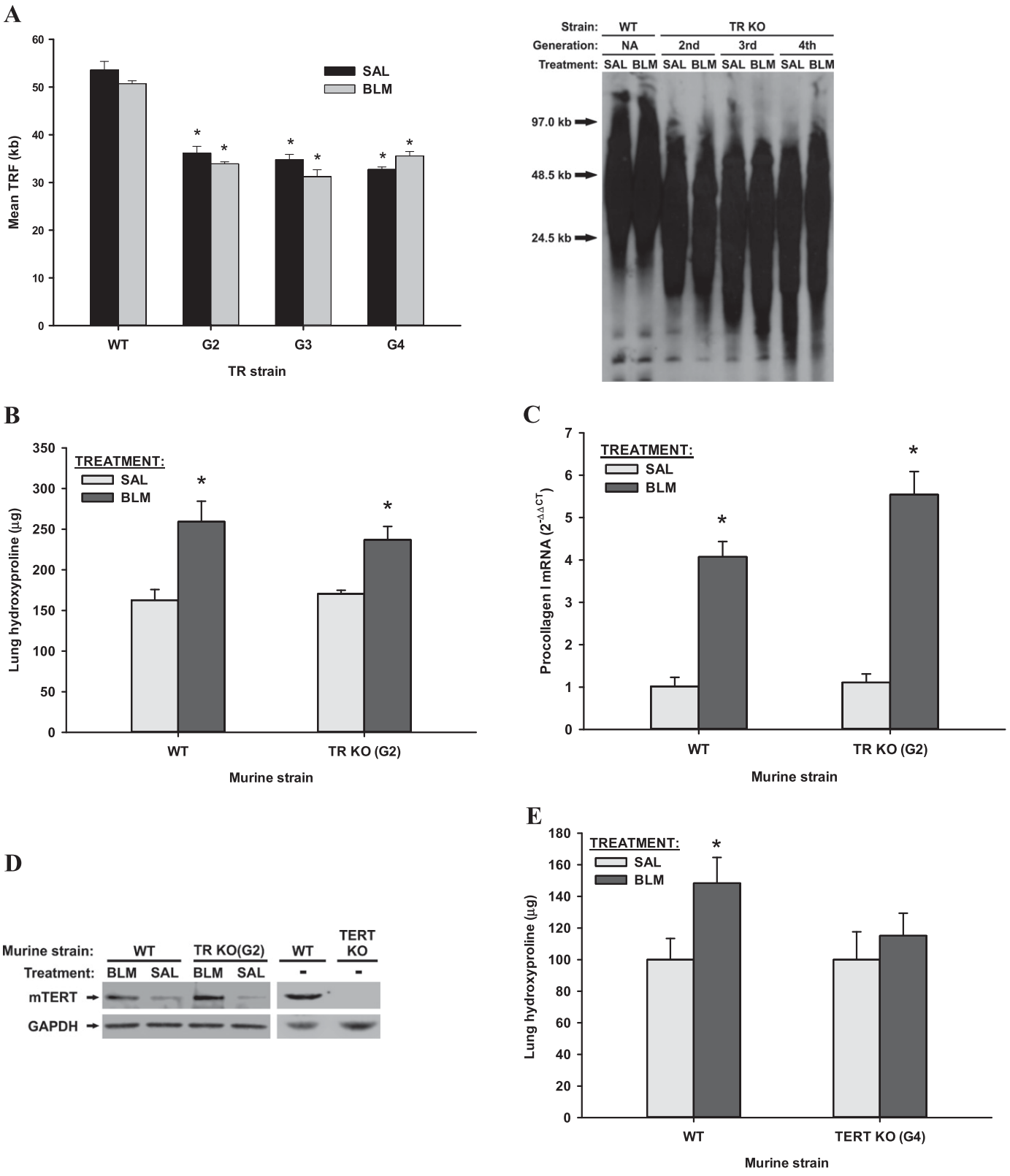


Figure 2. Measurement of telomere length in peripheral blood leukocytes. Genomic DNA (1 μg) was extracted from peripheral blood leukocytes and used for telomere length assessment. Average telomere length was measured by performing mean TRF analysis and Southern blotting. (*A*) Representative blots for IPF and control samples are shown, with the *asterisk* indicating a sample exhibiting a shortened telomere. "Hi" Lad, DNA ladder control with high telomere length; "Lo" Lad, DNA ladder control with low telomere length. (*B*) Telomere length was plotted versus age. The control samples (*open circles*) were used to generate a regression line, with the predicted 10th and 90th percentile indicated by the *lower* and *upper dashed curves*, respectively. The total number of samples per group (*upper right*) is indicated, as well as the percentage of samples in each group that exhibited telomere length lower than the 10th percentile (*upper left*). (*C*) The proportions of the shortest telomere signals in the three groups are shown. Mean values were not significantly different between all three groups. The average TRF is indicated for each group.



and TR KO mice (Figure 3C). Lung histopathology also revealed no noticeable differences between WT and KO mice (Figure E2). Analysis of fibrosis in third-generation and fourth-generation TR KO mice yielded similar negative results (data not shown), indicating that this degree of telomere shortening exerted no significant impact on pulmonary fibrosis. TR deficiency did not exert a noticeable effect on TERT expression or its induction by BLM treatment (Figure 3D). Thus the lack of effect for TR deficiency on fibrosis was reflected in this lack of effect on TERT expression, previously found to be essential for fibrosis. In contrast to this lack of effect for TR deficiency on fibrosis, fourth-generation TERT KO mice exhibited reduced fibrosis, which was comparable to that in second-generation KO mice (8) as assessed by total lung HYP content (Figure 3E).

Figure 3. Effects of telomerase RNA component (TR) or telomerase catalytic reverse transcriptase (TERT) deficiency on telomere length, TERT expression, and pulmonary fibrosis. (*A*) Genomic DNA from lung fibroblasts were isolated from bleomycin (BLM)–treated or saline (SAL)–treated wild-type (WT) or TR knockout (KO) mice, and analyzed for telomere length by TRF assay, followed by Southern blotting. The quantitative analysis is shown at *left* ($n = 3$, $^*P < 0.05$), and a representative blot is shown at *right*. G, generation. The effect of TR deficiency on fibrosis is shown as lung hydroxyproline content in whole-lung homogenates on Day 21 after BLM or SAL administration (*B*) and as procollagen I mRNA concentrations (*C*). Data are presented as means ± SEs, with $n = 5$ animals per group. *Asterisks* indicate a statistically significant difference ($P < 0.05$) from the corresponding SAL control groups. (*D*) The effect of TR deficiency on the BLM induction of TERT was analyzed. Lung-tissue lysates from WT and TR KO mice on Day 21 after BLM or SAL treatment were analyzed for TERT protein expression by Western blotting. Representative blots are shown. The *lane* on the *right* shows a negative control for the TERT antibody, using TERT KO lung lysate. (*E*) The effects of prolonged TERT deficiency on pulmonary fibrosis were analyzed by hydroxyproline assay in SAL-treated or BLM-treated G4 TERT KO mice. Data are expressed as percentages of respective SAL-treated groups ($n = 5$ mice per group). An *asterisk* indicates a statistically significant difference ($P = 0.012$) from the corresponding SAL-treated control group.

BLM exerted a minor (15% increase above saline control samples) but statistically insignificant effect on lung HYP in fourth generation TERT KO mice, in contrast to the significant induction (48% increase over saline control samples) in WT mice. Although the mean value for KO BLM lungs was lower than that in WT BLM lungs, the difference between them was not statistically significant ($P = 0.16$). This reduced responsiveness to BLM in TERT KO mice was also reflected in lung collagen I protein concentrations (Figure E3). Similar to TR KO mice, TERT KO mice exhibited gradual telomere shortening as a function of generation, which was not affected by BLM treatment (Figure E4). Thus telomere length shortening failed to affect reduced fibrosis significantly in TERT KO mice. Taken together, these findings suggest that pulmonary fibrosis in this BLM-induced model was dependent on TERT, but was independent of changes in lung fibroblast telomere length.

Histone Acetylation in the Induction of TERT Gene Expression

To better understand the mechanisms by which TERT expression differed in IPF from normal control samples, we investigated histone acetylation status in TERT gene expression. Consistent with the induction of telomerase activity, hTERT gene expression was increased in HLFs from patients with IPF relative to those from control subjects (Figure 4A), which was significantly correlated with H3K9Ac ($R^2 = 0.64$, $P < 0.001$; Figure 4B). The correlation was substantially decreased ($R^2 = 0.37$) in control samples alone (Figure 4C), suggesting that induced TERT expression in IPF cells may be attributable to increased H3K9 acetylation.

To evaluate this possibility, the effect of the histone deacetylase inhibitor, Trichostatin A (TSA), on TERT expression was analyzed. The results showed that TSA induced TERT mRNA expression in HLFs in a time and dose-dependent manner (Figure 5A). The TERT mRNA concentrations showed a dramatic induction with a greater than 30-fold peak increase by 2 μM TSA at 12 hours of treatment, and with a significant increase as early as 4 hours. The increase in expression remained high (i.e., a fivefold increase) at 24 hours. Consistent with mRNA induction, TERT protein was also induced by TSA treatment at 24 hours, which was associated with an increased level of global H3K9 acetylation (Figure 5B). A ChIP assay was performed next to detect the specific H3K9 acetylation status at the hTERT promoter region. The results revealed that the binding of H3K9Ac at the hTERT promoter was 60% higher in IPF cells with induced telomerase activity than in control cells with undetectable telomerase activity (Figure 6A). Moreover, when cells from patients with IPF were treated with TSA, a dose-dependent stimulation of H3K9Ac binding at the hTERT promoter resulted in a greater than 10-fold increase at a dose of 2 μM (Figure 6B). Thus the induction of TERT was associated with the increased binding of H3K9Ac specifically at the hTERT promoter.

The up-regulation of TERT expression by histone acetylation was also confirmed in MLF cultures in BLM-induced pulmonary fibrosis when the MLF cultures were treated with TSA (Figures 7A and 7B, respectively). The higher concentration of mouse TERT (mTERT) in cells from BLM-treated mice was associated with higher concentrations of H3K9Ac (Figure 7C). As in HLFs, increased H3K9Ac binding to the mTERT promoter was also observed when the murine cells were treated with TSA (data not shown). These observations, taken together, are consistent with a regulatory role for histone acetylation in the induction of TERT and telomerase activity in cells from lungs undergoing fibrosis.

DISCUSSION

Tissue injury and associated repair/remodeling responses are linked with some degree of telomerase induction. Telomerase played an essential role in an animal model of pulmonary fibrosis (5, 8–10). In this study, we present evidence that telomerase activity was induced in the majority of lung fibroblast cultures from patients with IPF, in contrast to less than 30% of control samples, indicating some degree of specificity for fibrotic ILD. Because the induced telomerase activity in lung fibroblasts declines with passage number in tissue culture (10, 19), these are likely underestimates of the actual incidence of telomerase induction. In contrast, telomere length shortening was a low-frequency ($\sim$ 6%) event in IPF and HP, indicating that such shortening was not specific for IPF or fibrotic ILD. These findings indicated a greater association between IPF and telomerase induction than telomere shortening. Moreover, telomere lengths were not significantly altered in the murine BLM model of pulmonary fibrosis, and shortened telomere lengths did not affect fibrosis significantly in this model. These data are in agreement with one study, in which the shortened telomere length in TR or TERT deficiency mice did not predispose them to enhanced BLM-induced lung fibrosis (20). In the present study, however, TERT deficiency impaired fibrosis independent of telomere length, whereas TR deficiency exerted no effect on fibrosis. The basis for these discrepant results on the effect of TERT deficiency in pulmonary fibrosis is unclear, but may be attributable to the different TERT KO strains used in these studies (21, 22). Nevertheless, both studies showed that telomere length exerts no significant effect on pulmonary fibrosis in this animal model. Finally, TERT activation in both IPF cells and murine cells from the BLM model appeared to be regulated by histone (H3K9) acetylation, similar to that seen in other cell types (23, 24), indicating that the epigenetic regulation of the TERT gene by histone acetylation may be involved in the induction of telomerase during fibrotic pathogenesis.

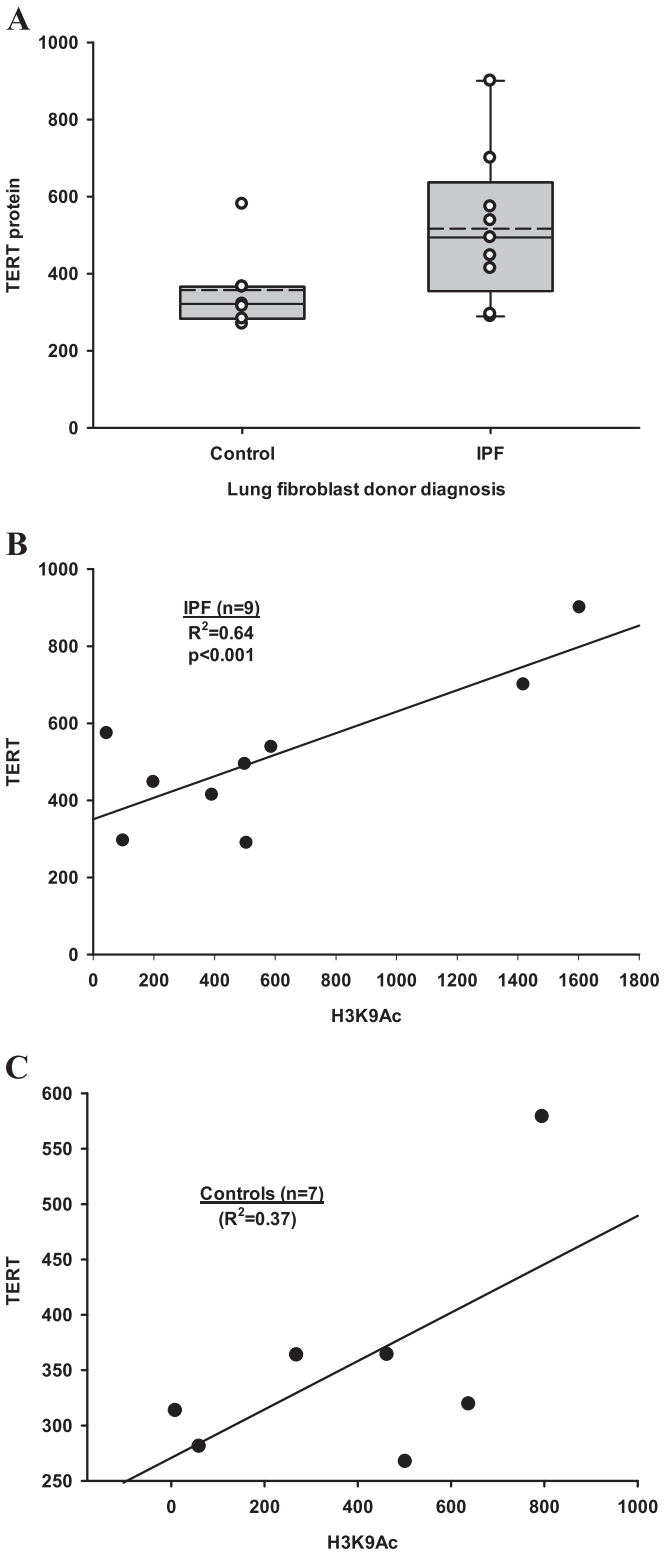


Figure 4. Human lung fibroblast TERT expression and histone acetylation. (*A*) Lung fibroblasts isolated from patients with IPF and control subjects (at passages 6–10) were analyzed for TERT protein expression by Western blotting. Glyceraldehyde 3–phosphate dehydrogenase (GAPDH) signals were used as a loading control. Data are expressed as relative integration units, and are normalized as percentages of GAPDH signals ($n = 7$ per group). Each data point represented result from cells from a single donor, and the significance of the *bars* and *lines* in the *box plot* is as described in the legend to Figure 1B. The global histone acetylation at H3K9 were detected by acetylated H3K9 antibody using Western blotting, as already described, and the correlation between the global acetylated H3K9 and TERT concentrations in lung fibroblasts from patients with IPF (*B*, $n = 9$) or control subjects (*C*, $n = 7$) were analyzed by linear regression and the results shown in the graph. Representative blots are shown in Figure E5 of the online supplement.

The pathogenic significance of induced telomerase activity in cells from patients with IPF or iNSIP is unclear, but such activity may afford survival advantages for these cells, and may thus contribute to the persistence of the mesenchymal response. Telomerase is also known to be reactivated during tissue injury and remodeling, in addition to its presence in germ cells and cancerous cells. In response to hypoxia and during hypertension, telomerase is selectively activated in human and rat vascular smooth muscle cells, and its down-regulation suppresses their proliferative response (25, 26). Telomerase activity is also induced in atherosclerotic coronary arteries, a finding correlated with the induction of TERT (27). Some inflammatory stimuli, including LPS and TNF-α, could activate telomerase and TERT expression, via NF-κB signaling in response to LPS in macrophages of human atherosclerotic lesions (28). Thus telomerase may be important in the fibroproliferative response during tissue remodeling. Moreover, our findings suggest the possibility that the induction of telomerase in lung fibroblast cultures, perhaps obtained from lavage or transbronchial biopsy samples, may constitute a useful biomarker for either fibrotic ILD and/or the progression/exacerbation of disease.

Because adult somatic cells usually lack telomerase activity, the basis for the telomerase activity in the control samples remains unclear. Proximity to tumor margins in the control samples from tumor resections was found to be associated with telomerase activity, but other samples from pneumothorax resections or trauma victims also expressed telomerase activity. However, fibroblasts obtained from these surgical resections may proceed from areas with pathological abnormalities. Thus, for example, a wide variety of pulmonary and pleural histological changes is recognized in the setting of spontaneous pneumothorax, including an epithelial, inflammatory, and fibrotic reaction with the up-regulation of a number of hypoxia-related, apoptosis-related, and inflammation-related genes upon microarray analysis (29, 30).

Despite the activation of telomerase in IPF and iNSIP fibroblasts, the distribution of telomere lengths in these cells was not significantly affected. Along with the dichotomy in the effect of TERT versus TR deficiency on fibrosis, this would argue that the significance of activation of telomerase may not be related to its telomere maintenance function, which would not be unprecedented. The function of TERT in the proliferation of hair-follicle stem cells is independent of its telomere maintenance role (31, 32). Transgenic TERT expression in mice induces a marked up-regulation of int/Wingless (Wnt) signaling during the promotion of podocyte proliferation and de-differentiation in adult kidneys (33). Interestingly, Wnt–β-catenin signaling is implicated in the pathogenesis of IPF (34).

This activation of telomerase in lung fibroblast cultures from patients with fibrotic ILD seems conceptually in conflict with the suggested importance of telomere length shortening associated with variable rate of TERT/TR mutations reported in patients with IPF, with as many as 37% for the familial type and 25% for sporadic cases (12, 14–16). However, one study showed no evidence of telomere shortening (0%) in BM mesenchymal stem cells from patients with IPF or in those with rheumatoid arthritis–related usual interstitial pneumonia (UIP) (35). The different estimates for the incidence of telomere shortening

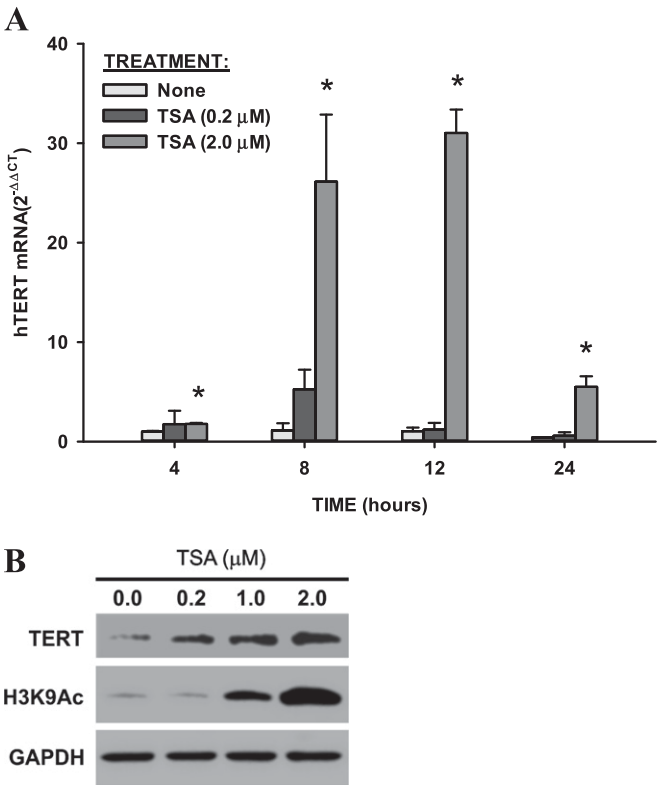


Figure 5. Trichostatin A (TSA) induction of human TERT (hTERT) in human lung fibroblasts. (*A*) Lung fibroblasts from a patient with IPF were treated with the indicated doses of TSA for the indicated times, and then analyzed for TERT mRNA by quantitative PCR. Untreated cells at 4 hours were used as the calibrator for the calculation of $2^{-\Delta\Delta CT}$. *Asterisks* indicate statistically a significant difference ($P < 0.01$) from the corresponding untreated control samples. Representative results are shown from at least four separate experiments with IPF cells from individual donors. Cells were also analyzed by Western blotting for TERT and acetylated H3K9 (H3K9Ac) protein concentrations after 24-hour treatment with the indicated doses of TSA. (*B*) Representative blots.

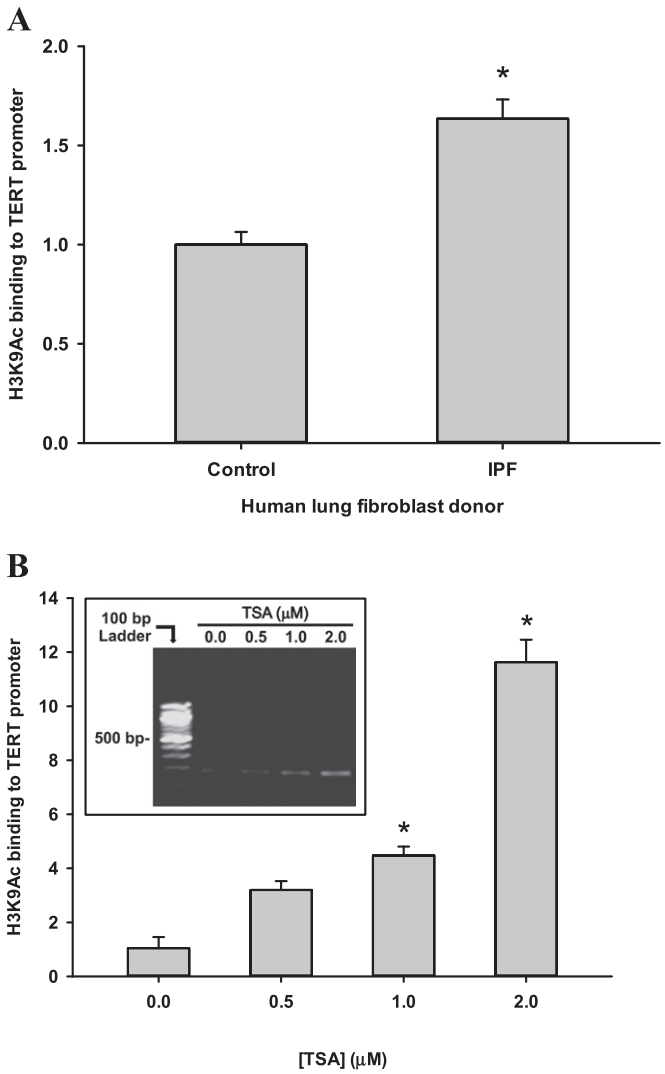


Figure 6. Chromatin immunoprecipitation (ChIP) assay of H3K9 acetylation status at the human TERT promoter. (*A*) The ChIP quantitative PCR analysis was performed in cells from human control and IPF samples, and the results are shown as means ± SE ($n = 3$). The *asterisk* indicates a statistically significant difference ($P = 0.005$) from control cells. (*B*) The cells as already described were treated with the indicated doses of TSA for 12 hours, and then subjected to ChIP assay. The cell DNA immunoprecipitated by acetylated H3K9 antibody was amplified by quantitative PCR. One tenth of the supernatant before immunoprecipitation was used for the DNA input control. Data are expressed as fold changes over untreated cells. The representative result is shown from at least three separate experiments. The *inset* depicts a typical gel image of quantitative PCR products. bp, base pairs. *Asterisks* indicate statistically significant differences ($P < 0.02$) from untreated cells.

may reflect the different populations studied. The incidence of shortening in the samples reported here was less than 6%, which may reflect the different population samples studied. The present study investigated the telomere lengths in a Mexican cohort, whereas other studies reported on samples collected in the United States or Canada (12, 36). The patients with IPF in this study were of the sporadic type, with no genetic information available. Only one patient with IPF (with a normal lung fibroblast mean TRF) had a known family history, but with genetic information unknown. The different estimates for the incidence of telomere shortening may also be attributable to the different methods used for telomere length measurement. Nevertheless, the majority of sporadic cases with telomere shortening failed to show any mutations in their TERT or TR genes. Indeed, one study of familial IPF failed to show any mutations in TERT or TR (37), confirming genetic heterogeneity, and demonstrating that telomere shortening is not always associated with TERT/TR mutations. Readily detectable mutations of *hTERT* and *hTR* in sporadic idiopathic interstitial pneumonias (IIPs) have been rare (1–3%), but 10–23% of sporadic IIPs involved shortened telomeres, even when mutations were not detected (12, 16). A recent study reported that 3 of 38 familial probands and none of 50 sporadic cases studied manifested TERT/TR mutations. The three familial *TERT* mutation carriers exhibited shortened telomeres, but telomere lengths in all other samples from subjects without mutations were not measured (36). These reports indicate that a minority of cases with IPF exhibit telomere shortening, most of which is not associated with TERT or TR mutations. Furthermore, experimental evidence is lacking to explain how shortened telomeres could affect the development of fibrosis, although the impairment of tissue repair and regeneration of alveolar epithelium may conceivably be a factor in promoting fibrosis. On the other hand, the degree of telomere shortening that may be required to induce susceptibility to the development of IPF remains unclear. Shortening above 10% of predicted, based on normal control samples, may be sufficient, but the information is insufficient to allow such a conclusion. A

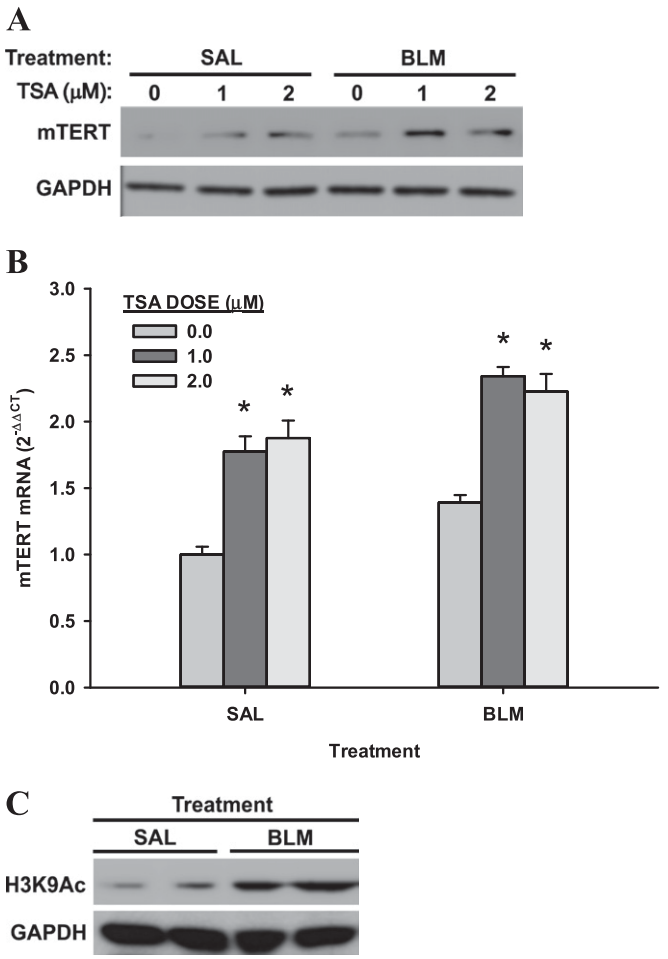


Figure 7. Mouse TERT (mTERT) regulation by histone H3K9 acetylation. Mouse lung fibroblasts were isolated 14 days after BLM or SAL treatment and analyzed for inductions of TERT protein at 24 hours (*A*) and mRNA at 8 hours (*B*) after TSA treatment by Western blotting and quantitative PCR, respectively. *Asterisks* indicate statistically significant differences ($P < 0.01$) from the corresponding untreated cells. (*C*) Acetylated H3K9 protein induction was detected in Day 14 BLM-treated lung tissue by Western blotting.

possibility that has not been excluded from such association studies holds that the telomere shortening noted may represent an effect of the increased cell proliferation/turnover attributable to active disease, rather than a causative factor for fibrosis. This is especially germane because in most cases with telomere shortening, no mutations in TERT or TR could be identified. Moreover, it remains unclear whether telomerase activity itself is detectable or altered in those previous studies showing shortened telomeres in patients with IPF.

A possible resolution of this apparent conflict may involve the opposing significance of telomerase and/or telomere length in epithelial versus mesenchymal cells, wherein the improved survival attributable to telomerase is beneficial in epithelial cells for regeneration and successful repair, but is detrimental if activated in mesenchymal cells. Thus the significance of telomerase may have to be viewed in the context of the cell in which it is expressed, and should not be simplistically viewed as either good or bad in the context of IPF. This is of importance, because how it is viewed in this context will exert consequences on how a therapeutic strategy can or should be crafted to either activate or inhibit telomerase. To target it selectively in specific cell types to achieve an effective therapy may be necessary.

Author disclosures are available with the text of this article at www.atsjournals.org

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