Original Article

Telomerase in pulmonary fibrosis

A link to alveolar cell apoptosis and differentiation

Argyris Tzouvelekis[1], Andreas Karameris[2], Evangelos Tsiambas[2], Anastasios Koutsopoulos[3], Rodoula Tringidou[4], Marios Froudarakis[1], Dimitrios Mikroulis[5], George Zacharis[1], Paschalis Steiropoulos[1] Ioanna Bazdiara[6], Constantinos Tsatalas[6], Ioannis Kotsianidis[6], Demosthenes Bouros[1]

[1]Department of Pneumonology, University Hospital of Alexandroupolis, Medical school, Democritus University of Thrace, Greece
[2]Department of Pathology, VA Hospital (N.I.M.T.S), Athens, Greece
[3]Department of Pathology, University Hospital of Heraklion, Crete
[4]Department of Pathology, Sotiria General Hospital, Athens, Greece
[5]Department of Cardiothoracic surgery, University Hospital of Alexandroupolis, Medical school, Democritus University of Thrace, Greece
[6]Department of Hematology, University Hospital of Alexandroupolis, Medical school, Democritus University of Thrace, Greece

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Correspondence to:
Argyris Tzouvelekis, MD, Clinical Research Fellow, Medical School, Democritus University of Thrace, Department of Pneumonology, University Hospital of Alexandroupolis, Alexandroupolis 68100
Tel.: +30 25510 75096, Fax: +30 25510 76106
E-mail: atzouvelekis@yahoo.gr

SUMMARY. Introduction: Telomerase is crucial for extended life span and differentiation and is linked to immortality. Therefore, its role may be crucial in the pathogenesis of pulmonary fibrosis. Our objective was to implicate telomerase in the pathogenesis of idiopathic fibrotic lung disease. **Patients and Methods:** Assessment of telomerase activity and expression was carried out using TRAP detection kit and qRT-PCR. Experimental procedure was enhanced by a series of immunostainings and fluorescence in situ hybridization analysis in tissue microarrays constructed with tissue samples from patients with idiopathic pulmonary fibrosis (IPF) and cryptogenic organizing pneumonia (COP). **Results:** We demonstrated significant downregulation of telomerase expression and activity in patients with fibrotic lung disease compared to controls. Immunolocalization studies coupled by FISH analysis revealed the presence of two subpopulations of type II AECs based on their telomerase expression levels: telomerase positive type II AECs, mainly overlying areas of active fibrosis and telomerase negative type II AECs, mainly localized in areas of established fibrosis. **Conclusions:** Downregulation of telomerase expression and activity in IPF may indicate a causal relationship between low telomerase expression and disease pathogenesis. The duality phenomenon in telomerase expression suggests that telomerase may regulate the fate of AECs towards either an apoptotic or a mesenchymal phenotype contributing directly to fibrosis. ***Pneumon 2010, 23(3):224-239.***

INTRODUCTION

Telomerase is a specialized reverse transcriptase that synthesizes TTAGGG telomere DNA at the ends of eukaryotic chromosomes, compensating for telomere erosion that normally occurs with each cell division and contributing to the stability, function and replication of chromosomes[1,2]. It has two essential components: a catalytic one, telomerase reverse transcriptase (h-TERT) and an RNA component (hTR)[3]. Telomerase has been shown to be crucial for extended life span, cell proliferation and differentiation and

has been linked to immortality[4]. Studies have provided us with abundant evidence that expression or induction of telomerase activity is not restricted to immortalized cancerous or transformed cells and it is also associated with tissue injury[5]. Despite this compelling evidence for the inducibility of telomerase activity in noncancerous cells[6,7] and its association with injured[8] and inflamed tissues[9], its role in noncancerous pathologic processes, characterized by increased proliferation or survival of cells, remains unclear. Idiopathic pulmonary fibrosis (IPF) is one such process, which is characterized by increased alveolar cell apoptosis, clusters of highly proliferative and immortal fibroblasts called fibroblastic foci (FF) comprising the histopathologic pattern of usual interstitial pneumonia (UIP)[10]. Recent evidence supports the concept that active fibrosis of IPF mirrors the abnormal wound repair in response to multiple sites of ongoing alveolar epithelial injury whereas parallels between IPF and cancer biology have also been drawn[10-12]. While irreversible fibrosis is the hallmark of the disease other types of pulmonary fibrosis such as cryptogenic organizing pneumonia (COP) characterized by distinct fibromyxoid lesions called Masson bodies (MB) that are susceptible to even complete reversal present with excellent prognosis and clinical course[13,14].

Experimental findings have demonstrated that telomerase activity was selectively induced in fibroblasts isolated from bleomycin (BLM) -injured rat lungs presumably before their differentiation to myofibroblasts[15-17]. The latter observation was further supported by the evidence that the loss of telomerase activity is closely associated with myofibroblast differentiation and possibly functions as trigger for this procedure[18,19]. Furthermore a dismal effect of low telomerase activity in bleomycin-induced alveolar cell apoptosis has been recently reported[20] while a causal effect-relationship between telomerase deficiency in murine lung fibroblasts and BLM-induced PF has also been demonstrated[21]. However, human and mouse telomerases are reported to differ in both their functional properties and their regulation whereas bleomycin-animal model is not fully representative of IPF[15].

Hence a human study is sorely needed. Recently published genetic association studies in patients with familial IPF[22,23] described a primary role for mutant telomerase components in the pathogenesis of IPF suggesting that the presence of short telomeres in alveolar epithelial cells (AECs) may lead to cell apoptosis and trigger the fibrogenic process through epithelial-mesenchymal interactions. Although, familial IPF is clinically indistinguishable from sporadic IPF except earlier age of onset (55 years vs. 67 years)[24], however there are no reports in the literature assessing telomerase activity, expression and localization in lung samples from patients with various forms of pulmonary fibrosis. To address this issue, we constructed tissue microarrays (TMAs) and demonstrated downregulation of telomerase in representative tissue samples from patients with sporadic IPF and COP compared to control subjects indicating a causal relationship between low telomerase activity and expression and pulmonary fibrosis. Most intriguingly, immunolocalization studies revealed for the first time the presence of two subpopulations of type II AECs based on their telomerase expression levels: telomerase positive type II AECs, mainly overlying areas of active fibrosis and exhibiting high levels of differentiation markers and telomerase negative type II AECs mainly localized in areas of established fibrosis and characterized by increased levels of apoptotic activity. Some of our results reported here have previously been reported in the form of abstracts[25,26].

MATERIALS AND METHODS

Subjects

In total, 45 newly diagnosed patients with idiopathic interstitial pneumonias (IIPs) of two different histopathologic patterns including 25 patients with IPF/UIP and 20 with COP were recruited in our study. The diagnosis was based on the consensus statement of the ATS/ERS (2002)[13]. Paraffin-embedded surgical lung specimens (open lung biopsy or by video assisted thoracoscopic surgery-VATS) from two different fibrotic regions of each individual were sampled. All patients were fully informed and signed an informed consent form where they agreed to the anonymous usage of their lung and blood samples for research purposes. All information from the patients' record was handled with the appropriate care to ensure that medical confidentiality was observed. Approval by the local ethical committee was also obtained (Protocol number: 1669). Twenty control paraffin blocks obtained from the normal part of lungs removed for benign lesions were collected from the archives of the Department of Pathology of three different institutions (Table 1).

Quantitative Real-Time reverse transcriptase-chain reaction (qRT-PCR)

h-TERT mRNA expression in 20 IPF and 10 control whole lung samples was measured using a quantitative

TABLE 1. Demographic and spirometric characteristics of patients with idiopathic pulmonary fibrosis/usual interstitial pneumonia (IPF/UIP) and cryptogenic organizing pneumonia (COP) and control subjects. Values are expressed as mean ± SD, and age as median (range).

Characteristics	IPF/UIP	COP/OP	Control subjects
Number	25	20	20
Sex: Male/Female	19/6	12/8	10/10
Age: median (yr)	66 (43-72)	50 (38-62)	39 (26-60)
Smokers/non smokers	20/5	6/14	13/7
FVC: % predicted	71 ± 3 **	78 ± 4 **	104 ± 12
TLC: % predicted	54 ± 4 **	72 ± 3 **	101 ± 11
K_{CO} % predicted	53 ± 5 **	68 ± 5 **	90 ± 6

Abbreviations: COP/OP: Cryptogenic organizing pneumonia/ organizing pneumonia, FVC: Forced Vital Capacity, IPF/UIP: Idiopathic pulmonary fibrosis/Usual interstitial pneumonia, Kco: Carbon monoxide transfer coefficient, TLC: Total Lung Capacity, *p<0.05, **p<0.001.

real-time RT-PCR assay with SYBR-Green I QPCR Master Mix. Primer sequences were as follows: for h-TERT; for: 5'- TGA CAC CTC ACC TCA CCC AC-3' and hTERT REV: 5'- CAC TGT CTT CCG CAA GTT CAC -3' and for 18S rRNA; 5'-TGC GAA TGG CTC ATT AAA TCA GTT 3' and 18S rRNA REV: 5'-CCG TCG GCA TGT ATT AGC TCT AG 3' (see online supplement for details).

Telomerase Activity

Telomerase activity was assayed in a total of 15 lung samples (8 IPF and 7 control) using a telomeric repeat amplification protocol (TRAP) assay in accordance with the manufacturer's protocol (TeloTAGGG Telomerase PCR ELISA plus detection kit; Roche Molecular Biochemicals, Mannheim, Germany)[27]. Briefly, 10 μg of tissue extract was suspended in 30 μl of reaction mixture (containing biotinylated telomerase substrate, optimized anchor primer, nucleotides, Taq polymerase, and a 216-bp internal standard DNA). The resulting mixture was subjected to polymerase chain reaction (PCR). Telomerase activity was quantified by measuring the absorbance of the PCR products at 450 nm. The level of telomerase activity in a given sample was determined by comparing the signal from the sample to the signal obtained using a known amount of control template. Based on the negative controls, samples with absorbance less than 0.2 were considered negative. Details on this methodology can be found in an online supplement.

Tissue microarray (TMA) construction

A total of 65 tissue samples consisting of 25 IPF, 20 COP and 20 control lung specimens derived from the normal part of lungs removed for benign lesions were used for TMA construction. Three representative areas of 1.5 mm in diameter each were identified and punched-out from each paraffin block by using a precision instrument (TMA-100, Chemicon, USA) and re-embedded into a new paraffin block as previously described[28]. Ultimately we constructed two TMA blocks comprising of 100 tissue elements each. Details on this methodology can be found in an online supplement.

Immunohistochemistry analysis

Immunohistochemistry for h-TERT antigen was carried out on using the anti-h-TERT mouse monoclonal antibody (clone 44F12-Novocastra Laboratories Ltd, Newcastle United Kingdom). To further analyze the cellular localization of telomerase expression in type I and II AECs and vis-ü-vis myofibroblast phenotype, double immunohistochemistry analysis for h-TERT and SP-A or a-smooth muscle actin (a-SMA), respectively, was undertaken. To assess differentiation of AECs in IPF and COP lung TMAs were immunostained with specific antibodies against N-cadherin (rabbit polyclonal antibody, GeneTex, GTX12221). Details on these methodologies can be found in an online supplement.

Determination of apoptosis

Apoptosis was assessed by immunohistochemistry analysis using specific antibody against DNA fragmentation factor of (DFF)[29] (Novocastra Laboratories Ltd, Newcastle United Kingdom) (see online supplement for details).

Fluorescence *in situ* hybridization (FISH) for telomerase messenger RNA expression

Detection of telomerase messenger RNA (mRNA) expression was undertaken using FISH. This was carried out using specific hTR probe which hybridizes to regions within the 451 base hTR component (DakoCytomation, Y1441). Levels of mRNA expression were automatically assessed by using the high-resolution DUET, BioView scanning system for FISH, morphology and immunocytochemistry applications. Interpretation of telomerase mRNA signal results was based on Zymed's Evaluation Chart for FISH. According to this guide, two mRNA copies per nucleus demonstrate normal telomerase gene pattern,

whereas 6–10 or small clusters characterize a low-level gene amplification. Finally, high gene amplification level is characterized by the presence of more than 10 mRNA copies or large clusters of them per nucleus in more than 50% of the examined cells (see online supplement for details).

Evaluation of results by Computerized Image Analysis

In order to evaluate the immunohistochemistry results not in a qualitative way but in a more accurate and reliable way, we performed computerized image analysis by using a semi-automated system (Matrox II Card Frame Grabber, Camera Microwave Systems, Microscope Olympus BX-50) allowing us to assess staining intensity in a 256 level scale - 0 (black)-255(white). Staining intensity values were then converted to reverse percentages {reverse staining intensity= (1-staining intensity/256) ×100 } (see online supplement for details).

STATISTICAL ANALYSIS

Statistical analysis was carried out using SPSS 13.0 software. Results are expressed as mean ± SD, or median (range), unless otherwise indicated. The unpaired t-test was used to compare the staining intensity of different immunostained factors between different forms of pulmonary fibrosis and between patients and controls. The paired t-test was used to compare staining intensity in epithelial cells, fibroblasts and myofibroblasts in IPF/UIP, COP and control samples. A p-value of <0.05 was considered as statistically significant.

RESULTS

Decreased telomerase expression and activity in fibrotic lungs compared to control samples

Because no information is available as to the expression of telomerase in human pulmonary fibrosis we assessed telomerase expression in mRNA and protein level using qRT-PCR, FISH and immunohistochemistry analysis in IPF, COP patients and control lung samples, respectively. Experimental procedure was further extended by assessing telomerase activity in IPF and control frozen lung samples, using a PCR ELISA detection kit. To expedite and standardize experimental procedures we constructed two TMA blocks consisting of 100 tissue cores each from 25 IPF, 20 COP and 20 control lung samples. TMA blocks were immunostained with anti-h-TERT antibody and analyzed quantitatively by computerized image analysis as described above. As expected, qRT-PCR showed a significant downregulation of telomerase expression in whole lung samples from IPF patients compared to controls (p<0.0001) indicating an association of low telomerase activity with pulmonary fibrosis (Figure 1A). In accordance with this, assessment of telomerase activity revealed almost undetectable enzymatic activity in lung extracts derived from IPF patients compared to control lung samples. Moreover, immunohistochemical studies coupled with semi-quantitative image analysis showed strong positive staining in the alveolar epithelium of

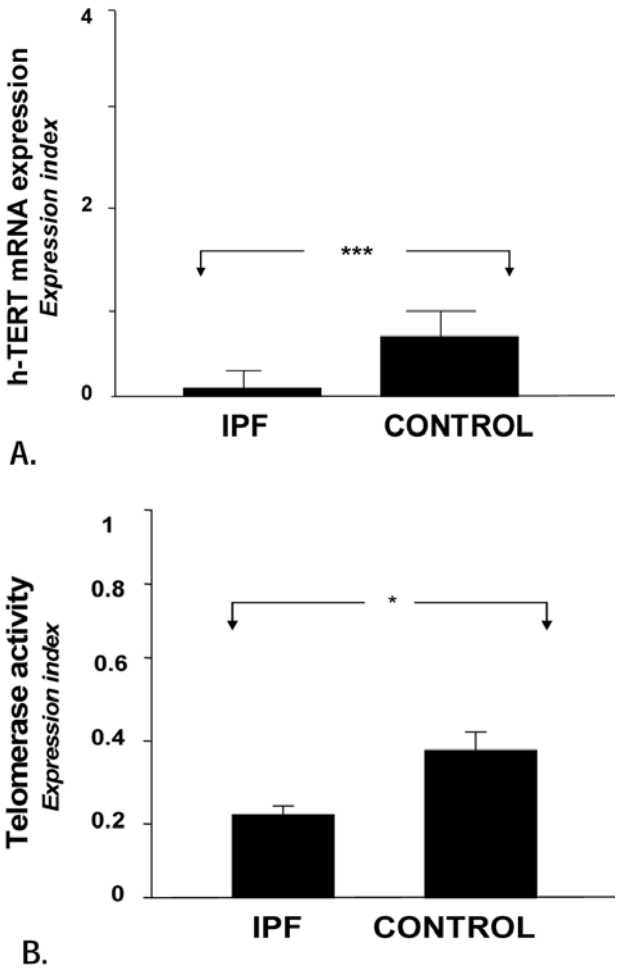

FIGURE 1. Decreased telomerase expression and activity in IPF compared to control lung samples. Real time qRT-PCR demonstrated statistically significant downregulation of telomerase expression in whole lung samples from IPF patients compared to control subjects (*** p<0.0001). In line with this, mean telomerase activity percentage, as assessed by TRAP assay, was found to be downregulated in IPF compared to control lung samples (*p<0.05).

control lung compared to the fibrotic lungs of two different histopathologic patterns, IPF ($p<0.001$) and COP ($p<0.001$) (Figure 2).

Telomerase is present in AECs surrounding areas of active fibrosis in IPF lung

To further analyze the cellular localization of tel-

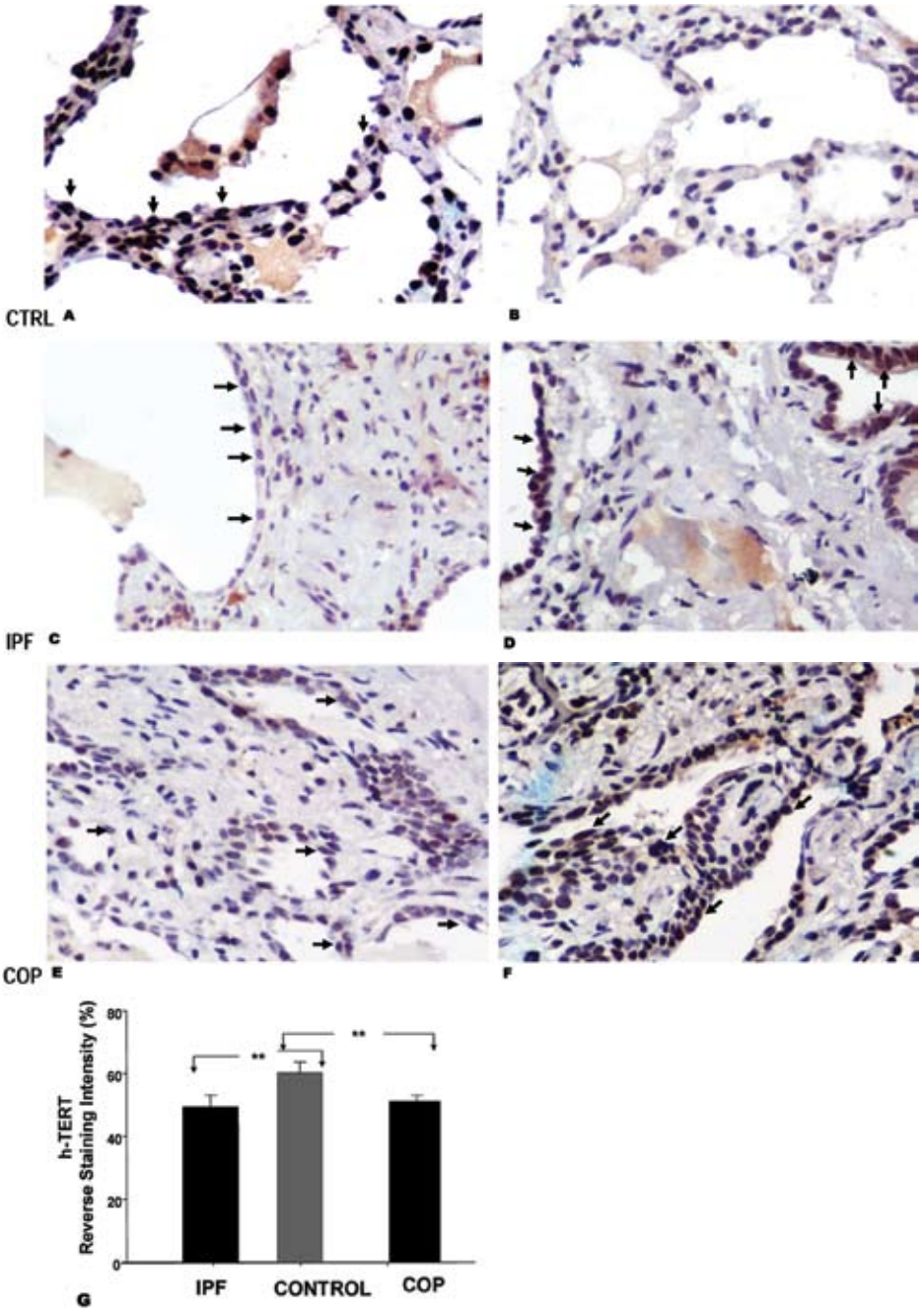

FIGURE 2. Decreased telomerase expression in fibrotic lungs compared to normal lung samples. Immunohistochemistry for h-TERT revealed positive nuclear staining (black arrows) in control lung (A) compared to isotype negative control (B), IPF (C, D) and COP lung (E, F). In IPF lung telomerase was mainly localized within areas of active fibrosis and in the alveolar epithelium surrounding them (D) whereas in COP lung positive staining was present within areas of alveolar filling (F). Semi-quantitative computerized immunohistochemical analysis (G) demonstrated significant differences in telomerase expression between fibrotic lungs and control samples (**$p<0.001$). Original magnifications: A-F: ×40.

omerase expression we performed dual immunostaining analyses and demonstrated absence of telomerase expression in type II AECs [telomerase negative (tel-) AECs] mainly overlying areas of established fibrosis and architectural distortion in IPF lung ($p<0.001$). In addition in COP lung the majority of type II AECs surrounding areas of organizing pneumonia showed minimal telomerase expression (Figure 3). Surprisingly, we identified clusters of type II AECs surrounding areas of active fibrosis mainly in IPF ($p<0.001$) and to a lesser extent in COP lung ($p<0.02$) which exhibited high telomerase staining [telomerase positive- (tel+) AECs], suggesting that these cells may represent a subpopulation of type II AECs that are resistant to apoptosis and cell damage and exhibit high proliferative and differentiative activity (Figure 4). In line with this, FISH analysis showed high amplification of telomerase expression not only within normal epithelium but also in areas of epithelium immediately adjacent to fibroblastic-like cells within the IPF lung in 19 of 25 cases whereas low amplification was seen in alveolar cells in 15 of 20 COP patients (Figure 5).

Increased telomerase expression in fibroblasts/ myofibroblasts in IPF compared to COP lung

To identify potential differences in the telomerase expression profiles exhibited by fibroblastic-like cells in patients with two different histopathologic patterns of pulmonary fibrosis we performed double immunohistochemistry analysis and demonstrated enhanced telomerase staining in fibroblastic/myofibroblastic foci within the IPF lung compared to Masson bodies that characterize the pattern of COP (Figure 6) ($p<0.001$). Furthermore, telomerase was also visualized within nuclei of pulmonary vascular cells that expressed aSMA in the IPF lung compared to COP subjects. Immunohistochemistry data was further extended by FISH analysis which revealed areas of high telomerase amplification within the fibrotic interstitium in IPF lung (17 of 25 cases) (Figure 5C) compared to COP patients where we detected low amplification of telomerase RNA component (18 of 20 cases) (Figure 5D). These findings may provide us with useful insights regarding differences between IPF and COP patients in disease progressiveness and treatment responsiveness and explain the resolution of lesions in COP lung in response to corticosteroids and the maintenance of fibroblastic foci despite of treatment.

Expression pattern of apoptosis and differentiation overlaps that seen for telomerase within fibrotic lungs

To confirm whether telomerase expression profile is followed by a similar apoptotic pattern the expression pattern of caspase-activated DNA fragmentation factor (DFF or caspase activated DNase-CAD) was determined. Enhanced apoptosis was clearly observed in the alveolar epithelium in IPF ($p<0.001$) and COP patients ($p<0.02$) compared to control subjects. In addition, as previously observed with telomerase negative AECs, we noticed strong positive staining in AECs surrounding areas of established fibrosis and architectural distortion (honeycomb cysts) suggesting that these cells where more likely to undergo apoptosis due to lack of telomerase expression. Finally increased apoptosis was also visualized within the fibrotic interstitium in COP lung compared to fibroblastic foci in IPF patients where apoptosis was minimal, further implicating telomerase in the "apoptotic paradox" characterizing IPF: apoptosis susceptibility in epithelial cells and apoptosis resistance of fibroblastic-like cells (Figure 7A-D). Since experimental data support a role of telomerase in cell differentiation we assessed whether the pattern of N-cadherin staining, a marker of mesenchymal differentiation, in the hyperplastic epithelium in IPF and COP lung overlaps that seen with telomerase. Intriguingly, N-cadherin was mainly localized in AECs ($p<0.01$) of morphologically altered phenotype immediately adjacent to fibroblastic foci suggesting that telomerase positive type II AECs need telomerase activity not only to resist to apoptosis but also to undergo a type of metaplasia, the epithelial – mesenchymal transition (Figure 7E-H).

DISCUSSION

To the best of our knowledge this is the first study in the literature investigating telomerase expression and activity in patients with idiopathic fibrotic lung disease and suggesting a causal relationship between low telomerase expression and pulmonary fibrosis as it happens with the phenotype in dyskeratosis congenita[30,31], a rare syndrome characterized by mutations in the telomerase complex and pulmonary fibrosis resembling IPF. Our study design was as follows: We used real time qRT-PCR and showed downregulation of telomerase expression in IPF lung compared to control samples. The latter was further supported by TRAP assay findings which revealed almost undetectable telomerase activity in IPF compared to control lung samples. In addition, we constructed two TMA

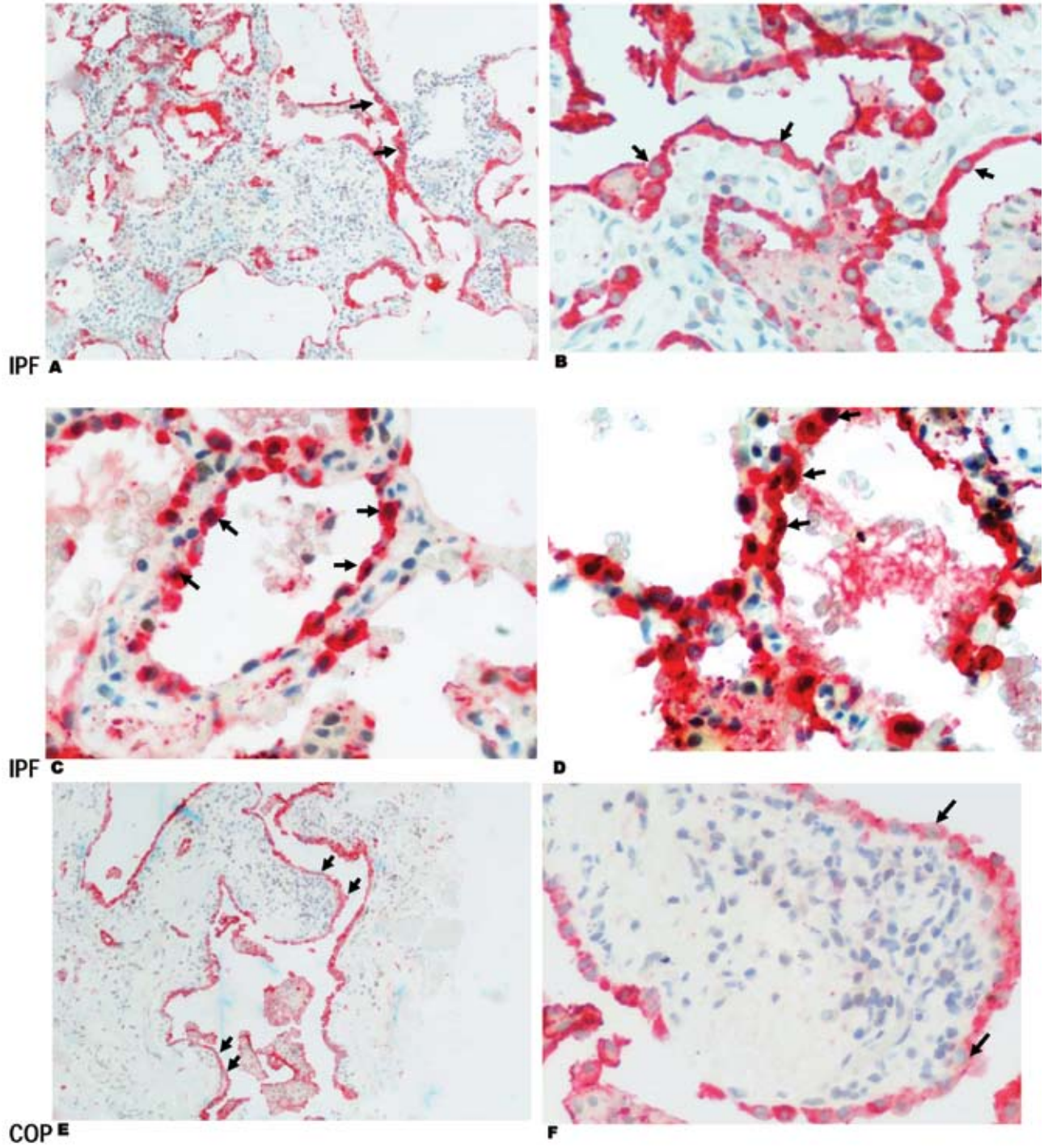

FIGURE 3. Telomerase was absent in the alveolar epithelium surrounding areas of established fibrosis in IPF lung. Dual immunostaining analysis for h-TERT and SP-A revealed absence of telomerase (white nuclei) from type II AECs (red cytoplasm) in areas of chaotic reepithelization and established fibrosis in IPF lung (A, B) whereas positive nuclear staining (arrows) was present in areas of alveolar epithelium that appeared histologically normal within the IPF lung (C, D). In addition in COP lung the majority of type II AECs surrounding areas of organizing pneumonia showed minimal telomerase expression (black arrows) (E, F). Original magnifications: A and E: ×20, B-D and F: ×40.

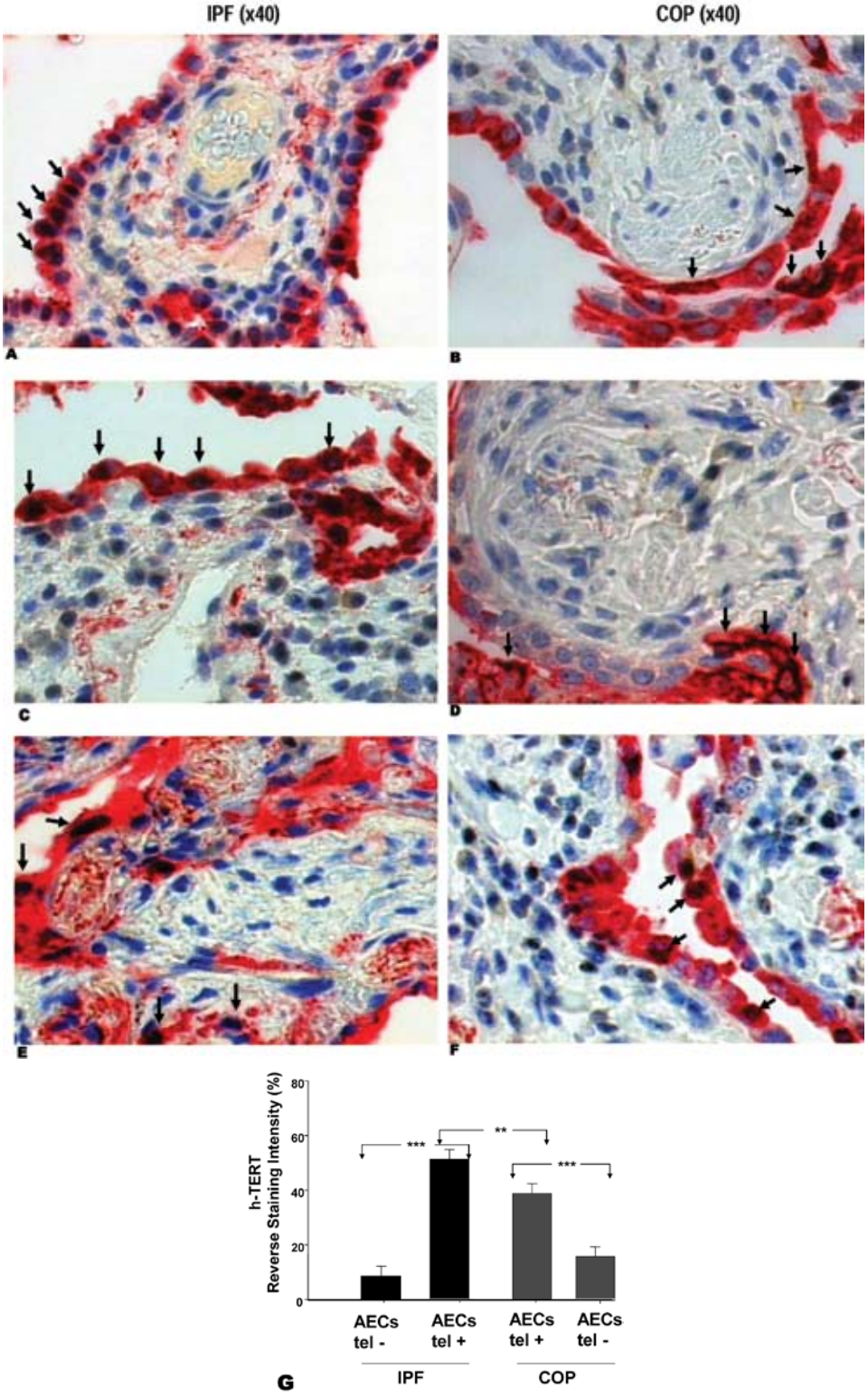

FIGURE 4. Telomerase co-localized with type II AECs overlying areas of active fibrosis within fibrotic lungs. Dual immunohistochemistry coupled with semi-quantitative image analysis revealed co-localization of telomerase (black nuclei) with type II AECs (red cytoplasm) immediately adjacent to areas of active fibrosis mainly in IPF lung (fibroblastic foci) (A, C and E) and to a lesser extent in COP lung (Masson bodies) (B, D and F) (**p<0.001). Original magnifications: A-F: ×40.

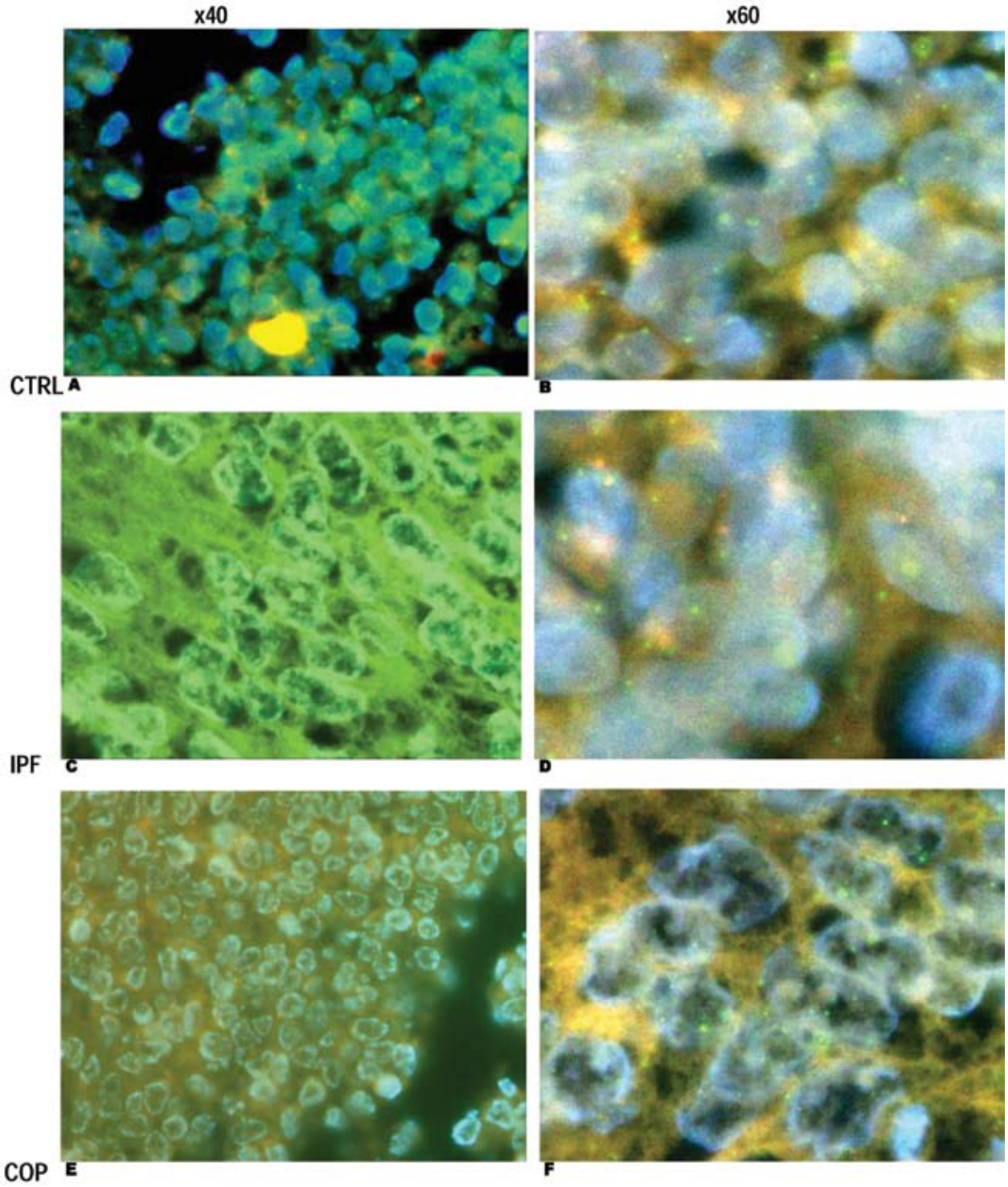

FIGURE 5. High amplification of telomerase mRNA expression in AECs overlying fibroblastic foci in IPF lung. FISH analysis revealed increased telomerase expression (mRNA copies indicated as green intracellular dots) in the alveolar epithelium of normal lung (A, B) compared to the fibrotic lung (C-F). Localization analyses revealed clusters of AECs immediately adjacent to areas of active fibrosis overexpressing telomerase mRNA mainly in IPF (D) and to a lesser extent in COP lung (E, F). Telomerase mRNA was also present in fibroblasts within the IPF lung (D) whereas low amplification was noticed within Masson bodies in COP lung (E). Original magnification: A, C and E: ×40, B, D and F: ×60.

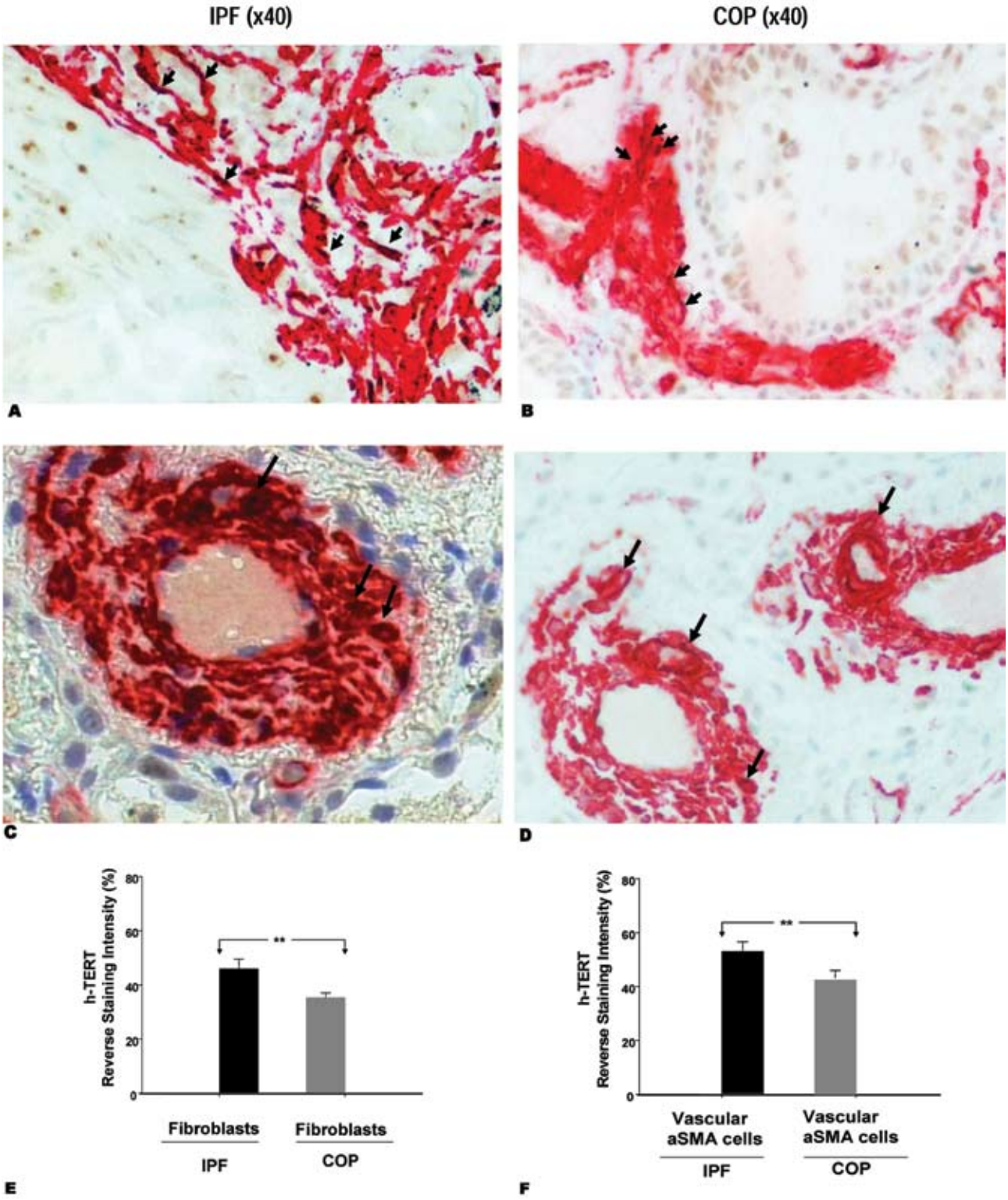

FIGURE 6. Telomerase co-localizes with a-smooth muscle actin (a-SMA) in fibroblastic foci in IPF lung. Dual immunostaining computerized image analysis for h-TERT and a-SMA revealed telomerase co-localization (black nuclei) within areas of active fibrosis (red cytoplasm) (arrows) in IPF (A) compared to COP lung (B). Telomerase was also present in pulmonary vascular smooth muscle cells that expressed aSMA in IPF (C) compared to COP lung (D). Original magnifications: A-D: ×40.

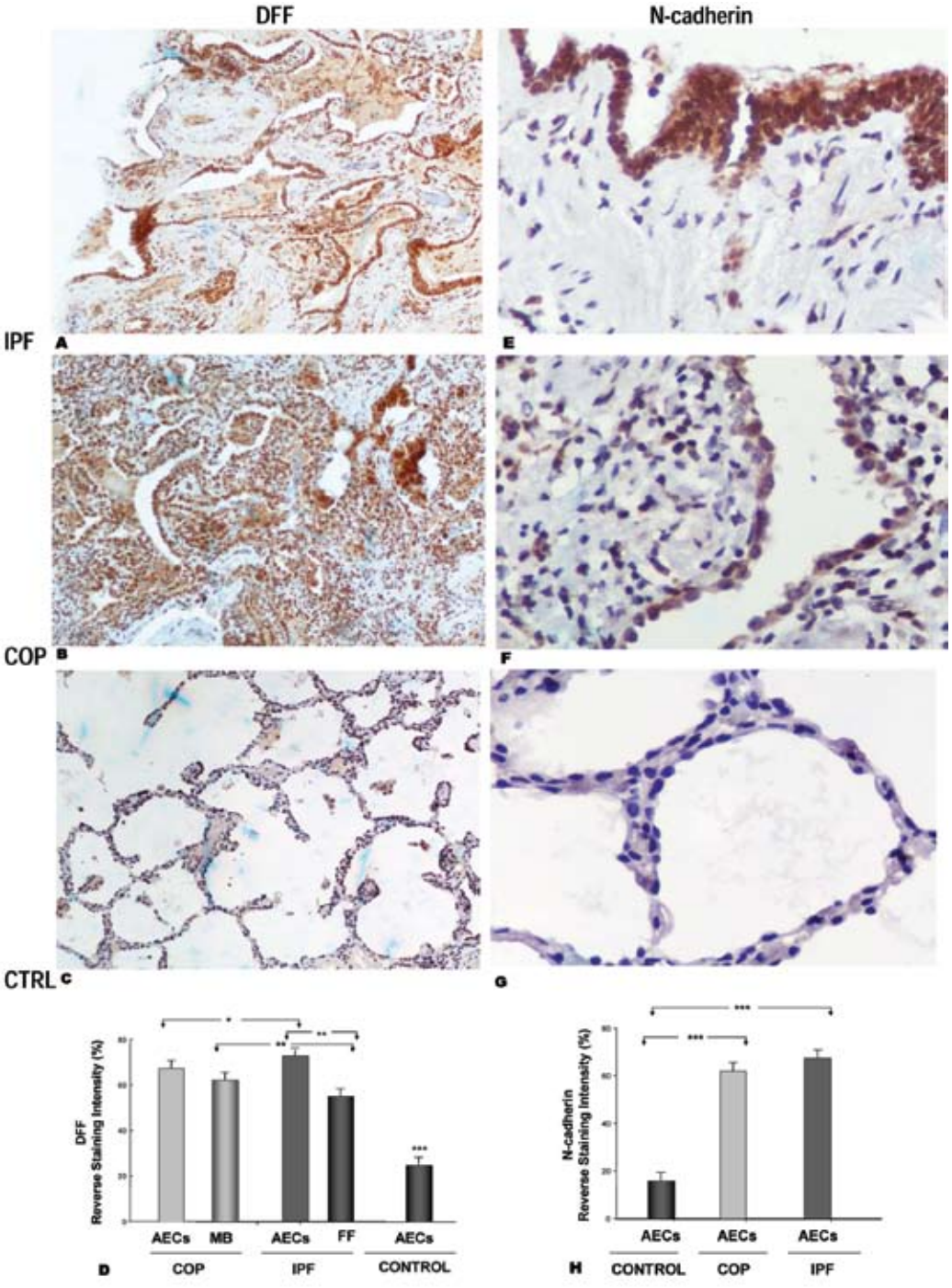

FIGURE 7. Expression pattern of apoptosis and differentiation overlaps that seen for telomerase within fibrotic lungs. Increased apoptosis as assessed by positive DFF immunostaining was observed in the hyperplastic alveolar and bronchiolar epithelium within the IPF lung (A) whereas in COP lung DFF was also visualized in areas of organizing pneumonia and Masson bodies (B). Moreover, intense staining for N-cadherin was present and most prominent in areas of abnormal reepithelization and broncholization (arrows) surrounding fibroblastic foci in the IPF lung (E), while in COP lung N-cadherin was in the epithelium overlying areas of alveolar filling and organizing pneumonia (F), areas that stained intensely for telomerase. No staining for DFF (**p<0.001) and N-cadherin (**p<0.001), as assessed by computerized image analysis was visualized within intact, normal alveolar structures (C, G). Original magnifications A-C and E: x20, F and G: ×40.

blocks comprising of 100 representative tissue samples from patients with IPF and COP and control subjects. Immunostaining studies coupled with computerized image analysis revealed decreased telomerase expression in IPF and COP lung samples compared to controls. We then chose to analyze the cellular localization of telomerase expression both by dual immunohistochemistry and FISH which absence of telomerase in type II AECs surrounding areas of established fibrosis and architectural distortion within the IPF lung. Interestingly, we found strong nuclear staining not only within areas of active fibrosis but also in regenerative type II AECs immediately adjacent to them, as well as in pulmonary vascular cells, mainly in IPF lung and to a lesser extent in COP samples. Most intriguingly, regional heterogeneity in telomerase expression, in both fibrotic lungs, resulted in similar expression patterns of apoptotic and differentiation markers, as assessed by immunolocalization analyses.

In IPF/UIP, areas of accumulated fibroblasts and myofibroblasts mainly distributed in subepithelial areas of the intersititium represent the pathogenetic hallmark of fibrogenesis and have been used as part of the histopathologic criteria for the diagnosis of UIP[10]. The presumed persistence as assessed by prolonged survival and increased proliferation, of these cells can potentially be the basis for progressive fibrosis instead of resolution. While IPF clinical course seems to be unfavorable other members of the same disease group such as COP present with excellent prognosis and treatment responsiveness[19]. Several studies have attributed these differences to distinct pathogenetic characteristics reflected by regional differences in apoptotic[32-34] and angiogenic profiles[35-37]. Moreover, while the alveolar epithelium has been thought as a passive bystander in the process of pulmonary fibrosis, the past few years the notion that repeated epithelial injury induces regenerative AECs to transition to a mesenchymal phenotype (epithelial-mesenchymal transition-EMT) ,thus contributing directly to fibrogenesis, has dragged much of attention[38-40].

Recent studies focusing on telomerase expression in fibroblasts derived from experimental models of pulmonary fibrosis revealed a role of telomerase activity in cell differentiation. In particular, Phan et al. suggested that induction of telomerase activity in rat lung fibroblasts may be due to mediators of their proliferation while its loss by inhibitory molecules triggers their differentiation to myofibroblasts[15,21-23]. In addition, Fridlender et al. implicated low telomerase activity in robust apoptosis of AECs isolated from mice lungs after treatment with bleomycin further suggesting that the transient elevation of telomerase activity in lung epithelial cells in vivo may represent an active defense mechanism against immediately adjacent fibroblastic/myofibroblastic foci that may promote AECs apoptosis as part of the fibrotic process[24]. Finally and more recently, a causal-effect relationship between low telomerase activity and pulmonary fibrosis was reported by Liu et al. who demonstrated in TERT-deficient mice reduced lung fibrosis accompanied by decreased fibroblast proliferation and increased apoptosis. Conversely, restoration of telomerase activity in BLM-injured lungs was associated with increased survival of lung fibroblasts favouring the development of lung fibrosis[21]. However, human and mouse telomerases are reported to differ in both their functional properties and their regulation and additionally bleomycin-animal model is not fully representative of IPF due to its self-limiting nature, rapidity of its development and the close association with inflammation that accompanies the lung injury. In addition, at the disease level the net effects of telomerase activity versus inhibition on the development and progression of human pulmonary fibrosis are so far largely unknown[23].

Therefore, we investigated for the first time in the literature, telomerase expression and activity in tissue samples from patients with two different forms of fibrotic lung disorders, IPF and COP. We used the pioneering technology of tissue microarrays, which allowed us the simultaneous analysis of up to 200 tissue blocks in a single experiment under highly standardized conditions, meaning all tissue specimens were analyzed in an identical, unbiased fashion, with minimal tissue damage, less cost and precise positioning of arrayed samples which facilitates manual interpretation of the staining[28] and serves as an ideal basis for automated analysis of specific cell lines. Data interpretation revealed reduced telomerase expression and activity in IPF compared to control lung samples suggesting a causal relationship between low telomerase expression and human pulmonary fibrosis. Results from qRT-PCR and TRAP assay were extended by immunohistochemical studies coupled with semi-quantitative image analysis which evaluated signal staining intensity in an unbiased fashion and in a 256-level scale (conventional microscopes identify signal differences in a 30-level scale)[41,42] and showed strong positive staining in normal epithelium compared to the fibrotic one. Most intriguingly, immunolocalization studies coupled by FISH analysis further supporting seminal observations[43], demonstrated two distinct subpopulations of type II AECs

based on their telomerase expression levels; morphologically altered telomerase positive cells overlying clusters of active fibrosis and exhibiting increased levels of differentiation markers such as N-cadherin mainly within the IPF lung and to a lesser extent in COP lung and telomerase negative cells visualized in areas of architectural distortion in IPF lung and immediately adjacent to areas of organizing pneumonia in COP tissue samples. The latter cells seem to exhibit increased apoptotic activity as can be supported by similar expression patterns between telomerase and DNA fragmentation factor. Moreover, increased telomerase expression was also observed in areas of alveolar epithelium that appeared histologically normal within the IPF lung.

The duality phenomenon in telomerase expression in regenerative type II AECs provides us with useful pathogenetic insights regarding the role of telomerase in pulmonary fibrosis. It is therefore tempting to speculate that changes in telomerase activity may regulate the fate of AECs towards either an apoptotic or a mesenchymal phenotype, thus contributing directly to fibrosis (Figure 8). Recent evidence by two independent studies[22,23] associate mutant telomerase components with familial IPF suggesting that short dysfunctional telomeres may lead to alveolar cell apoptosis and generation of multiple fibrotic lesions. They also report that under multiple attacks bronchoalveolar epithelium responds with constant regeneration and this process relies on local progenitor cells that exhibit low survival rates due to short telomeres resulting from low telomerase activity. Our findings are consistent with this hypothesis. Telomerase negative progenitor cells are more likely to undergo apoptosis as assessed by increased expression of DNA fragmentation factor, thus contributing to the abnormal reepithelization characterizing disease pathogenesis. In line with this, the presence of increased telomerase staining in flattened, attenuated type II AECs (Figures 7B and 7E) expressing markers of cell differentiation may suggest a need for telomerase activity to promote EMT[38,44,45].

In addition, telomerase presence within clusters of highly proliferative fibroblastic-like cells in IPF compared to COP samples indicates a mechanism through which IPF fibroblasts of altered phenotype exert their resistance to apoptosis and may potentially explain the resolution of lesions in COP lung in response to corticosteroids and the maintenance of FF resulting in dismal prognosis despite of treatment[46,47]. The unremitting recruitment and maintenance of the altered fibroblast phenotype with generation and proliferation of immortal fibroblasts coupled with the EMT phenomenon is reminiscent with the transformation of cancer cells and metaplasia. Whether mutations in telomerase genes are responsible either for reduced telomerase expression and shorten telomeres resulting in loss of AECs or increased expression leading to hyperplasia and transition to a mesenchymal phenotype, a form of metaplasia, is so far largely unknown. What is already known is that mutated telomerase components may lead to genomic instability, as it happens during the stepwise progression of normal cells into cancer cells. Telomerase activation after onset of genomic instability coupled with an injurious pro-fibrotic microenvironment, promotes acquisition of transformed cells through a process known EMT (Figure 8). Searching for telomerase mutations in AECs and fibroblasts isolated from patients with different forms of pulmonary fibrosis at different disease stages may provide a way forward and help clinicians to identify high-risk groups that are unlikely to respond to immunosuppressive agents.

In addition, localization of telomerase within pulmonary vascular cells expressing aSMA in IPF lung may explain the increase proliferation and remodeling characterizing these cells leading ultimately to major vasoconstriction and the development of pulmonary hypertension secondary to end-stage fibrosis, one leading cause of death in IPF patients worldwide[48]. However, future studies are required to corroborate this observation.

Despite relative enthusiasm arising from the above data, our study exhibits a number of limitations that should be addressed with caution. Firstly, based on semi-quantitative immunohistochemical analysis it is not definitive whether there is a correlation between telomerase staining and telomerase expression. However, immunohistochemistry data was supported by quantitative FISH analysis and corroborated results from qRT-PCR and TRAP assay. Therefore, it is conceivable that strong associations using different techniques can be supportive of our hypothesis. Secondly, based on our approach it is rather unclear whether there is an association between loss of telomerase expression and activity and apoptosis or induction and differentiation of epithelial cells to mesenchymal phenotypes. Nevertheless, our study represents the first attempt to correlate telomerase with alveolar cell apoptosis, differentiation and increased proliferation of immortalized fibroblasts in the pathogenetic model of pulmonary fibrosis. Taken together, we can assume that novel therapeutic approaches aiming at preventing loss of alveolar cells through induction of telomerase activity may be proven more beneficial than current anti-fibrotic

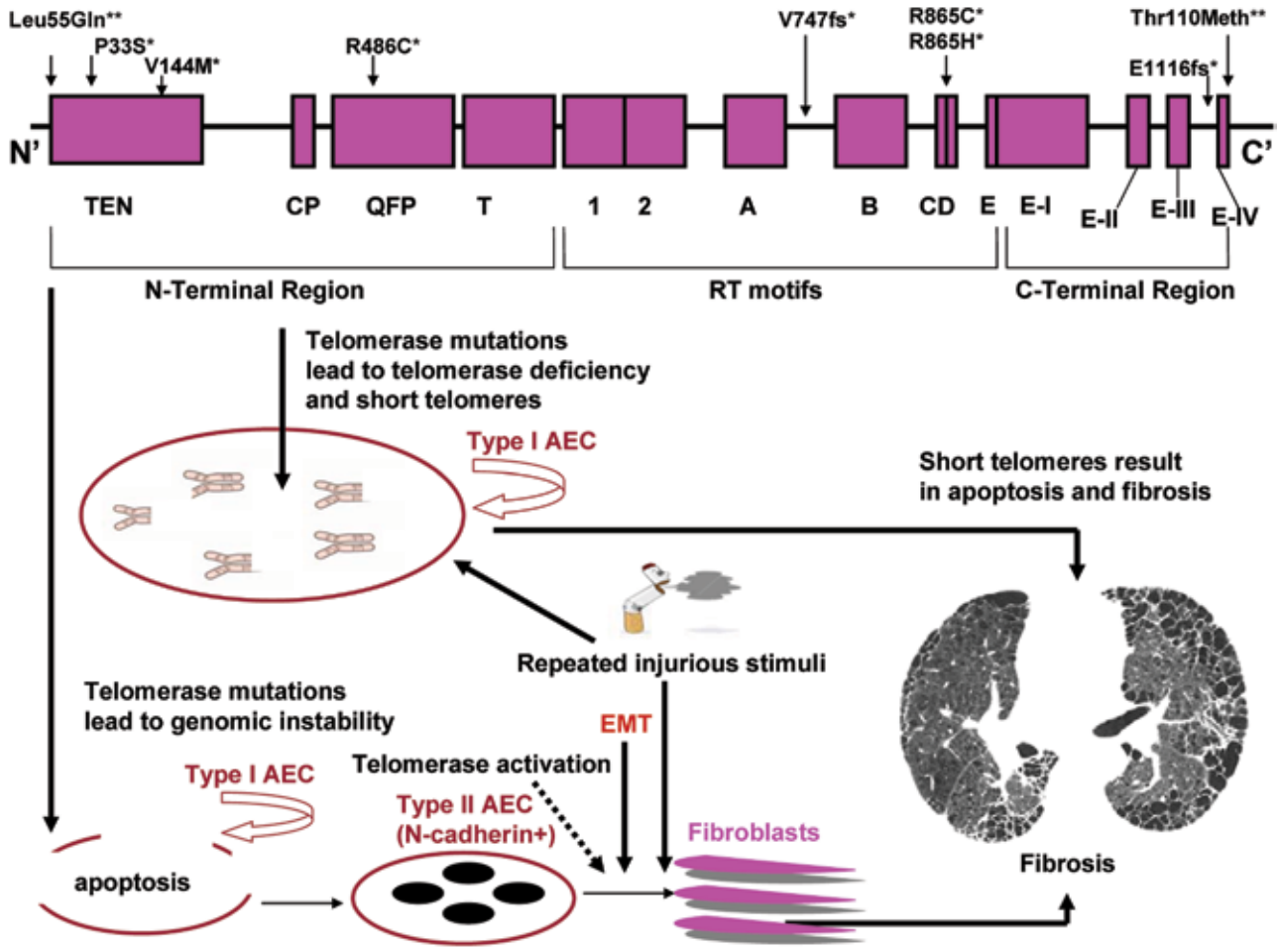

FIGURE 8. Telomerase mutations, telomerase activation, epithelial-mesenchymal transition, apoptosis and pulmonary fibrosis. Schematic representation of the conserved domains of hTERT with missense mutations, as indicated by Tsakiri K et al. (23) (*) and Armanios M (22) et al. (**). Mutations of hTERT catalytic subunit may lead to telomerase deficiency and short dysfunctional telomeres in type I alveolar epithelial cells (AECs). The latter coupled with repeated injurious stimuli (i.e. cigarette smoke) could potentially promote AEC apoptosis triggering the fibrogenic process. Furthermore, mutated telomerase components may lead to genomic instability, as it happens during the stepwise progression of normal cells into cancer cells. Apoptosis of type I AECs leads to compensatory proliferation of type II AECs. Telomerase activation after onset of genomic instability coupled with an injurious pro-fibrotic microenvironment, promotes acquisition of transformed cells through a process known as epithelial – mesenchymal transition (EMT). During this process, type II AECs instead of differentiating to type I AECs, undergo a form of metaplasia towards a mesenchymal phenotype (N-cadherin+), thus contributing directly to pulmonary fibrosis, as indicated by honeycombing changes in high resolution computed tomography (HRCT).

or immunomodulatory agents[49].

In conclusion, we have shown reduced telomerase expression in idiopathic fibrotic lung disorders compared to control samples implicating low telomerase activity in the pathogenesis of pulmonary fibrosis. This downregulation of telomerase expression that was followed by similar expression patterns of apoptosis was marked in AECs surrounding areas of end-stage lung disease. Surprisingly elevated telomerase staining was not only present within highly proliferative fibroblastic/myofibroblastic foci where detection of apoptosis was minimal but also within areas of hyperplastic epithelium surrounding them indicating that telomerase may regulate the fate of alveolar cells towards either an apoptotic or a differentiative - mesenchymal phenotype, thus contributing directly to fibrosis. Finally differential telomerase expression profiles in IPF and

COP may explain differences in disease progressiveness and treatment responsiveness. Future studies in patients with different forms of interstitial pneumonias coupled with experimental data potentially using telomerase null mice may help us to elucidate the role of telomerase in IPF and highlight novel therapeutic targets.

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