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Endothelial Cell Senescence Exacerbates Pulmonary Fibrosis Potentially Through Accelerated Endothelial to Mesenchymal Transition

RISA RAMADHIANI[1,2], KOJI IKEDA[1,3,*], KEN-ICHI HIRATA[2], and NORIAKI EMOTO[1,2,*]

[1]*Laboratory of Clinical Pharmaceutical Science, Kobe Pharmaceutical University, 4-19-1 Motoyamakitamachi, Higashinada, Kobe 658-8558, Japan;*

[2]*Division of Cardiovascular Medicine, Department of Internal Medicine, Kobe University Graduate School of Medicine, 7-5-1 Kusunoki, Chuo, Kobe 6500017, Japan;*

[3]*Department of Epidemiology for Longevity and Regional Health, Kyoto Prefectural University of Medicine, 465 Kajii, Kawaramachi-Hirokoji, Kamigyou, Kyoto 6028566, Japan*

**Corresponding author*

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Idiopathic pulmonary fibrosis (IPF) is a devastating lung disease characterized by progressive lung fibrosis and obliteration of normal alveolar structures. Myofibroblasts play a central role in the progression of IPF by producing excess amount of extracellular matrix, and these myofibroblasts show heterogenous origins including resident fibroblasts, epithelial cells via epithelial to mesenchymal transition (EMT) and endothelial cell (EC) via endothelial to mesenchymal transition (EndMT). Although lung aging has been considered as essential mechanisms through abnormal activation of epithelial cells and fibroblasts, little is known about a role of EC senescence in the pathogenesis of IPF. Here, we reveal a detrimental role of EC senescence in IPF by utilizing unique EC-specific progeroid mice. EC-specific progeroid mice showed deteriorated pulmonary fibrosis in association with an accelerated EndMT in the lungs after intratracheal bleomycin instillation. We further confirmed that premature senescent ECs were susceptible to EndMT *in vitro*. Because senescent cells affect nearby cells through senescence-associated secretory phenotype (SASP), we assessed a potential role of the EC-SASP in EMT and myofibroblastic transition of resident fibroblasts. EC-SASP enhanced the myofibroblastic transition in resident fibroblasts, while no effect was detected on EMT. Our data revealed a previously unknown role of EC senescence in the progression of IPF, and thus rejuvenating ECs and/or inhibiting EC-SASP is an attracting therapeutic strategy for the treatment of IPF.

INTRODUCTION

Idiopathic pulmonary fibrosis (IPF) is the most common interstitial pneumonia, which is a progressive fibrotic interstitial lung disease with unknown etiology (1). IPF occurs in middle-aged and elderly adults, and most patients are older than 60 years at the time of diagnosis (2). The prevalence of the disease is increasing, especially in the United States, as its prevalence among adults over 65 years became doubled in 10 years between 2000 and 2011 (3). The prognosis of IPF is poor, with a median survival of 3.8 years among adults over 65 years in the United States. Recently, two medications, nintedanib and pirfenidone, have been developed for the treatment of IPF. Nintedanib is a tyrosine kinase inhibitor that blocks various growth factor signaling, while pirfenidone inhibits TGF-β production, collagen synthesis, and fibroblast proliferation (1). Both drugs slowed the respiratory function decline by ~50% over the course of 1 year; nonetheless it remains unclear whether these drugs can extend the life-span in patients with IPF.

Aberrant repair of injured alveolus and subsequent deposition of fibrils derived by fibroblasts is the core pathogenesis in IPF (1). Fibroblasts in the lungs of pulmonary fibrosis showed multiple origins including resident mesenchymal cells, bone marrow progenitor cells, epithelial cells, and endothelial cells (4-6). Epithelial to mesenchymal transition (EMT) is a dynamic change in cellular organization from epithelial to mesenchymal phenotype (7). During EMT, epithelial cells lose epithelial characters and acquire mesenchymal phenotype, which leads to functional changes in cell migration and invasion (7). EMT is crucially involved in the pathogenesis of IPF by providing sources for fibroblasts. On the other hand, endothelial to mesenchymal transition (EndMT) is a process in which endothelial cells (ECs) adopt a mesenchymal phenotype (8). During

Phone: +81-78-441-7537 Fax: +81-078-441-7538 E-mail: ikedak-circ@umin.ac.jp

EndMT, ECs lose endothelial characters, and express mesenchymal cell-specific genes, which leads to phenotypic shift to the higher motility and contractile properties (8). Less is known about a role of EndMT in the pathogenesis of IPF than that of EMT; however, crucial involvement of EndMT in pulmonary fibrosis has been revealed recently (4,5,9). TGF-β signaling plays a central role in both EMT and EndMT processes (6-8) (10). TGF-β is produced by a wide variety of cell types such as macrophages, neutrophils, epithelial cells, ECs, and fibroblasts, and it promotes the differentiation of quiescent fibroblast into active myofibroblast that highly express the extracellular matrix proteins (11).

It has been suggested that accelerated aging in the lungs due to smoking, viral infections, and microaspiration is causally involved in IPF; however, biological processes that link aging to IPF remain to be elucidated. In this study, we explored a potential role of EC senescence in IPF using unique EC-specific progeroid mice that we recently generated (12,13). We revealed a detrimental role of EC senescence in the progression of IPF potentially through enhancing EndMT.

MATERIALS AND METHODS

Antibodies

Antibodies used are; anti-von Willebrand factor (Abcam #Ab9378), anti-actin α-smooth muscle, FITC-labelled (Sigma-Aldrich # F3777), and Alexa Fluor 594-labelled (Life Technologies # A21207) donkey anti-rabbit IgG.

Animal study

All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Kobe Pharmaceutical University. Transgenic mice that overexpress the dominant negative form of the telomere repeat-binding factor (TRF)-2 in ECs (VEcad-TRF2DN-Tg) were generated (C57/BL6J background) as described previously (12,13). Transgenic mice and their littermates were regularly used for experiments at the age of 12–15 weeks.

Pulmonary fibrosis was induced by intratracheal administration of bleomycin (10 mg/kg) as described previously (6). Briefly, mice were anesthetized with 1–2% isoflurane (Pfizer, NY), and the trachea was exposed through midline cervical incision. Bleomycin in 60 μl of sterile 0.9%s saline (vehicle) was administered via a 22-gauge cannula, and then the mice were briefly connected to ventilator unit (Muromachi Kikai, Tokyo, Japan) for uniform distribution of bleomycin before closing the skin incision.

Lung mechanics measurements

Two weeks after bleomycin instillation, the mice were anesthetized with 2,2,2–tribromoethanol (Sigma-Aldrich), tracheostomized, then paralyzed with 30 μg of pancuronium bromide (Sigma-Aldrich). Subsequently, lung mechanic parameters were measured by using flexiWare (flexiVent, SCIREQ, Quebec, Canada). Each maneuver was then recorded.

Histological analysis

The lung was inflated, and fixed with 4% paraformaldehyde, followed by embedding in paraffin. The sections were cut into 4 μm, and stained with Masson's trichrome to assess the fibrotic area. The five randomly chosen images were captured using Keyence BZ-X800.

Immunostaining was performed to quantify the αSMA positive area, and to detect the endothelial-mesenchymal transition (EndMT) process. Briefly, the lung sections were deparaffinized, incubated in antigen unmasking solution (Vector Laboratories), and then blocked in 5% skim-milk in PBS containing 0.2% Triton-X. The sections were incubated with first antibody at 4^{o}C for overnight, followed by incubation with fluorescence-labelled secondary antibody. After washing with PBS, sections were covered with VECTASHIELD mounting medium with DAPI (Vector Laboratories #H-1200), and observed under fluorescent microscopy (Keyence BZ-X800).

Mouse lung fibroblast isolation

The isolation of lung fibroblasts was performed according to the previous protocol (14). Briefly, the lung tissues obtained from C57/BL6J mice were minced and digested with 1 mg/ml collagenase IV (Gibco) in Hanks' Balanced Salt Solution (HBSS) (Gibco). The digested tissues were filtered through 100 μm cell strainer (Falcon, Corning, NY), followed by centrifugation at 1500 rpm for 10 min. After washing with Ack Lysing Buffer (Gibco), cells were cultured in low-glucose DMEM supplemented with 10% FBS.

Preparation of senescent ECs and their conditioned medium

Premature senescence was induced by retrovirus-mediated gene transfer of the TRF2DN in human pulmonary artery ECs (PAECs) as previously described (12,15). PAECs infected with retrovirus carrying GFP were used as young control cells. RNAs were extracted from cells 72 h after retrovirus infection. To prepare the conditioned medium, RPMI medium containing 1% FBS medium was given to cells 72 h after retrovirus infection, and the conditioned medium was collected after incubation for 24 h. After removal of floating cells by centrifugation, the conditioned medium was stored in -80^{o}C until use.

Induction of EndMT, EMT, and myofibroblast transition

PAECs were cultured in HuMedia-EG2 (Kurabo). The EndMT was induced by the treatment with TGF-β1 (10 ng/ml) and IL-1β (10 ng/ml) for 6 days, as previously described (10). For EMT, A549 cells were treated with 10 ng/ml TGF-β1 and 10 ng/ml TNFα in the conditioned medium prepared from young control and premature senescent PAECs for 24 h. For myofibroblast transition, mouse lung fibroblasts were treated with 10 ng/ml TGF-β1 in the conditioned medium prepared from young control and premature senescent PAECs for 24 h.

Quantitative PCR

RNAs were extracted from cells and mouse tissues using RNAiso plus (TAKARA), followed by purification using NucleoSpin RNA clean-up kit (Macherey-Nagel). The cDNA was synthesized using PrimeScript RT Reagent Kit with gDNA eraser (TAKARA). Qualitative PCR was performed using LightCycler96 (Roche Applied Science). The mRNA expression levels were normalized to 18S expression levels, and the relative gene expressions are presented in arbitrary units. The primers used are shown in Table I.

Statistical analysis

All data are presented as mean ± SEM. Statistical analysis was performed using Graphpad Prism 9. The difference between 2 groups was analyzed using student *t*-test, while differences between groups more than 3 was analyzed using one-way ANOVA with Tukey's post hoc test, as indicated. The number of experiments and animals per group were indicated in the figure legends.

RESULTS

Endothelial progeroid mice show exacerbated pulmonary fibrosis

To investigate a role of EC senescence in age-related diseases, we have generated EC-specific progeroid mice (12). ECs-specific progeria has been achieved by overexpressing the dominant negative form of telomere repeat-binding factor 2 (TRF2) in ECs under the control of the VE-cadherin promoter (VEcad-TRF2DN-Tg mice) (13). We explored a potential role of EC senescence in the pathogenesis of IPF by generating bleomycin-induced pulmonary fibrosis model using these EC-specific progeroid mice. At the base line, the body weight was similar, and there was no apparent difference in lung structures and fibrosis between wild-type (WT) and VEcad-TRF2DN-Tg mice (Figure 1A and 1B). The body weight loss during 2 weeks after bleomycin instillation was also similar between these mice (Figure 1C). Of note, *in vivo* lung mechanics analysis showed an increase in tissue damping of the lungs in VEcad-TRF2DN-Tg mice after bleomycin-instillation, which suggest severe lung fibrotic changes (Figure 1D). Furthermore, histological analysis exhibited the deteriorated pulmonary fibrosis in VEcad-TRF2DN-Tg mice after bleomycin-instillation (Figure 1E). These data revealed a detrimental role of EC senescence in the development of pulmonary fibrosis.

EndMT is enhanced in the lungs of EC-specific progeroid mice after bleomycin-instillation

In the lungs of VEcad-TRF2DN-Tg mice, significantly more cells that express the myofibroblast/mesenchymal marker α-SMA were detected than in WT mice after bleomycin-instillation (Figure 1F). Moreover, mesenchymal marker genes expressions were robustly increased in the lungs of VEcad-TRF2DN-Tg mice comparing to those in WT mice (Figure 1G). Of note, endothelial marker gene expression was reduced, while epithelial cell marker gene expression was increased in the lungs of VEcad-TRF2DN-Tg mice (Figure 1G). Furthermore, ECs that express mesenchymal marker gene appeared to increase in the lungs of VEcad-TRF2DN-Tg mice after bleomycin-instillation (Figure 2). These data collectively suggest that EndMT is enhanced in the lungs of bleomycin-treated VEcad-TRF2DN-Tg mice, which leads to augmented myofibroblast emergence and worsened pulmonary fibrosis.

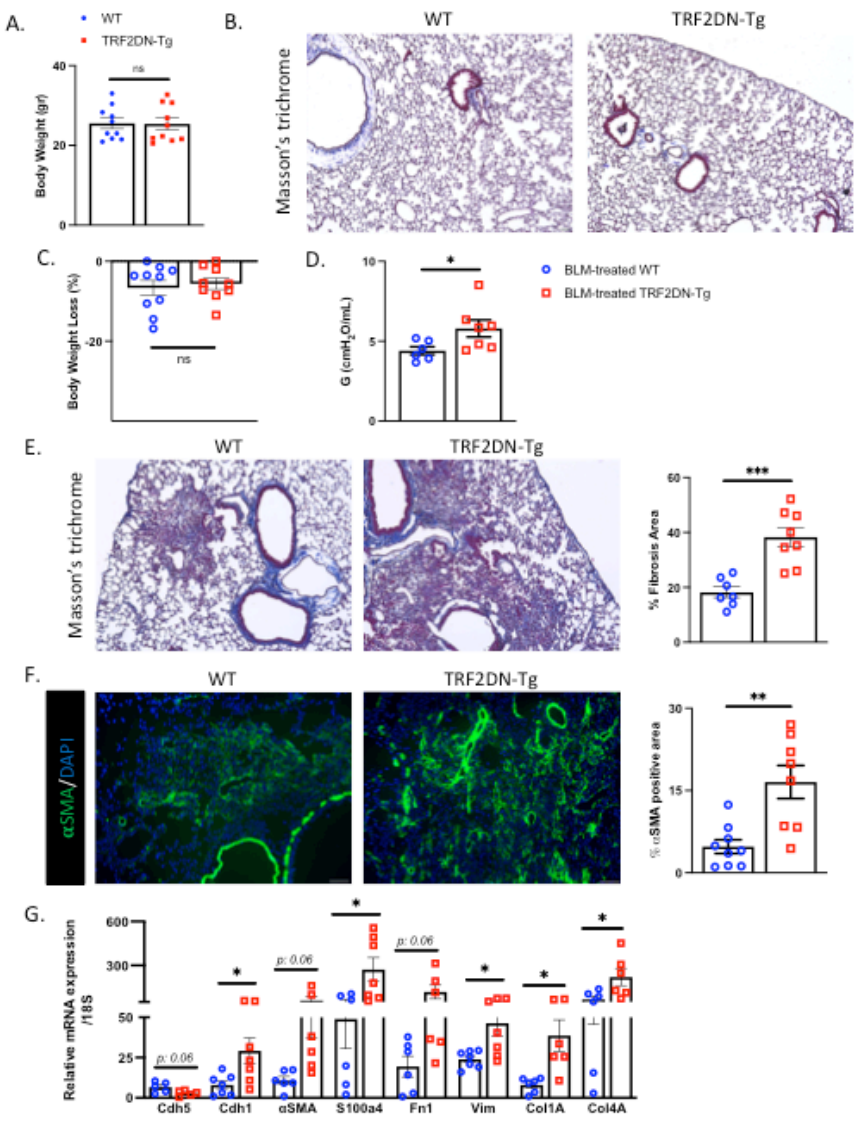

Figure 1. EC-specific progeria exacerbates pulmonary fibrosis in mice.
(A, B) Body weight (A) and representative images of the lung sections stained with Masson's Trichrome (B) in WT and the Tg mice in normal condition. (C) Loss of body weight in WT and the Tg mice during 2 weeks after bleomycin (BLM)-instillation. (D) Tissue damping of the lungs in WT and the Tg mice after BLM-instillation. (E) Representative images of the lung sections stained with Masson's trichrome. Lungs were isolated from WT and the Tg mice 2 weeks after BLM-instillation. Fibrotic area was quantified. (F) Representative images of immunohistochemistry for αSMA in the lung sections of BLM-treated WT and the sTg mice. αSMA-positive area was quantified. (G) Quantitative PCR analysis for endothelial and mesenchymal marker genes in the lungs of BLM-treated mice. Genes analyzed are cadherin-5 (Cdh5), cadherin-1 (Cdh1), α-smooth muscle actin (αSMA), S100 calcium binding protein A4 (S100a4), fibronectin 1 (Fn1), vimentin (Vim), collagen type-1 α1 chain (Col1A), and collagen type-4 α1 chain (Col4A). Data are presented as mean ± SEM. Two-tailed student's *t*-test was used for the statistical analysis of the differences between two groups. The number of samples analyzed was: n = 6–9 for WT and n = 7–9 for Tg. Scale bars: 50 μm. **P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.*

Senescent ECs are highly susceptible to EndMT

To explore the correlation between EC senescence and EndMT, we prepared premature senescent pulmonary artery ECs (PAECs) by retrovirus-mediated gene transfer of TRF2DN. Cellular senescence was confirmed by the increased expression of cyclin-dependent kinase inhibitors (p16, p19 and p21) and inflammatory cytokines compared to those in the control young cells transfected with GFP (Figure 3A). When induced EndMT using TGF-β and IL-1β, loss of endothelial marker and gain of mesenchymal marker were accentuated in senescent PAECs comparing to those in young control cells (Figure 3B). These data strongly suggest that cellular senescence causes high susceptibility to EndMT in ECs.

Senescent ECs enhance myofibroblastic transition of resident fibroblasts through the SASP

Senescent cells affect nearby cells by secreting numerous soluble factors including cytokines, growth factors, and matrix metalloproteinases, which is called senescence-associated secretory phenotype (SASP) (16). Since EC senescence caused augmented myofibroblast emergence in the lungs of bleomycin-treated mice, we examined whether senescent ECs affect EMT and/or myofibroblast transition through the SASP. Conditioned medium (CM) that is enriched with SASP factors was prepared using the young control and the premature senescent PAECs, and we treated A549 cells, human alveolar epithelial cells, with the CM in the presence or absence of EMT induction. Treatment with the CM of senescent PAECs did not affect the expression of either

epithelial or mesenchymal marker gene in A549 cells as compared to cells treated with CM of the control young PAECs both in the presence and absence of the EMT induction (Figure 4A).

On the other hand, when treated with the CM of senescent PAECs, mouse lung fibroblasts showed enhanced myofibroblastic transition induced by TGF-β (Figure 4B). These results suggest that senescent ECs enhance the myofibroblastic transition of resident lung fibroblasts through the SASP, which might contribute to the exacerbated pulmonary fibrosis in the EC-specific progeroid mice after bleomycin-instillation, in addition to the accelerated EndMT.

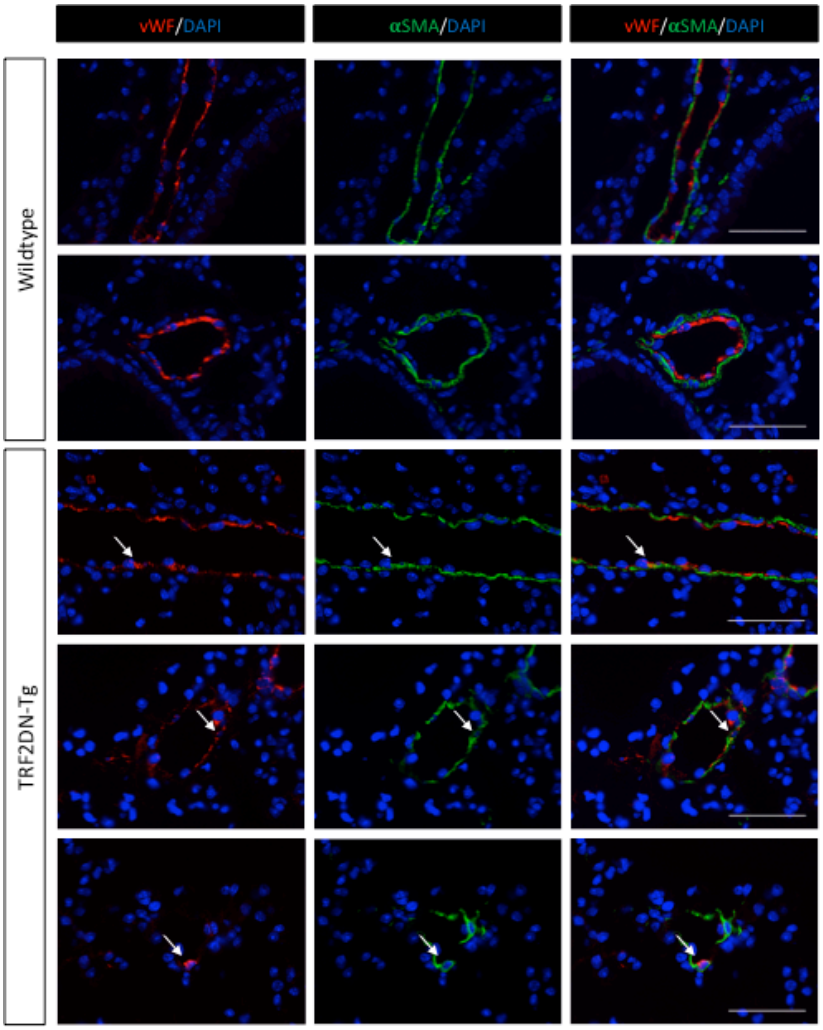

Figure 2. EC-specific progeroid mice show accelerated EndMT in the lungs.
Representative images of immunohistochemistry for endothelial marker (vWF) and mesenchymal marker (αSMA) in the lung sections of bleomycin-treated WT and the Tg mice. The number of mice analyzed was: n = 6–9. Arrows indicate ECs undergoing EndMT. Scale bars: 50 μm.

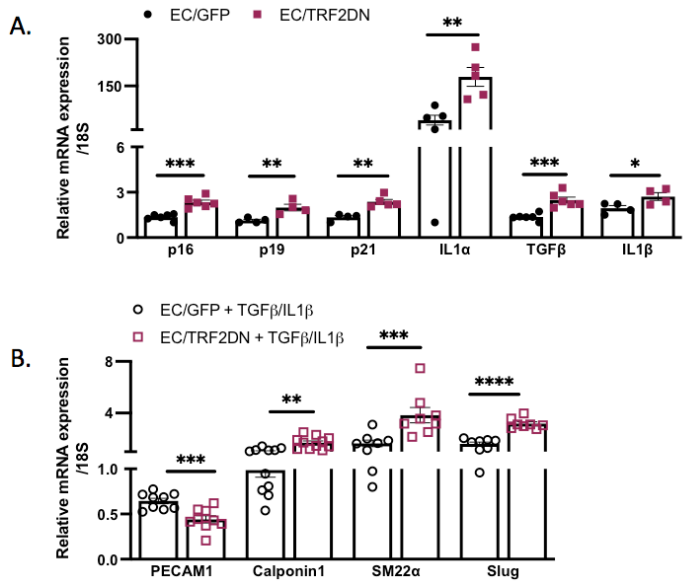

Figure 3. Senescent ECs are susceptible to EndMT.
(A) Quantitative PCR analysis for CDK inhibitors and SASP factors in pulmonary arterial endothelial cells (PAECs) transfected with either GFP (young control ECs) or TRF2DN (premature senescent ECs) (n = 4–6 each). (B) Quantitative PCR analysis for endothelial and mesenchymal marker genes in young control (GFP) and premature senescent (TRF2DN) PAECs after EndMT induction (n = 8–12 each). Data are presented as mean ± SEM. Two-tailed student's *t*-test was used for the statistical analysis of the differences between two groups. **P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.*

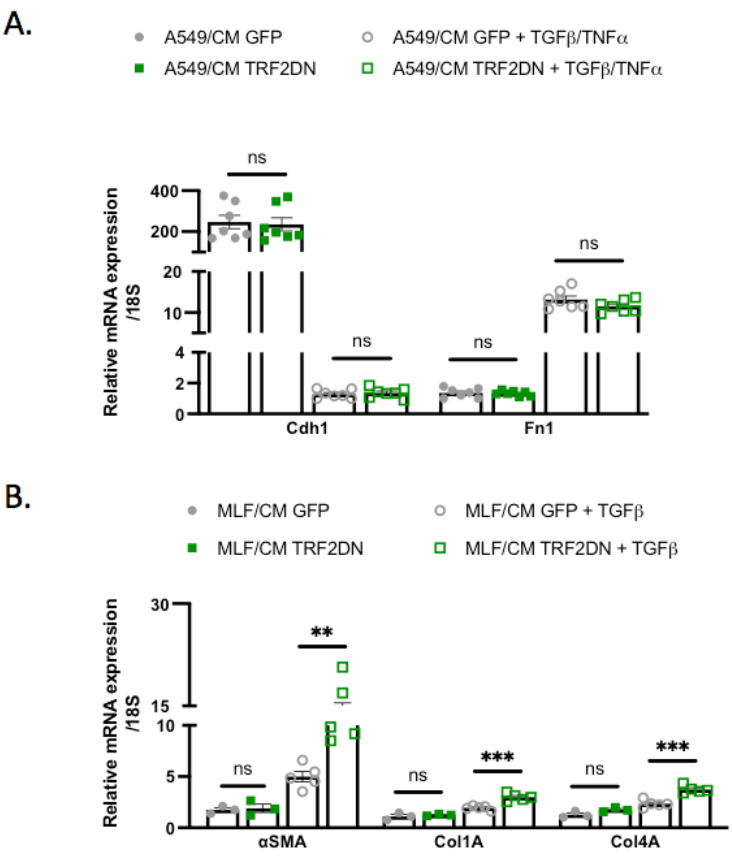

Figure 4. Senescent ECs enhances myofibroblastic transition in lung fibroblasts.
(A) Quantitative PCR analysis for epithelial and mesenchymal marker genes in A549 cells treated with conditioned medium (CM) derived from young control (GFP) and premature senescent (TRF2DN) PAECs (n = 7–8 each). Some cells were treated with TGF-β and TNF-α to induce EMT. (B) Quantitative PCR analysis for myofibroblast marker genes in mouse lung fibroblasts treated with CM derived from young control (GFP) and premature senescent (TRF2DN) PAECs (n = 3–5 each). Some cells were treated with TGF-β to induce myofibroblastic transition. Data are presented as mean ± SEM. One-way ANOVA was used for the statistical analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

DISCUSSION

IPF is a devastating lung disease with unknown etiology, and its incidence is increasing, especially in elderly population. It has been suggested that IPF may result from an acceleration of aging in the lungs, although its underlying molecular mechanisms remain to be elucidated (2). In the current study, we revealed a previously unknown mechanism that could link EC cellular senescence to the pathogenesis of IPF by utilizing unique EC-specific progeroid mice.

Dysregulated accumulation and/or deposition of collagen fibrils are the core pathogenesis in IPF. These collagen fibrils are mostly derived from myofibroblast, alternatively activated fibroblasts that highly express α-SMA. The source of increased myofibroblasts in fibrotic foci has been explained as; proliferating resident lung fibroblasts, epithelial cells through EMT, bone marrow-derived fibroblasts, and ECs through EndMT (11). EndMT is a process in which ECs acquire a mesenchymal phenotype such as high motility and contractility, instead of losing endothelial characteristics. EndMT has been reported to play important roles in various diseases including cancer, pulmonary hypertension, atherosclerosis, and cardiac and kidney fibrosis (8). Of note, EndMT is an emerging factor involved in IPF as an additional pathway to supply myofibroblasts in the fibrotic foci (5). TGF-β produced by macrophages, epithelial cells, and activated myofibroblasts during the wound healing process is probably involved in the mechanism for EndMT, while detailed molecular mechanisms underlying the EndMT during pulmonary fibrosis remain to be elucidated. Although its contribution has been considered to be less than EMT, our data suggest a crucial role of EndMT in the development of pulmonary fibrosis, especially in elderly patients.

Our data showed that senescent ECs accelerate myofibroblastic transition in lung fibroblasts through the SASP, while no significant effect was detected on EMT. Further analysis is required to identify SASP factor(s) that is causally involved in the enhanced myofibroblastic transition of lung fibroblasts. Considering a critical role of TGF-β in the transformation of fibroblast into myofibroblast, TGF-β is one of the most likely candidates for the SASP factor(s) that enhance myofibroblastic transition in lung resident fibroblasts. In fact, we observed that senescent ECs express TGF-β higher than in young control cells. TGF-β is also crucially involved in EndMT and EMT, while EC-mediated SASP did not show significant effect on EMT in our experimental settings. Therefore, autocrine pathway through the SASP factors may play a role in the higher susceptibility of senescent ECs to EndMT. Because combinations of some cytokines such as TGF-β, IL-1β, and TNF-α are known to efficiently induce EMT and EndMT, senescent ECs might produce some partner cytokines in addition to TGF-β, which coordinately enhance myofibroblastic differentiation but not suitable for EMT-induction.

In this study, we mostly assessed the expression levels for CDK inhibitors, SASP factors, and markers for endothelial, epithelial, and mesenchymal cells at mRNA levels; however, analyses for the protein levels of these molecules are preferred to confirm their expressional changes.

Our present study demonstrate that EC senescence plays a detrimental role in the development of pulmonary fibrosis potentially through accelerated EndMT and enhanced myofibroblastic transition of resident lung fibroblasts. Senescent EC is an attracting pharmacotherapeutic target for the prevention and treatment of IPF, especially in elderly population.

Table I. Nucleotide sequences of the primers

Human		
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
p16	CACCAGAGGCAGTAACCATGCCCGC	GTAGGACCTTCGGTGACTGATGATC
p19	GGCAGTTCAAGAGGGTCACACTGCT	ACCATGTGGCCCTGCAGGATGTCCA
p21	GGAAGACCATGTGGACCTGTCACTG	AGATCAGCCGGCGTTTGGAGTGGTA
IL-1α	GGTCACCAAATTCTACTTCCAGGAGGAC	GTGACCAGGTTGTTGTGACGCCTTC
TGF-β	CCCAGCATCTGCAAAGCTC	GTCAATGTACAGCTGCCGCA
IL-1β	AGCTGTACCCAGAGAGTCCTGTGCTGA	AGGAGAGAGCTGACTGTCCTGGCTGATG
PECAM1	GCCGTGGAAAGCAGATACTCTA	GGAGCAGGGCAGGTTCATAAAT
Calponin1	AGCATGGCGAAGACGAAAGGAA	CCCATCTGCAGGCTGACATTGA
SM22α	CCTGGCTAGGGAAACCCACCCT	TCTGGGGAAAGCTCCTTGGAAGT
Slug	CATTTCAACGCCTCCAAGA	CTGCCGACGATGTCCATAC
Cdh1	TGCACCAACCCTCATGAGTG	GTCAGTATCAGCCGCTTTCAG
Fn1	TCGCCATCAGTAGAAGGTAGCA	TGTTATACTGAACACCAGGTTGCA
Mouse		
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Cdh5	CCAAAGTGTGTGAGAACGCTGT	GCCGTGTTATCGTGATTATCCGTG
Cdh1	GACCACTATGCATGCTGCACACGTC	TGGCACTCCAGTCTCTCTCAGAGAAC
αSMA	ACAGAGGCACCACTGAACCCTAAG	ACAATCTCACGCTCGGCAGTAGTC
S100A4	CCTGTCCTGCATCGCCATGATGTGT	CTTGCTCAGCATCAAGCACGTGTCTG
Fn1	TGTGACAACTGCCGTAGACC	GACCAACTGTCACCATTGAGG
Vim	TAGCAGGACACTATTGGCCG	CTGTTGCACCAAGTGTGTGC
Col1A	CCGTGCTTCTCAGAACATCA	AGCATCCATCTTGCAGCCTTG
Col4A	CTTCATTCCTGGTAACCCTGGTG	GCAACGGTACAAAGGGAGAGAG

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