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Role for Telomerase in Pulmonary Hypertension

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Abstract

Background—Cells exhibiting dysregulated growth may express telomerase reverse transcriptase (TERT), the dual function of which consists of maintaining telomere length, in association with the RNA template molecule TERC, and controlling cell growth. Here, we investigated lung TERT in human and experimental pulmonary hypertension (PH) and its role in controlling pulmonary artery smooth muscle cell (PA-SMC) proliferation.

Methods and Results—Marked TERT expression or activity was found in lungs from patients with idiopathic PH and from mice with PH induced by hypoxia or serotonin-transporter overexpression (SM22-*5HTT*$^{+}$ mice), chiefly within PA-SMCs. In cultured mouse PA-SMCs, TERT was expressed on growth stimulation by serum. The TERT inhibitor imetelstat and the TERT activator TA65 abrogated and stimulated PA-SMC growth, respectively. PA-SMCs from PH mice showed a heightened proliferative phenotype associated with increased TERT expression, which was suppressed by imetelstat treatment. *TERC*$^{-/-}$ mice at generation 2 and *TERT*$^{-/-}$ mice at generations 2, 3, and 4 developed less severe PH than did wild-type mice exposed to chronic hypoxia, with less distal pulmonary artery muscularization and fewer Ki67-stained proliferating PA-SMCs. Telomere length differed between *TERC*$^{-/-}$ and *TERT*$^{-/-}$ mice, whereas PH severity

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None.

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was similar in the 2 strains and across generations. Chronic imetelstat treatment reduced hypoxia-induced PH in wild-type mice or partially reversed established PH in SM22-*5HTT*$^{+}$ mice while simultaneously decreasing TERT expression. Opposite effects occurred in mice treated with TA65.

Conclusions—Telomerase exerts telomere-independent effects on PA-SMC growth in PH and may constitute a treatment target for PH.

Keywords

hypertension, pulmonary; muscle; telomerase; vascular remodeling

Pulmonary arterial hypertension (PAH), whether idiopathic (iPAH) or associated with an underlying disease, is a proliferative disorder in which excessive growth of constitutive pulmonary vascular cells leads to hypertrophic remodeling of the arterial wall.[1,2] Pulmonary artery smooth muscle cells (PA-SMCs) are the main targets of this growth disorder. PA-SMCs from patients with pulmonary hypertension (PH) exhibit an abnormal proliferative phenotype that shares several common features with tumor cells.[3-5] We recently reported the acquisition of this proliferative phenotype during PH progression in experimental animals, indicating that dysregulated growth of PA-SMCs is an abnormality shared by human and experimental PH.[6]

Dysregulated proliferation of nontumor and tumor cells is associated with overexpression of the enzyme telomerase reverse transcriptase (TERT), which adds the DNA repeat TTAGGG to telomere ends, thereby maintaining telomere length during genome replication.[7,8] To perform this canonical function, TERT requires the RNA template molecule TERC and a number of telomerase-associated proteins.[7] In humans, telomerase is active during embryogenesis but is repressed in most adult tissues, with the exception of stem cells, germ cells, and certain rapidly proliferating cells.[8] Telomerase reactivation is a prerequisite for cell immortalization, and marked telomerase upregulation is found in 90% of human tumors.[9] A growing body of evidence also indicates that telomerase may exert biological functions independently of its enzymatic activity in telomere maintenance.[8,10,11] Thus, recent studies suggest that telomerase may promote cell growth and protect against cell apoptosis through pathways unrelated to its telomere elongation function.[8]

Telomerase can be expressed by vascular smooth muscle cells (SMCs) on stimulation by growth factors or exposure to hypoxia.[12,13] In vitro studies of cultured vascular SMCs showed that telomerase inhibition was associated with diminished SMC growth.[12] In animal models, telomerase expression has been shown to be associated with neointimal formation after vascular injury, supporting a role for telomerase in dysregulated growth of vascular cells.[14-16] Whether telomerase is involved in the pathogenesis of PH remains unknown.

Here, we tested the hypothesis that telomerase was involved in the excessive PA-SMC growth underlying the progression of human and experimental PH. To this end, we evaluated TERT expression and localization in lung tissue from patients with iPAH and in mice developing PH, and we investigated hypoxic PH severity in *TERT*$^{+/-}$ and *TERC*$^{-/-}$ mice at different generations. To explore the potential of telomerase as a therapeutic target for limiting PA-SMC proliferation, we investigated whether pharmacological telomerase

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inhibition or stimulation affected PH in chronically hypoxic wild-type (WT) mice and in normoxic SM22-*5HTT*$^{+}$ mice characterized by spontaneous PH. Moreover, we studied cultured mouse PA-SMCs to investigate telomerase expression and activity in response to growth stimulation, as well as the effects of telomerase inhibition and stimulation.

Methods

Patient Characteristics and Measurements

Human lung tissue was obtained from 6 patients with iPAH who underwent lung transplantation at the Universitaire Ziekenhuizen Leuven, Leuven, Belgium. Table I in the online-only Data Supplement reports their hemodynamic and clinical characteristics. The collection protocol was approved by the Institutional Ethics Committee of the University Hospital of Leuven, and all participants gave written informed consent.

Control lung tissue was obtained from 6 patients undergoing lung resection surgery for localized lung tumors at the Institut Mutualiste Montsouris (Paris, France). The control subjects had forced expiratory volume in the first second of expiration/forced vital capacity values >70%; none of the patients with iPAH or control subjects had chronic cardiovascular, hepatic, or renal disease or a history of cancer chemotherapy. The collection protocol was approved by the institutional review board of the Henri Mondor Teaching Hospital (Creteil, France), and all control subjects signed an informed consent document before study inclusion. Lung tissue was snap-frozen and then stored at −80°C until use. PA-SMCs were isolated from lung tissues of patients by enzymatic collagenase/elastase digestion.

Mice

Adult male mice (C57Bl/6j) were used according to institutional guidelines, which complied with national and international regulations. All animal experiments were approved by the Institutional Animal Care and Use Committee of the French National Institute of Health and Medical Research (INSERM)–Unit 955, Créteil, France. Transgenic mice with 5-HTT overexpression in SMCs under the control of the SM22 promoter (SM22-5HTT^{+}) were produced and bred as previously described.[17] SM22-5HTT^{+} mice are fertile and have a normal life span and normal growth but spontaneously develop PH. Mice with deletion of the *TERT* (*TERT*$^{-/-}$) or *TERC* (*TERC*$^{-/-}$) gene were obtained from The Jackson Laboratory (Bar Harbor, ME) or donated by M.A. Blasco (CNIO, Madrid, Spain), respectively. Only male mice, 50 *TERT*$^{-/-}$ mice and 30 *TERC*$^{-/-}$ mice, were used for the experiments. The telomerase inhibitor imetelstat was administered in a dose of 15, 30, or 45 mg·kg^{-1}·d^{-1},[18] and the telomerase activator TA65 was given in a dose of 25 mg·kg^{-1}·d^{-1} by intraperitoneal injection.[19] Ten mice were usually studied for each treatment condition. At treatment completion, the lungs were removed and prepared for histological or Western blot analyses.

Exposure to Chronic Hypoxia

Male mice 15 to 20 weeks old were exposed to chronic hypoxia (9% O_2) in a ventilated chamber (Biospherix, New York, NY) as previously described.[20] Normoxic mice were kept in the same room with the same light/dark cycle.[20]

Assessment of PH

Hypoxia-exposed and SM22-*5HTT*$^{+}$ mice were anesthetized with ketamine and xylazine.[20] After incision of the abdomen, a 26-gauge needle connected to a pressure transducer was inserted into the right ventricle (RV) through the diaphragm, and RV systolic pressure (RVSP) was recorded immediately. Then, the thorax was opened, and the lungs and heart were removed. The RV was dissected from the left ventricle plus septum, and these dissected samples were weighed for determination of the Fulton index (RV/left ventricle plus septum). The lungs were fixed by intratracheal infusion of 4% aqueous buffered formalin. A midsagittal slice of the right lung was processed for paraffin embedding. Sections 5 μm in thickness were cut and stained with hematoxylin-phloxine-saffron stain for examination by light microscopy. In each mouse, a total of 20 to 30 intra-acinar vessels accompanying either the alveolar ducts or alveoli were examined by an observer blinded to treatment or genotype. Each vessel was categorized as nonmuscular (no evidence of vessel wall muscularization), partially muscular (SMCs identifiable in less than three fourths of the vessel circumference), or fully muscular (SMCs in more than three fourths of the vessel circumference). The percentage of muscular pulmonary vessels was determined by dividing the sum of partially and fully muscular vessels by the total number of vessels in the relevant group of animals. Ki67-stained cells, terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)–positive cells, and p16- and p21-stained cells were assessed in the distal pulmonary vessel walls and expressed as the number of stained nuclei over the total number of nuclei counted in the media of at least 20 muscular vessels per mouse.

Biological Measurements in Mouse Tissues and Cultured PA-SMCs

Western blotting was used to detect and quantify TERT, p53, and p21 in mouse tissues and PA-SMCs. The antibodies used were anti-TERT (Santa-Cruz Biotechnology, Nanterre, France), anti-p53 (Cell Signaling Technology, Boston, MA), and monoclonal anti-p21Waf1/Cip1 (Cell Signaling Technology) antibodies. Levels of TERT, p21, and Bax mRNAs in lung tissue were determined by use of a real-time quantitative polymerase chain reaction assay (PCR). Total mRNA was extracted from tissues with the RNeasy Mini Kit (Qiagen, ZA Courtaboeuf, France). Real-time quantitative PCR was performed in a 7900HT real-time PCR system (Applied Biosystems, ZA Courtaboeuf, France), using SYBR Green Mix (Invitrogen) as described previously.[20] The amounts of mRNA were normalized for the amount of the control gene, *18s*, which was chosen among 4 tested housekeeping genes on the basis of its stable expression in all samples (data not shown). Because the control gene might amplify genomic DNA, contaminating the RNA samples, we checked the absence of such contamination by performing the reverse-transcriptase step with and without reverse transcriptase.

The endogenous enzymatic activity of telomerase in mouse lung and cultured cells was quantified with the teloTAGGG telomerase PCR Elisaplus kit (Roche Diagnostics, Meylan, France).

Immunohistochemistry

Lung tissue sections for immunolabeling were prepared as previously described.[20] Slides were incubated for 60 minutes in 1% BSA and 5% goat serum in PBS and then incubated

overnight with anti-p21 mouse antibody (Santa Cruz Biotechnology Inc, Santa Cruz, CA), anti-p16 mouse antibody (Thermo Fisher Scientific, Waltham, MA), and anti-Ki67 rabbit antibody (Abcam, Cambridge, UK). We used the ABC Vectastain kit (Vector Labs, Burlingame, CA) to mark the primary antibodies according to the user's guide. The staining substrate was diaminobenzidine (FastDAB, Sigma-Aldrich, St. Louis, MO), and the sections were counterstained with methyl green. For TUNEL stain, epitopes were retrieved by treatment with DNase I digestion for 10 minutes at room temperature. After endogenous peroxidase blockade with H_2O_2, tissues were incubated in equilibration buffer and treated with terminal deoxynucleotidyl transferase to detect TUNEL-positive nuclei, as suggested by the manufacturer (Roche Diagnostics). Tissues were then incubated with peroxidase-conjugated anti-digoxigenin antibodies and revealed with DAB. After counterstaining with methyl green, sections were mounted with coverslips. For immunofluorescence studies of human lung tissues, slides were incubated overnight at 4°C with anti-TERT mouse antibody (Ab176364, Abcam), anti–α-smooth muscle actin (SMA) rabbit antibody (Ab5694, Abcam), anti-CD44 antibody (Ab6124, Abcam), or anti–proliferating cell nuclear antigen (ab29, Abcam) and then exposed to anti-mouse Alexa Fluor (Cell Signaling Technology) and anti-rabbit Alexa Fluor (Invitrogen, Life Technologies, Carlsbad, CA) antibodies. Nuclei were stained with Hoechst.

Extraction and Culture of PA-SMCs

Mouse PA-SMCs were obtained from pulmonary arteries as previously described.[20] To assess the effects of drugs on PA-SMC proliferation, we exposed PA-SMCs to imetelstat (0.05–1 μmol/L), TA65 (1–10 μmol/L), or vehicle in serum-free medium and then to 10% FCS. Then, the cells were incubated with a BrdU-labeling reagent (final concentration, 10 μmoL/L) for 48 hours before detection of BrdU-labeled cells (BrdU Labeling and Detection Kit I, Roche, Penzberg, Germany). BrdU incorporation was quantified by spectrophotometry at 520 nm, expressed in optical density units, and taken as an indicator of the cell count. BrdU values were usually obtained from 20 to 30 wells of cells from independent experiments.

To assess the effects of TERT inhibition on PA-SMC apoptosis, cells were trypsinized and resuspended in binding buffer 1X, incubated with annexin V/FITC–conjugated antibody, and stained with propidium iodide as previously described.[20] Apoptotic cells were propidium iodide–positive cells and annexin V/propidium iodide–positive cells. To assess the effects of TERT inhibition on cell senescence, we determined the percentage of β-galactosidase–positive cells after 48 hours of incubation with imetelstat with or without FCS.

Chemicals and Drugs

Imetelstat and TA65 were purchased from Sergei Gryaznov (Geron Corp, Menlo Park, CA) and diluted in vehicle (NaCl 0.9%) for treatment of the mice. Mice received daily intraperitoneal injections of imetelstat or TA65 for 21 days.

Telomere Length Measurement

Telomere length was assessed with real-time quantitative PCR. Briefly, the ratio of the telomere repeat copy number to the single-gene copy number was determined by use of an

Applied Biosystems 7900HT thermocycler (Life Technologies) in a 384-well format and the comparative Ct method (ratio of the telomere repeat copy number to the single-gene copy number=2−ΔΔCt). Genomic DNA was extracted from PA-SMCs with the QIAamp DNA Kit (Qiagen) and quantified with a spectrophotometer. Each sample (30 ng DNA) was run in triplicate with the SYBR Green method (Invitrogen). The sequences and final concentrations of the primers for the telomere and 36B4 (acidic ribosomal phosphoprotein PO, a single-copy gene used for normalization) were as follows:

Tel1, 5′-CGGTTTGTTTGGGTTTGGGTTTGGGTTTGGGTTTG GGTT-3′, 300 nmol/L; Tel2, 5′-GGCT TGCCTTA CCCTTACCCTTAC CCTTACCCTTACCCT-3′, 300 nmol/L; 36B4F, 5′-CAGCAAGTGG GAAGGTGTAATCC-3′, 300 nmol/L; and 36B4R, 5′-CCCATTCTAT CATCAACGGGTACAA-3′, 300 nmol/L.

Telomere length was expressed as the ratio of the telomere repeat copy number over the single-gene copy number (36B4 gene).[21] Triplicate values of each sample had a ΔCt SD <0.1.

Statistical Analysis

Quantitative variables from human studies are reported as median and individual values. These variables were compared by use of the non-parametric Mann-Whitney test. Data from animal and cell studies are reported as mean±SEM. Parametric tests were used after verification that the variables in each group were normally distributed. One-way ANOVA was performed to compare the effects of drugs, to compare hypoxia-exposed vehicle-treated mice with normoxia-exposed control mice, to compare transgenic mice with WT mice during normoxia or hypoxia, and to compare generations of mice. One-way ANOVA for repeated measures was used to assess the time or dose dependency of treatment effects on TERT expression and activity, as well as on the proliferation of PA-SMCs. Values of $P<0.05$ were considered significant for the initial ANOVA, and the Bonferroni correction was then applied for multiple comparisons. Data were analyzed with GraphPad Prism 5.0 statistical software (San Diego, CA).

Results

Increased Lung Telomerase Expression in Patients With iPAH and Mice With PH

Lung TERT protein and activity levels were increased in explanted lungs from patients with iPAH compared with control subjects ($P<0.05$; Figure 1A and 1B). Immunofluorescence staining for TERT protein in iPAH lung tissues predominated in PA-SMCs in the outer hypertrophied media of pulmonary vessels, as shown by immunofluorescence staining for TERT, α-SMA, and CD44 of paraffin-embedded lung sections (Figure 1C). TERT staining, which was not found in contractile and nondividing PA-SMCs expressing α-SMA, predominated in PA-SMCs expressing CD44 and located in the outer media. No TERT staining was found in control lung tissues. Cultured human PA-SMCs expressing CD44 and α-SMA are shown in Figure 1D, with TERT staining found in dividing proliferating cell nuclear antigen–positive cells stimulated with FCS (Figure 1E). In mice exposed to 21 days of hypoxia and developing PH, we found increased lung TERT activity ($P<0.01$), mRNA

($P<0.01$), and protein levels ($P<0.01$) compared with normoxic mice (Figure 2A). Similarly, SM22-*5HTT*$^{+}$ mice with spontaneous PH exhibited increased lung TERT activity ($P<0.001$), mRNA ($P<0.01$), and protein levels ($P<0.01$) compared with control WT mice, despite being studied under normoxic conditions (Figure 2B). No TERT mRNA, protein, or activity was detected in lungs from *TERT*$^{-/-}$ mice exposed to normoxia or hypoxia (see the representative TERT protein gel shown in Figure 2A).

Telomerase in Cultured Mouse PA-SMCs: Relationship With Cell Growth

To establish that cultured PA-SMCs expressed TERT protein, we studied mouse cells in the quiescent state and after stimulation with 10% FCS. TERT mRNA, protein, and activity levels, which were low in quiescent cells, increased on cell-growth stimulation with FCS (Figure 3A). The TERT protein level peaked 24 to 48 hours after FCS stimulation ($P<0.01$) and then returned to baseline values on day 6, at which time the cells reached confluence and stopped growing without undergoing senescence or apoptosis. TERT activity 48 hours after FCS stimulation was inhibited by imetelstat ($P<0.01$) and stimulated by TA65 ($P<0.01$; Figure 3B). No TERT mRNA, protein, or activity was detected in cells from *TERT*$^{-/-}$ mice (Figure I in the online-only Data Supplement). PA-SMC exposure to imetelstat dose-dependently inhibited FCS-induced PA-SMC proliferation (Figure 3C) without inducing cell senescence or apoptosis, as shown by the β-galactosidase–positive or annexin V/propidium iodide–positive cell counts below 5% (Figure II in the online-only Data Supplement). Treatment with TA65, in contrast, stimulated cell growth (Figure 3D). PA-SMCs from *TERT*$^{-/-}$ mice, which grew more slowly than did PA-SMCs from WT mice ($P<0.05$), were unaffected by imetelstat or TA65 treatment (Figure 3C and 3D).

Dysregulated Growth of Mouse PA-SMCs in PH and Telomerase Function

We then investigated whether cultured PA-SMCs from chronically hypoxic mice or SM22-*5HTT*$^{+}$ mice maintained an abnormal proliferative phenotype in vitro and exhibited alterations in TERT expression. Under stimulated conditions, PA-SMCs in both mouse PH models contained higher levels of TERT protein than did those from normoxic WT mice (Figure 4A). Moreover, the rate of FCS-induced growth of PA-SMCs from chronically hypoxic mice or SM22-*5HTT*$^{+}$ mice was higher than in control mice ($P<0.05$), and this increase was abolished by imetelstat treatment (Figure 4B and 4C).

Effects of *TERT* or *TERC* Gene Deletion in Chronically Hypoxic Mice

Our finding of TERT overexpression in PH prompted us to test whether *TERT* or *TERC* deletion affected PH development in mice. After 21 days of hypoxia exposure, RVSP was lower ($P<0.01$) and RV hypertrophy was less severe ($P<0.01$) in *TERT*$^{-/-}$ and *TERC*$^{-/-}$ mice than in control WT mice (Figure 5A). Furthermore, the distal pulmonary vessels were less muscular in mutant than in WT mice ($P<0.01$), with a smaller percentage of dividing Ki67-positive pulmonary vascular cells ($P<0.01$) and a higher percentage of p21- and p16-positive cells ($P<0.01$; Figure 5A and 5B). PH severity was not significantly different between *TERT*$^{-/-}$ and *TERC*$^{-/-}$ mice or across generations of mutant mice. Furthermore, the percentage of p21- and p16-positive cells did not differ between *TERT*$^{-/-}$ and *TERC*$^{-/-}$ mice.

Both *TERT*$^{-/-}$ and *TERC*$^{-/-}$ mice had shorter lung telomeres compared with WT mice (P<0.01; Figure 5C). In addition, telomeres were shorter in *TERC*$^{-/-}$ mice than in *TERT*$^{-/-}$ mice (P<0.01) and in *TERT*$^{+/-}$ mice at generation 3 compared with *TERT*$^{+/-}$ mice at generation 1 (data not shown; P<0.05). Telomere length was not affected by exposure to hypoxia in WT or transgenic mice. Thus, no correlation was found between lung telomere length and PH severity. Although lung TERT expression did not differ between *TERC*$^{-/-}$ and WT mice during normoxia, it increased to a lesser extent in *TERC*$^{-/-}$ mice than in WT mice after hypoxia exposure (P<0.01; Figure 5D).

Effects of Pharmacological TERT Inhibition or Activation in Chronically Hypoxic Mice

Intraperitoneal injection of 15 to 45 mg·kg^{-1}·d^{-1} of the TERT inhibitor imetelstat dose-dependently blunted PH development as assessed by RVSP, RV/left ventricle plus septum, and distal pulmonary artery muscularization in chronically hypoxic mice (Figure 6A and Figure III in the online-only Data Supplement) but had no effect in normoxic mice. The protective effect of imetelstat was accompanied by decreased PA-SMC proliferation manifesting as a decrease in the percentage of Ki67-positive cells (P<0.01) and an increase in senescent p21- and p16-positive cells (P<0.01) without apoptosis induction (Figure 6A and 6B). In contrast, treatment with 25 mg·kg^{-1}·d^{-1} TA65 increased PH severity in chronically hypoxic mice (P<0.025) but did not affect physiological parameters in normoxic mice (Figure 6A). The aggravating effect of TA65 was associated with an increase in Ki67-positive cells (P<0.01) and with decreases in the percentages of p21- (P<0.025), p16- (P<0.025), and TUNEL- (P<0.01) positive cells compared with vehicle-treated hypoxic mice (Figure 6A and 6B).

Imetelstat treatment decreased lung TERT mRNA (P<0.01) and protein (P<0.025) in hypoxic, but not normoxic, mice. These effects in hypoxic mice were associated with increased lung p53 (P<0.025) and p21 (P<0.025) protein levels without changes in p21 mRNA levels (Figure 6C and 6D). Conversely, TA65 treatment increased lung TERT mRNA levels during hypoxia (P<0.025) without altering TERT protein levels. TA65 treatment failed to alter the levels of p53 and p21 proteins but decreased the expression of 2 p53 downstream genes, as shown by decreases in lung p21 (P<0.01) and Bax mRNA levels (P<0.01) during hypoxia (Figure 6C and 6D). Imetelstat 45 mg·kg^{-1}·d^{-1} from day 15 to 30 partially reversed PH in chronically hypoxic mice (Figure IV in the online-only Data Supplement). Neither imetelstat nor TA65 affected RVSP, RV/left ventricle plus septum, or distal pulmonary artery muscularization in chronically hypoxic *TERT*$^{-/-}$ mice (Figure V in the online-only Data Supplement).

Effects of Pharmacological TERT Inhibition or Activation in SM22-5HTT^{+} Mice

We studied SM22-*5HTT*$^{+}$ mice to determine whether telomerase inhibition reversed established PH and whether lung TERT expression was altered in a normoxic PH model. Imetelstat partially reversed PH in SM22-5HTT^{+} mice, as shown by decreases in RVSP (P<0.01), RV hypertrophy (P<0.01), and pulmonary vessel muscularization (P<0.01); a decrease in Ki67-positive proliferating PA-SMCs (P<0.01); and increases in p21- (P<0.01), p16- (P<0.01), and TUNEL- (P<0.025) positive cells within pulmonary vessels (Figure 7A and 7B). In contrast, TA65 treatment worsened PH severity in SM22-*5HTT*$^{+}$ mice,

increasing the percentage of muscular pulmonary vessels ($P<0.025$) and Ki67-positive cells ($P<0.01$) and decreasing the percentage of TUNEL- ($P<0.025$), p21- ($P<0.025$), and p16- ($P<0.01$) positive cells (Figure 7A and 7B). Imetelstat treatment decreased lung TERT mRNA ($P<0.025$) without altering protein levels while increasing lung p53 protein levels ($P<0.025$), lung p21 mRNA ($P<0.025$) and protein ($P<0.01$) levels, and lung Bax mRNA levels ($P<0.01$; Figure 7C and 7D). TA65 had nearly opposite effects, with increases in TERT protein levels ($P<0.025$) and decreases in p21 mRNA ($P<0.025$), p21 protein ($P<0.01$), and Bax mRNA ($P<0.01$) levels (Figure 7C and 7D).

Discussion

By combining studies of human and mouse lung tissues, mouse cells, and transgenic mouse models, we demonstrated that TERT activation in PA-SMCs played a major role in PH pathogenesis. TERT, the protein component of telomerase, was upregulated in PA-SMCs from remodeled pulmonary vessels in both patients with iPAH and mice with experimentally induced PH. *TERT* or *TERC* gene deletion or pharmacological TERT inhibition prevented or reversed PH in mice. Taken together with our findings of TERT induction in proliferating cultured PA-SMCs and of altered PA-SMC growth in response to TERT inhibition or activation, these results support a substantial role for telomerase in PA-SMC growth and PH via functions independent of telomere elongation. Telomerase may therefore hold promise as a novel pharmacological target for the treatment of PH.

Increasing attention is being focused on the role of telomeres and telomerase in proliferative disorders and cancer.[8] In adult tissues, telomerase is not expressed in somatic cells or quiescent cells but is highly expressed in rapidly dividing cells and cancer cells. Recent work also indicates that telomerase may promote cell growth and protect against cell apoptosis through pathways unrelated to telomere elongation.[8] Previous studies documented telomerase expression in systemic vascular SMCs stimulated by mitogenic agents or stress and indicated that telomerase was required for SMC proliferation.[12-16]

Here, we focused on the role for telomerase in PH and its involvement in PA-SMC proliferation. TERT expression is the limiting factor for telomerase activity because TERC is constitutively expressed in most tissues; therefore, we assessed TERT expression in human and mouse lung samples. We found marked TERT expression in lungs from patients with iPAH and in mice with experimental PH. Interestingly, only small amounts of TERT were detected in normal lungs, whereas TERT mRNA, protein, and activity were elevated in lungs from mice with established PH. Lung TERT was increased in both hypoxic and SM22-5HTT$^+$ mice, indicating that PH, and not hypoxia, was the main factor responsible for telomerase expression.[13] In lungs from patients with iPAH, telomerase was expressed predominantly by PA-SMCs expressing CD44 in the outer media and was not expressed by nondividing contractile PA-SMCs expressing α-SMA. These observations are consistent with a role for CD44-expressing SMCs in the pathogenesis of vascular lesions.[22]

These findings prompted us to test whether *TERT* or *TERC* gene deletion modified the development of PH in mice. To determine whether any effect on PH development was related to telomere length, we determined lung telomere length in several generations of

mice. *TERT*$^{-/-}$ and *TERC*$^{-/-}$ mice were similarly protected against PH, independently of generation. Telomere length was considerably shorter in *TERC*$^{-/-}$ than in *TERT*$^{-/-}$ mice,[23] in accordance with the fact that heterozygous TERC$^{+/-}$ mice, but not heterozygous *TERT*$^{-/-}$ mice, have shorter telomeres than their respective nonmutant counterparts.[23] The similar protection against PH afforded by *TERT* or *TERC* deletion was therefore independent of telomere length. In the absence of TERC, TERT maintains catalytic activity but is unable to elongate the telomeres.[8] *TERC* and *TERT* deletion leading to similar protection against PH might suggest that protection against PH was related to loss of the telomere elongation function of telomerase. However, *TERT* expression was markedly reduced in *TERC*$^{-/-}$ mice, in keeping with previous studies documenting major regulatory effects of *TERC* on the expression of *TERT*.[24] Importantly, telomere length in *TERT*$^{-/-}$ mice decreased from generation 1 to 3, but the severity of hypoxic PH was similar across generations, further suggesting that telomerase deficiency, and not telomere attrition, altered experimental PH progression.

To further investigate the mechanisms involved in the effects of telomerase in PH, we used imetelstat as a pharmacological inhibitor and TA65 as a pharmacological stimulator.[18,19] We reasoned that any effect of these drugs given for only 3 weeks would reflect telomerase activity independently of effects on telomeres. Interestingly, imetelstat treatment protected against the development of hypoxia-induced PH, markedly diminishing the number of proliferating PA-SMCs. Moreover, imetelstat partially reversed PH in SM22-5HTT^{+} mice and hypoxic mice with established PH. On the other hand, TA65 treatment was associated with small but significant increases in PH severity and dividing PA-SMC counts in both chronically hypoxic and SM22-5HTT^{+} mice. Neither imetelstat nor TA65 affected PH in TERT$^{-/-}$ mice. These results constitute further evidence that the intensity of pulmonary vascular remodeling and the development of PH in mice are causally related to telomerase activity.

Recent studies suggest that telomerase may promote cell growth and protect against apoptosis through pathways unrelated to its telomere elongation function.[8] One major argument supporting such alternative functions of telomerase is the rapid occurrence of cell function changes in response to acute modifications in telomerase activity in the absence of associated changes in telomere length. To elucidate the mechanism by which telomerase inactivation protected against PH, we studied the effects of telomerase on cultured PA-SMCs. PA-SMC growth stimulation was associated with marked increases in telomerase expression and activity, and pharmacological inhibition or stimulation of telomerase activity inhibited or stimulated PA-SMC growth, respectively. These results are consistent with previous studies of systemic vascular SMCs documenting the involvement of telomerase in growth-rate control and the proliferating capacity of vascular SMCs.[12,13] Taken together, these data support an effect of telomerase on pulmonary vascular SMC growth via pathways independent of telomere elongation.

Treatment of mice with a telomerase inhibitor or activator was associated not only with altered cell growth but also with changes in the number of p21- and p16-positive cells and TUNEL-positive cells in the pulmonary vasculature. Moreover, TERT inhibition prevented the decrease in lung p53 protein nuclear fraction seen during PH progression and potentiated

the expression of p21 mRNA and protein. Opposite effects were obtained during stimulation with TA65. In recent studies, we found that treatment with nutlin-3a, an inhibitor of the interaction between p53 and the ubiquitin ligase Mdm2, markedly increased lung p53 levels and protected against PH by inducing PA-SMC senescence.[20] In contrast to findings with nutlin-3a, imetelstat treatment of cultured PA-SMCs decreased cell growth but did not induce cell senescence. However, cell senescence markers such as p16 and p21 were increased in PA-SMCs from hypoxic mice and, to a greater extent, in telomerase-deficient mice or imetelstat-treated WT mice. Thus, the protective effect of telomerase inactivation on PH was associated with a simultaneous decrease in PA-SMC proliferation, as assessed by the percentage of Ki67-positive cells, and with an increase in the number of senescent p21- and p16-positive PA-SMCs. These data are consistent with a dual effect of telomerase inactivation in vivo consisting of both inhibition of PA-SMC growth and induction of PA-SMC senescence.

Cultured PA-SMCs derived from patients with PH or from experimental animal models of PH are characterized by an increased growth capacity compared with non-PH cells.[3,6] We therefore investigated whether this proliferative PA-SMC phenotype was linked to an increase in TERT expression in cultured PA-SMCs in our 2 experimental models of PH. Compared with cells from normoxic WT mice, PA-SMCs from chronically hypoxic mice or SM22-*5HTT*$^{+}$ mice exhibited an increased growth rate, along with marked overexpression of TERT. Furthermore, TERT inhibition with imetelstat markedly inhibited FCS-induced PA-SMC growth, abolishing the difference between cells from mice with PH and control mice. These results are consistent with the possibility that the increased PA-SMC proliferation associated with PH development requires TERT overexpression.

Taken together, these results support the induction of telomerase expression and activity as a major feature associated with PA-SMC proliferation and PH progression. Telomerase exerts telomere-independent effects in PH and may represent a novel pharmacological target for the treatment of this disease.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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CLINICAL PERSPECTIVE

Pulmonary artery hypertension (PAH), whether idiopathic or associated with an underlying disease, is a proliferative disorder sharing several features with cancer. One characteristic of cells exhibiting dysregulated growth is expression of the enzyme telomerase reverse transcriptase (TERT), the dual function of which consists of maintaining telomere length, with the TERC molecule as an RNA template, and controlling cell growth. By combining studies of human and mouse lung tissues, mouse cells, and transgenic mouse models, we demonstrated that TERT activation in pulmonary artery smooth muscle cells (PA-SMCs) played a major role in pulmonary hypertension (PH) pathogenesis. TERT, the protein component of telomerase, was upregulated in PA-SMCs from remodeled pulmonary vessels in both patients with PAH and mice with experimentally induced PH. *TERT* or *TERC* gene deletion or pharmacological TERT inhibition prevented or reversed PH in mice. Taken together with our findings of TERT induction in proliferating cultured PA-SMCs and of altered PA-SMC growth in response to TERT inhibition or activation, these results support a substantial role for telomerase in PA-SMC growth and PH via functions independent of telomere elongation. Telomerase exerts telomere-independent effects in PH and may represent a novel pharmacological target for the treatment of this disease.

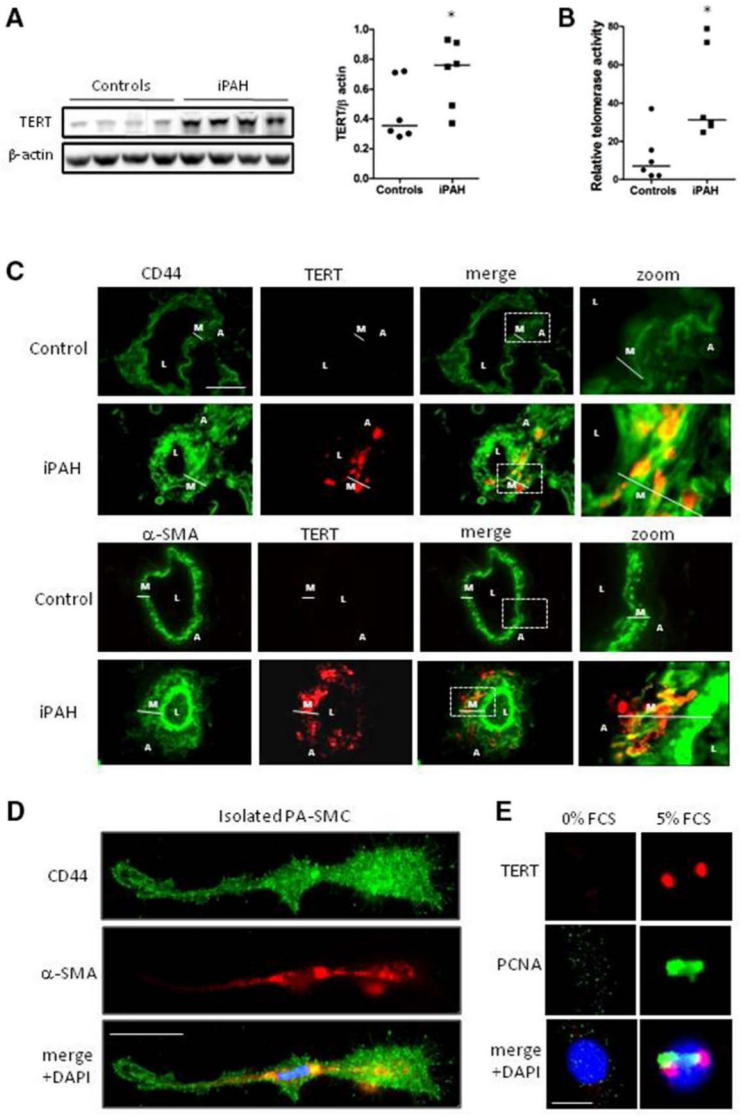

Figure 1.

Increased expression and activity of telomerase reverse transcriptase (TERT) in patients with idiopathic pulmonary artery hypertension (iPAH). **A**, TERT protein levels and activity were measured in total lung protein extracts from patients with iPAH and control subjects. Individual values and median (black bar) from 6 patients and 6 age- and sex-matched control subjects. **P*<0.05. **B**, Relative telomerase activity from patients with iPAH and control subjects. *p<0.05. **C**, Representative micrographs of lung immunofluorescence staining for TERT in pulmonary vessels from patients and control subjects. TERT was expressed predominantly in the outer media of remodeled pulmonary vessels and colocalized with CD44 in smooth muscle cells. No immunoreactivity was detected in nondividing contractile pulmonary artery smooth muscle cells (PA-SMCs) expressing α-smooth muscle actin (α-SMA). Bar, 50 μm. The area represented in the zoom panel is indicated on the merge image by a white square. **D**, Representative micrograph attesting colocalization of CD44 (green) and α-SMA (red) in isolated PA-SMCs. Bar, 25 μm. **E**, Representative micrograph showing

immunofluorescence staining for TERT (red) and proliferating cell nuclear antigen (PCNA; green) in quiescent (0% FCS) and proliferating (5% FCS) PA-SMCs. Proliferating cells were identified by labeling with PCNA. PCNA labels nuclear foci representing sites of ongoing DNA replication. Bar, 10 μm. A indicates adventitia; L, lumen; and M, media.

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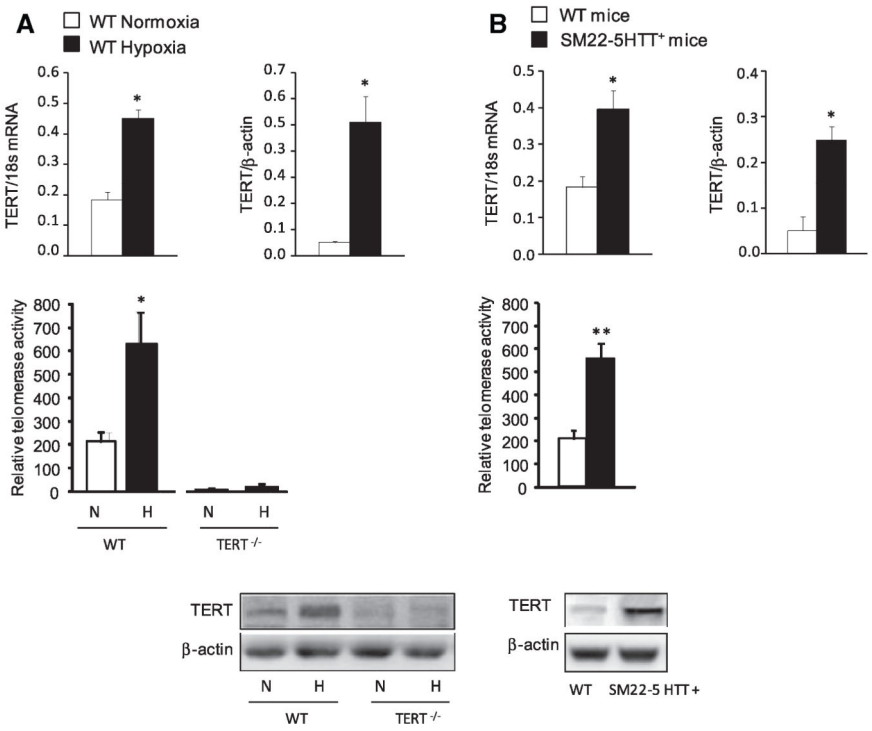

Figure 2.

Increased expression and activity of telomerase reverse transcriptase (TERT) in lungs from mice with hypoxia-induced pulmonary hypertension and from SM22-5HTT^{+} mice. **A**, TERT mRNA, protein, and activity in lungs from mice studied on day 21 after exposure to hypoxia (H) or normoxia (N). **B**, TERT mRNA, protein, and activity in lungs from SM22-5HTT^{+} mice and wild-type (WT) control mice. The figure showing telomerase activity during normoxia and hypoxia and the representative Western blot gels include negative controls from TERT$^{-/-}$ mice. Data are mean±SEM of 6 to 10 animals. *P<0.01, **P<0.001 vs control WT normoxic mice.

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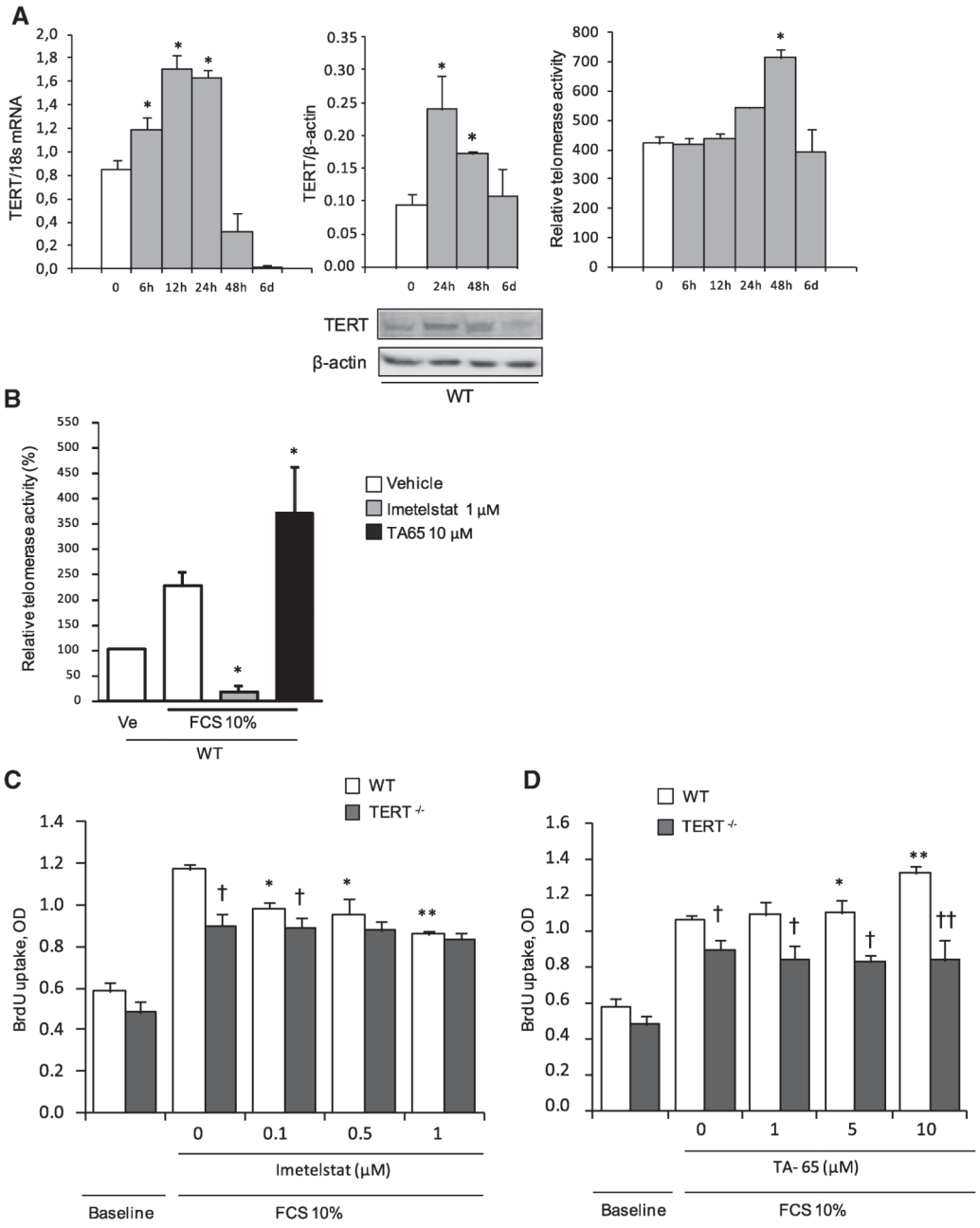

Figure 3.

Association of telomerase reverse transcriptase (TERT) expression and activity with cell proliferation in mouse pulmonary artery smooth muscle cells (PA-SMCs). **A**, TERT mRNA and activity levels were measured in PA-SMCs in the quiescent state and 6, 12, 24, and 48 hours and 6 days after stimulation with 10% FCS. TERT protein levels were measured by Western blot in PA-SMCs in the quiescent state and 24 and 48 hours and 6 days after stimulation with 10% FCS. **P*<0.01 vs PA-SMCs not treated by FCS (time 0; mean±SE; n=6 at each time point). **B**, Telomerase activity in PA-SMCs from wild-type (WT) mice studied in the quiescent state and 48 hours after stimulation with 10% FCS in the presence of imetelstat (1 µmol/L) or TA65 (10 µmol/L). **P*<0.01 vs vehicle (Ve)-treated PA-SMCs

stimulated by FCS (mean±SE; n=8). **C** and **D**, Effects of treatment with the TERT inhibitor imetelstat (**C**) or the TERT activator TA65 (**D**) on proliferation of cultured mouse PA-SMCs. The cells were starved of FCS for 48 hours and then exposed to the drugs according to different protocols. Proliferation of PA-SMCs from WT and *TERT*$^{-/-}$ mice was measured with the BrdU assay 48 hours after stimulation with 10% FCS or vehicle (mean±SE; n=30 at each time point). OD indicates optical density units. **P*<0.01, ***P*<0.001 vs vehicle-treated PA-SMCs stimulated by FCS; †*P*<0.05, ††*P*<0.01 vs corresponding values in cells from WT mice.

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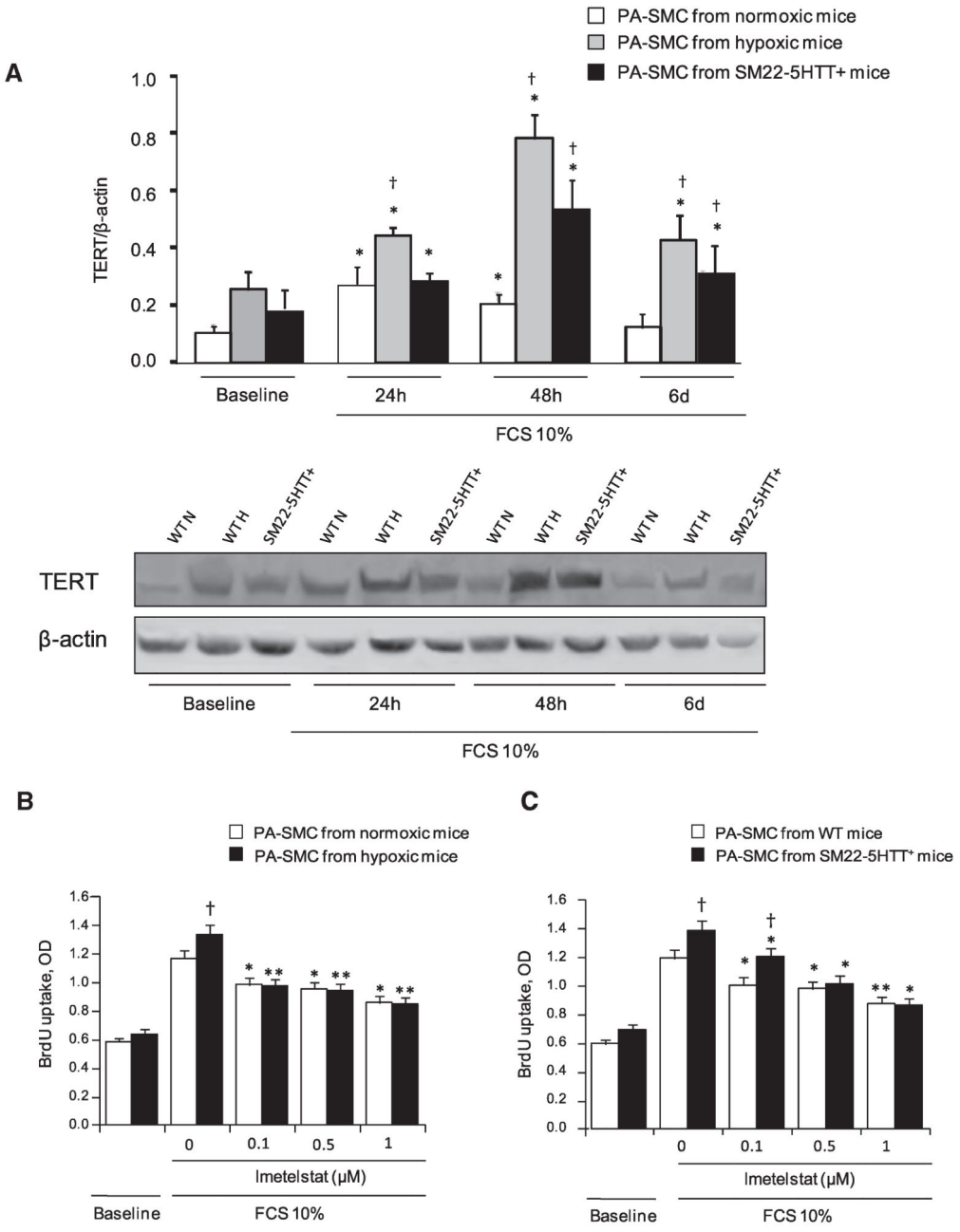

Figure 4.

Telomerase reverse transcriptase (TERT) expression and dysregulated growth of pulmonary artery smooth muscle cells (PA-SMCs) from mice with pulmonary hypertension. **A**, TERT protein levels measured by Western blot in PA-SMCs from wild-type (WT) normoxic mice (WT N), WT hypoxic mice (WT H), and SM22-*5HTT*$^{+}$ mice after stimulation with 10% FCS. *P<0.01 vs values recorded at time 0 in PA-SMCs not treated by FCS; †P<0.025 vs corresponding values in cells from WT normoxic mice (mean±SE; n=6 at each time point). **B** and **C**, Effects of treatment with the TERT inhibitor imetelstat on the proliferation of cultured PA-SMCs from chronically hypoxic mice (**B**) and SM22-*5HTT*$^{+}$ mice (**C**) compared with normoxic WT mice (mean±SE; n=30 at each time point). *P<0.01,

**$P<0.001$ vs vehicle-treated PA-SMCs stimulated by FCS; †$P<0.05$ vs respective values in PA-SMCs from WT normoxic mice.

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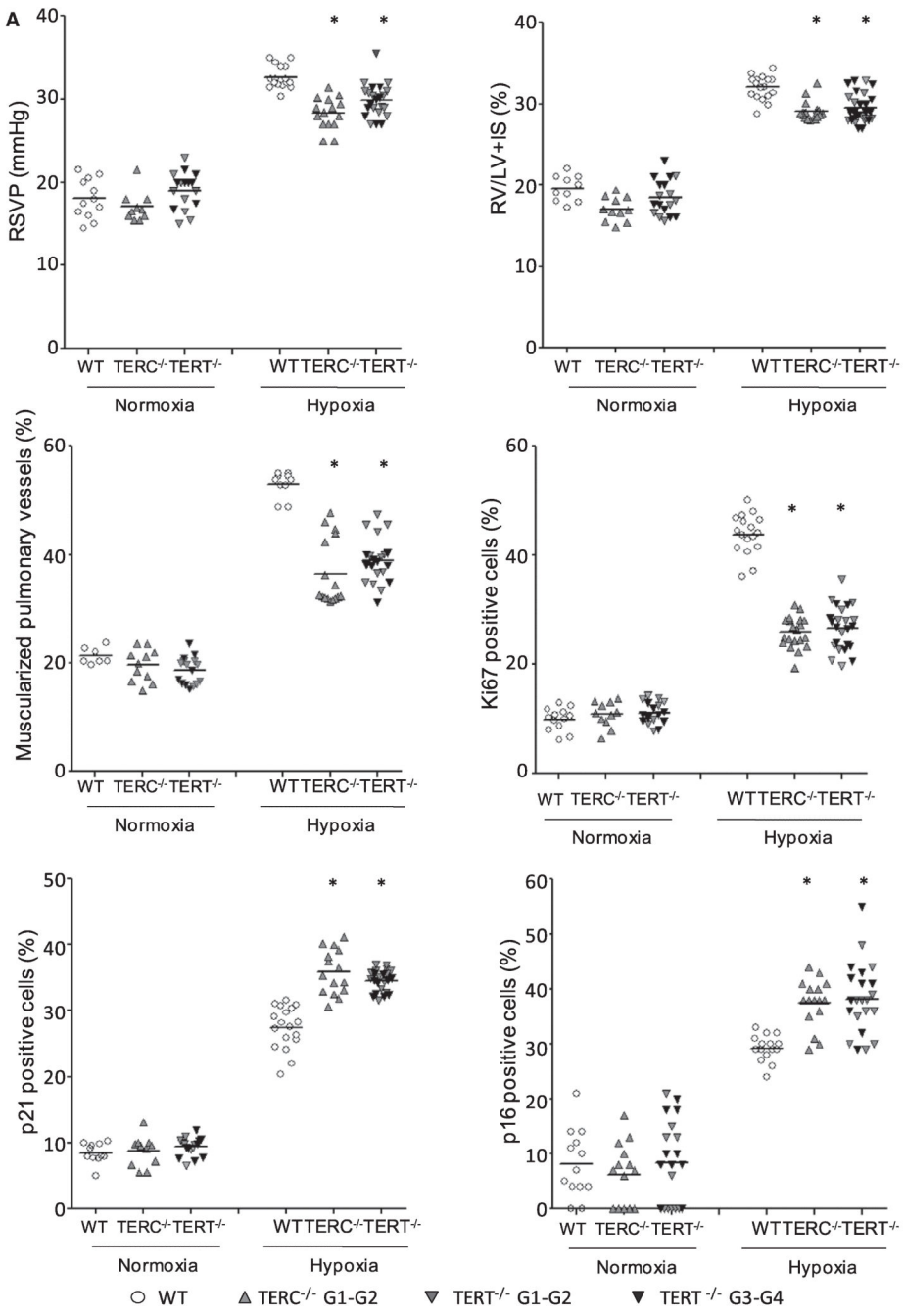


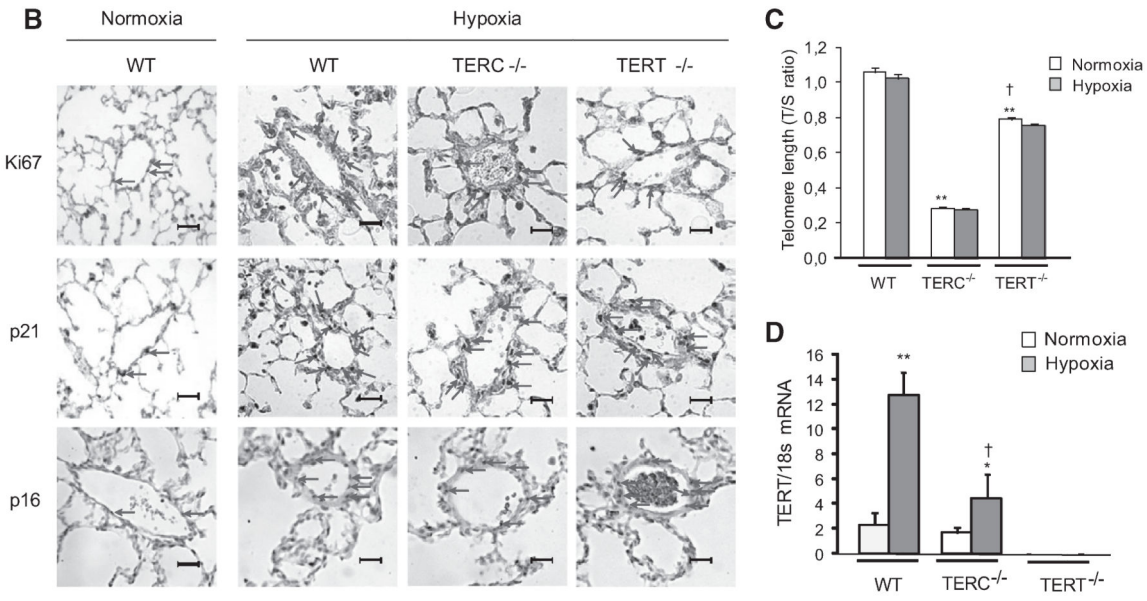


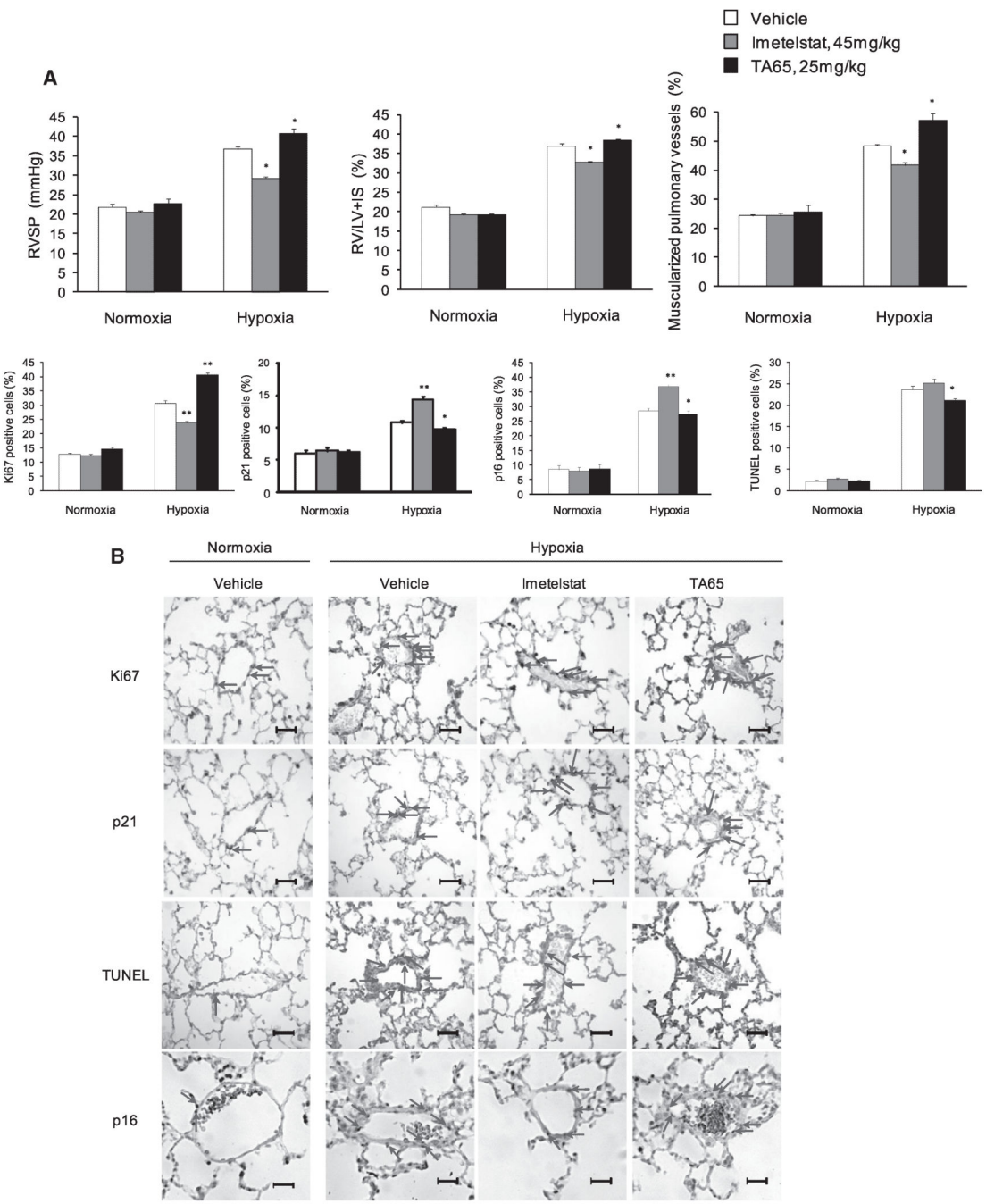
Figure 5.

TERT or *TERC* gene deficiency abrogates the development of hypoxic pulmonary hypertension in mice.

A, Graphs of right ventricular systolic pressure (RVSP), right ventricular (RV) hypertrophy index (RV/left ventricle plus septum [LV+S]) weight), percentage of muscularized pulmonary vessels, percentage of dividing Ki67-positive cells, and p21- and p16-stained cells in wild-type (WT) mice, $TERC^{-/-}$ mice, and $TERT^{-/-}$ mice studied at generations (G) 1, 2, 3, and 4 under normoxia and hypoxia. For all graphs, both individual and mean values are represented. *P<0.01 vs WT mice.

B, Representative micrographs of pulmonary vessels stained for Ki67, p21, or p16. Red arrows show Ki67-, p21-, and p16-positive nuclei. No immunoreactivity was detected in sections incubated with rabbit IgG control and secondary anti-rabbit antibody. Bar, 50 μm.

C, Telomere length measured in WT, $TERC^{-/-}$, and $TERT^{-/-}$ mice during normoxia and after 18 days of hypoxia. Data are mean±SEM of 10 to 25 animals. ***P<0.001 vs WT normoxic mice; †P<0.01 vs $TERC^{-/-}$ mice. **D**, Telomerase reverse transcriptase (TERT) mRNA levels measured by real-time polymerase chain reaction in lungs from WT, $TERC^{-/-}$, and $TERT^{-/-}$ mice studied on day 18 after exposure to normoxia or hypoxia. Data are mean ±SEM of 6 to 10 animals. ***P<0.001 vs normoxic mice; †P<0.01 vs WT mice.



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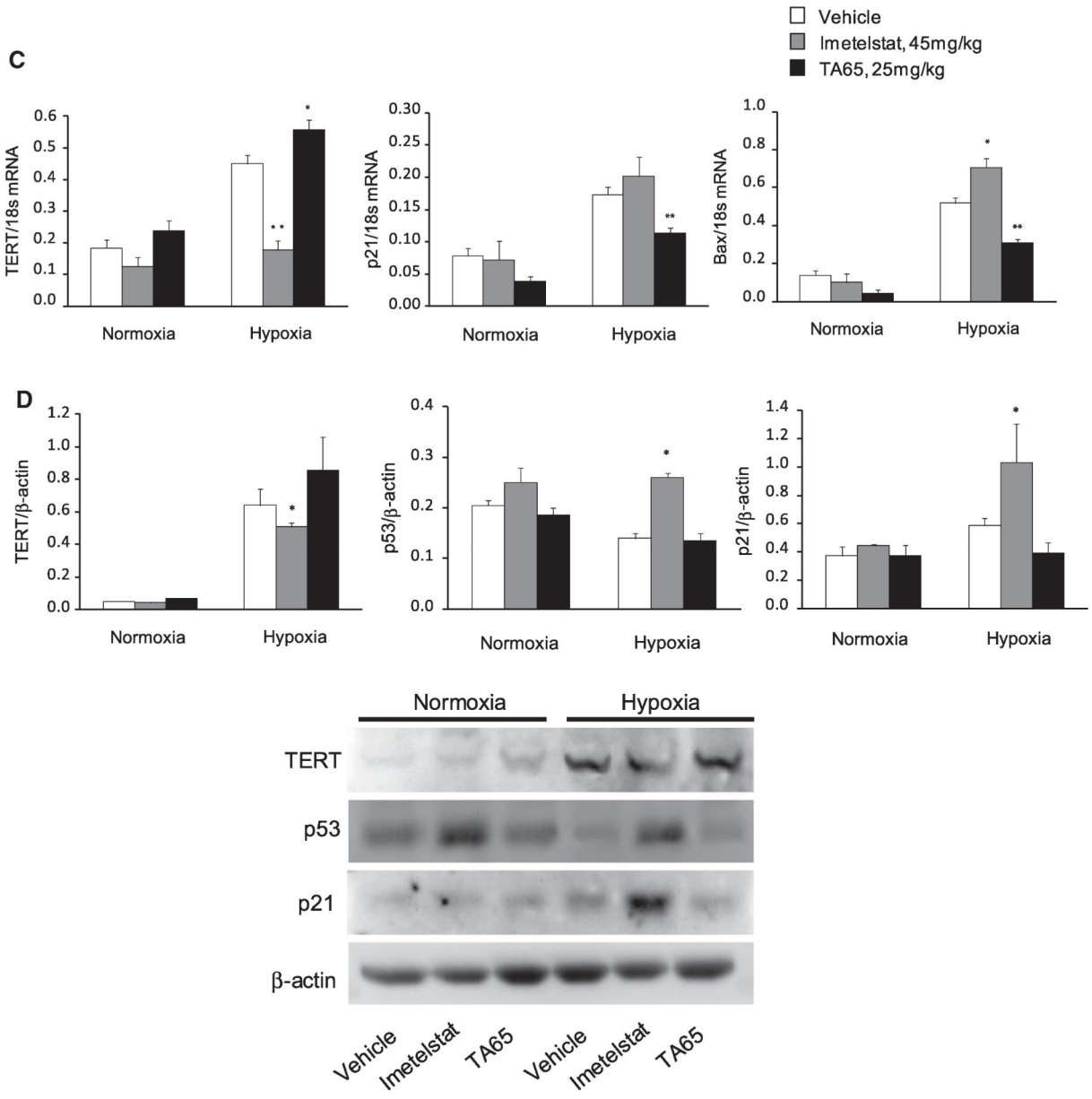

Figure 6.

Effects of treatment with imetelstat or TA65 on hypoxia-induced pulmonary hypertension in mice. **A**, Right ventricular systolic pressure (RVSP), right ventricular (RV) hypertrophy index (RV/left ventricle plus septum [LV+S] weight ratio), pulmonary vessel muscularization (percentage of partially and fully muscularized pulmonary vessels), and percentages of dividing Ki67-positive cells, p21- and p16-stained cells, and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)–stained cells in mice studied on day 21 of hypoxia or normoxia exposure and daily intraperitoneal treatment with vehicle, imetelstat, or TA65 **B**, Representative micrographs of pulmonary vessels stained for Ki67, p21,TUNEL, and p16. No immunoreactivity was detected in sections incubated with secondary anti-rabbit and anti-mouse antibody but no primary antibody. **C**, mRNA levels of

TERT, p21, and Bax measured by real-time polymerase chain reaction in lungs from mice on day 21 of hypoxia or normoxia exposure and daily intraperitoneal treatment with vehicle, imetelstat, or TA65. **D**, Lung protein levels of telomerase reverse transcriptase (TERT), p53, and p21 measured by Western blot in the same groups of mice. Data are mean±SEM of 6 to 10 animals. WT indicates wild-type. *P<0.025, **P<0.01 vs vehicle-treated mice after exposure to hypoxia.

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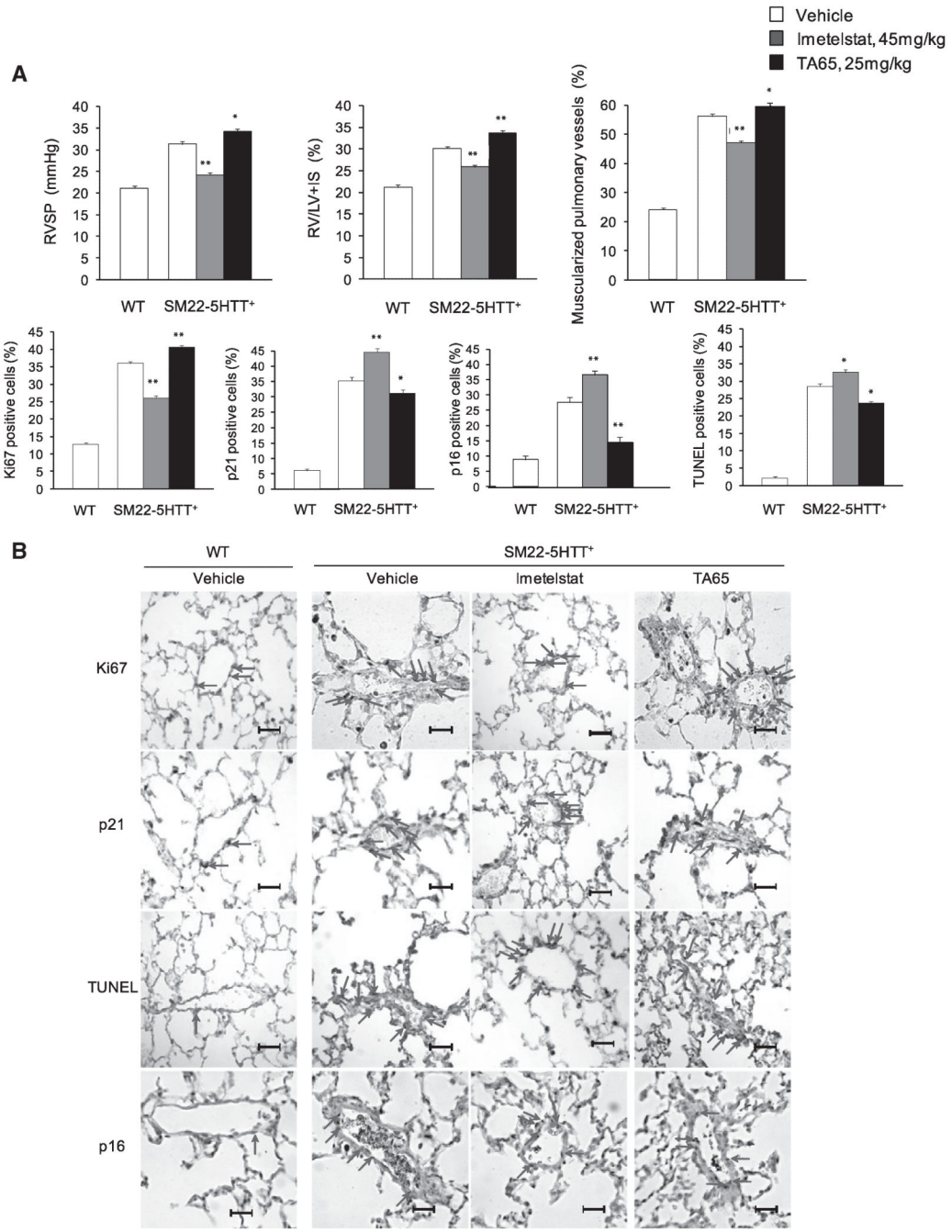

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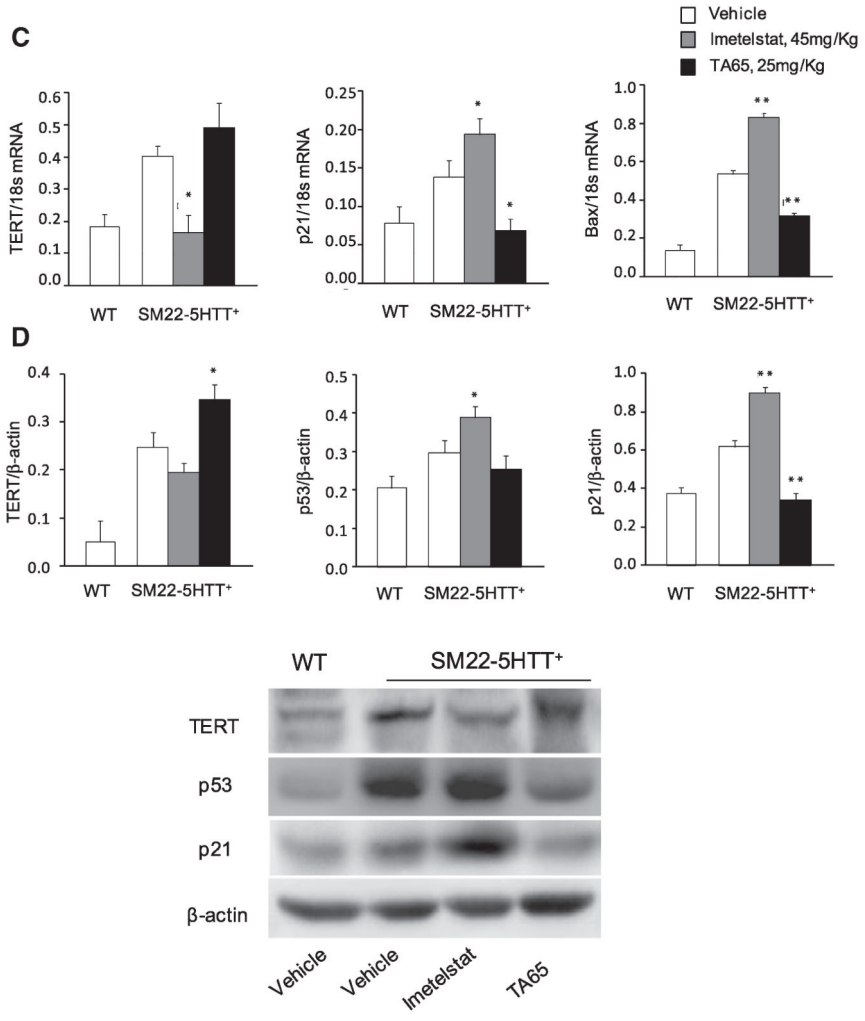

Figure 7.

Effects of treatment with imetelstat or TA65 on pulmonary hypertension in SM22-*5HTT*$^+$ mice. **A**, Right ventricular systolic pressure (RVSP), right ventricular (RV) hypertrophy index (RV/left ventricle plus septum [LV+S] weight ratio), pulmonary vessel muscularization (percentages of partially and fully muscularized pulmonary vessels), and percentages of dividing Ki67-positive cells, p21- and p16-stained cells, and terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL)-stained cells in SM22-*5HTT*$^+$ mice studied after 21 days of treatment with vehicle, imetelstat, or TA65 **B**, Representative micrographs of pulmonary vessels stained for Ki67, p21, TUNEL, or p16. No immunoreactivity was detected in sections incubated with secondary anti-rabbit and anti-mouse antibody but no primary antibody. **C**, Lung mRNA levels of telomerase reverse transcriptase (TERT), p21, and Bax measured by real-time polymerase chain reaction in wild-type (WT) mice and SM22-*5HTT*$^+$ mice studied after 21 days of treatment with vehicle, imetelstat, or TA65. **D**, Lung protein levels of TERT, p53, and p21 measured by Western blot in the same groups of mice. Data are mean±SEM of 6 to 10 animals. *P<0.025, **P<0.01 vs vehicle-treated SM22-*5HTT*$^+$ mice.

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