ORIGINAL RESEARCH

Unique and Redundant Functions of p70 Ribosomal S6 Kinase Isoforms Regulate Mesenchymal Cell Proliferation and Migration in Pulmonary Fibrosis

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Abstract

The p70 ribosomal S6 kinase (p70S6K) is a downstream substrate that is phosphorylated and activated by the mammalian target of rapamycin complex and regulates multiple cellular processes associated with pulmonary fibrogenesis. Two isoforms of the p70S6K have been identified (S6K1 and S6K2), but their relative contributions in mediating pulmonary fibrosis are unknown. To interrogate the roles of the p70S6K isoforms, we overexpressed transforming growth factor (TGF)-α in mice deficient for the S6K1 or S6K2 genes and measured changes in lung histology, morphometry, total lung collagen, lung function, and proliferation between wild-type and isoform-deficient mice. Deficiency of S6K1, but not S6K2, had a significant effect on reducing proliferation in subpleural fibrotic lesions during TGF-α–induced fibrosis. Migration was significantly decreased in mesenchymal cells isolated from the lungs of S6K1 knockout mice compared with wild-type or S6K2 knockout mice. Conversely, increases in subpleural thickening were significantly decreased in S6K2-deficient mice compared with wild type. Deficiency of S6K2 significantly reduced phosphorylation of the downstream S6 ribosomal protein in lung homogenates and isolated mesenchymal cells after TGF-α expression. However, deficiency of neither isoform alone significantly altered TGF-α–induced collagen accumulation or lung function decline *in vivo*. Furthermore, deficiency in neither isoform prevented changes in collagen accumulation or lung compliance decline after administration of intradermal bleomycin. Together, these findings demonstrate that the p70S6K isoforms have unique and redundant functions in mediating fibrogenic processes, including proliferation, migration, and S6 phosphorylation, signifying that both isoforms must be targeted to modulate p70S6K-mediated pulmonary fibrosis.

Keywords: pulmonary fibrosis; pleural disease; mammalian target of rapamycin; p70 ribosomal S6 kinase

Clinical Relevance

The p70 ribosomal S6 kinase (p70S6K) regulates multiple cellular processes associated with pulmonary fibrosis and is activated in human disease. The p70S6K is composed of two isoforms, yet the individual roles for each isoform in mediating pulmonary fibrogenesis is unknown. This study demonstrates that both isoforms must be targeted to modulate fibroproliferation.

Pulmonary fibrosis can be initiated and propagated through a number of distinct signaling pathways or cascades. Several of these signaling pathways overlap and converge to activate downstream pathways, which can regulate fibrogenic cellular processes, including mesenchymal proliferation and matrix deposition (1, 2). The p70 ribosomal S6 kinase (p70S6K) is a mitogen- and amino acid–sensitive serine–threonine kinase, which can be activated by upstream signaling pathways shown to mediate pulmonary fibrosis including mitogen-activated protein kinase/Erk, phosphoinositide 3-kinase

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(PI3K), and mammalian target of rapamycin (mTOR) (3, 4). Activation of mTOR, in complex with raptor (mTORC1), leads to phosphorylation of p70S6K and the eukaryotic initiation factor-4E–binding protein. The p70S6K contains two similar S6 kinase proteins (S6K1 and S6K2) encoded by two different genes that subsequently phosphorylate the 40S ribosomal protein S6 (S6) (5). The primary functions of both p70S6K and eukaryotic initiation factor-4E–binding proteins remain under investigation, but are known to include control of translation of specific mRNAs involved in cellular growth, proliferation, protein translation, and cell cycle regulation (6). Emerging evidence suggests that aberrant mTORC1–S6K signaling contributes to various pathologic conditions, including fibrosis, diabetes, obesity, organ hypertrophy, and cancer. Because of the strong association between S6 expression and cancer, highly selective and specific small-molecule inhibitors of p70S6K have been developed that demonstrate significant single-agent efficacy in glioblastoma and colon carcinoma xenograft models and are currently in advanced oncology trials (7, 8).

There are limited data on the contribution of p70S6K in mediating pathologic remodeling in the lung. The p70S6K is necessary for the proliferative responses of pulmonary artery adventitial fibroblasts in response to hypoxia (9). In cultured human airway smooth muscle cells, activation of S6K is required for transforming growth factor (TGF)-β– and endothelin-1–induced airway smooth muscle cell size enlargement (10). In pulmonary disease, Western blot for phosphorylated S6 demonstrated increased expression in three of five idiopathic pulmonary fibrosis (IPF) samples compared with no signal for either nondiseased control subjects or patients with chronic obstructive pulmonary disease (11). In a separate study, immunohistochemical analysis on lung tissues taken at transplantation revealed strong expression of phosphorylated S6 in myofibroblasts from IPF lung tissue compared with little or no expression in healthy lung tissue (12). Together, these findings indicate that the p70S6K pathway is up-regulated in at least a portion of patients with IPF, and emphasize the importance of developing a greater understanding on the relevance of this pathway in mediating fibroproliferative disease.

To further understand the role of the epidermal growth factor receptor (EGFR) pathway in pulmonary fibrosis, our laboratory previously generated doxycycline (Dox) regulatable transgenic mice overexpressing TGF-α under control of the lung epithelial-specific, 2.3-kb rat club cell secretory protein (CCSP) gene promoter. When TGF-α transgenic mice are administered Dox, progressive fibrotic lesions develop in the adventitial and subpleural regions of the lung characterized by mesenchymal proliferation with myofibroblast transformation, progressive migration of fibrotic lesions into the interstitium, extracellular matrix deposition, severe restrictive changes in lung mechanics, and cachexia (13, 14). We recently demonstrated that fibroproliferation in the TGF-α model is associated with increased phosphorylation of the p70S6K pathway immediately after TGF-α induction with localization of activity primarily seen in mesenchymal cells in the subpleural fibrotic regions (15). Administration of a selective small-molecule ATP competitive inhibitor of p70S6 kinase, which inhibits both S6K1 and S6K2 isoforms, prevented the development of extensive subpleural fibrosis and attenuated the progression of subpleural fibrosis when administered as a rescue therapy (3). These studies demonstrate a proof of concept that targeting the S6K pathway is feasible to effectively modify the progression of pulmonary fibrosis in the subpleural compartment of the lung *in vivo*. However, the role of the individual p70S6K isoforms in mesenchymal cell activation and pulmonary fibrosis is unknown.

Emerging data in oncology demonstrate that the S6K1 and S6K2 isoforms have overlapping, as well as unique, biologic functions (16). In the lung, it is unknown if these individual isoforms regulate distinct functional fibroproliferative processes. Identifying a specific role for each isoform in lung fibrogenesis would be informative to guide potential treatment strategies, as current p70S6K inhibitors target either the S6K1 or both isoforms (16). To interrogate the roles of the p70S6K isoforms, we overexpressed TGF-α in mice deficient for the S6K1 or S6K2 genes, and measured changes in pulmonary fibroproliferation between wild-type and isoform-deficient mice.

Materials and Methods

Transgenic Mice

Mice expressing the reverse tetracycline-responsive transactivator (rtTA) under control of the 2.3-kb rat CCSP gene promoter (CCSP/rtTA) were mated to $S6K1^{-/-}$ mice (17) (generously provided by Sara Kozma, Ph.D., University of Cincinnati, Cincinnati, OH), generating offspring with a genotype of $rtTA^{+/-}/S6K1^{+/-}$. In a separate mating, transgenic mice with the human TGF-α cDNA under control of the tetracycline operon plus a minimal cytomegalovirus promoter (tetO/TGF-α) were mated to $S6K1^{-/-}$ mice, generating offspring with a genotype of $tetO/TGF\text{-}\alpha^{+/-}/S6K1^{+/-}$. Offspring from both of these separate matings were crossed and mice selected by PCR to contain the CCSP/rtTA, tetO/TGF-α, and either $S6K1^{++}$ or $S6K1^{-/-}$ transgenes using primers and PCR conditions previously reported (17–19). To generate TGF-α–overexpressing mice in the absence of the S6K2 gene, the same mating strategy was employed using $S6K2^{-/-}$ mice (courtesy of Dr. Kozma). Mice deficient for either S6K1 or S6K2 have normal lungs, with no effect on viability or mating; however, loss of both isoforms results in lethality at embryonic stages, although the lungs demonstrate no gross anatomical defects (17, 19).

Separately, wild-type and S6K knockout (KO) mice were administered bleomycin (0.1 mg) daily in 0.05 ml saline solution intradermally 5 days per week for 4 weeks, as previously described (20).

Fibrosis Endpoints

Lungs were inflation fixed using 4% paraformaldehyde and paraffin sections stained with trichrome and α-smooth muscle actin (αSMA; Sigma, St. Louis, MO); the degree of fibrosis was histologically scored and subpleural thickness measured by MetaMorph (Molecular Devices, Sunnyvale, CA), as previously described (3, 15). Paraffin sections were immunostained with Ki67 antigen (Dako, Glostrup, Denmark) and the proliferation index determined (21, 22). Lung mechanics were assessed on mice with a computerized flexiVent system (SCIREQ, Montreal, PQ, Canada), and lung hydroxyproline levels determined as previously described (23).

Western Blots

Western blot analysis was quantified using the volume integration function on PhosphorImager software, Multigage (Fujifilm, Valhalla, NY). Antibodies used include phosphorylated Akt (Ser 473; Cell Signaling, Danvers, MA), phosphorylated

S6 (Ser 235/236; Cell Signaling), and C4 actin Mouse (Seven Hills Bioreagents, Cincinnati, OH).

Mesenchymal Cell Migration Assay

Mesenchymal cell migration was assayed using a high-content imaging system (Essen BioScience, Ann Arbor, MI). Primary lung mesenchymal cells were seeded overnight into a 96-well plate precoated with fibronectin 1 (1 μg/well). An automated wound maker generated wounds on cell monolayers to create cell-free zones. Cells were then treated with TGF-α or media and images taken every 2 hours to quantify the degree and rate of migration of cells into the cell-free scratch areas of the well.

Primary fibroblasts were isolated from the lung cultures of wild-type mice and cultured on polylysine-coated coverslips, fixed with 3.7% formaldehyde in PBS, and made permeable to stain F-actin using fluorescein–phalloidin. The fluorescence images of cells were analyzed using confocal microscopy, as described previously (24).

Statistical Analysis

All data were analyzed with Prism (Version 5; GraphPad Software, Inc., La Jolla, CA). Unpaired Student's *t* tests and one-way ANOVA with Tukey's multiple comparison post test were used to compare different experimental groups, and data were considered statistically significant at *P* values less than 0.05.

Results

Reduced Migration in Primary Lung Mesenchymal Cells from S6K1$^{-/-}$ Mice

To determine the roles of the p70S6K isoforms in migration, we isolated primary mesenchymal cells from the lung cultures of wild-type, S6K1$^{-/-}$, and S6K2$^{-/-}$ mice and quantified changes in migration using a real-time, high-content imaging system. Mesenchymal cells from S6K1$^{-/-}$ lungs had a lower migration capacity compared with wild type and S6K2$^{-/-}$ (Figure 1A).

To assess the role of the p70S6K isoforms on TGF-α–driven migration, mesenchymal cells were treated with and without TGF-α in low-FCS (0.1%) media. TGF-α treatment significantly enhanced migration in wild-type cells (Figure 1B). However, TGF-α–driven migration of mesenchymal cells was significantly diminished in S6K1$^{-/-}$ compared with wild-type and S6K2$^{-/-}$ cells (Figure 1B). Together, these data demonstrate that the migration capacity of mesenchymal cells was largely dependent of S6K1, and indicate differential regulation of mesenchymal cell migration by p70S6K isoforms.

We next assessed if inhibition of both isoforms altered migration. Wild-type mesenchymal cells pretreated with the dual S6K1 and S6K2 inhibitor, LY2584702, demonstrated inhibition of migration after TGF-α stimulation in low-FCS (0.1%) media with kinetics of inhibition similar to that of S6K1$^{-/-}$ cells (Figure 1C). F-actin polymerization is a critical step in the migration of cells, and was assessed using immunofluorescence in TGF-α–stimulated wild-type mesenchymal cells pretreated with and without LY2584702. We detected reduced F-actin when cells were pretreated with LY2584702 before TGF-α stimulation (Figure 1D).

Reduced Phosphorylated S6 Signaling in Lung Fibroblasts from S6K2$^{-/-}$ Mice

As we observed differential regulation of mesenchymal cell migration, we investigated if p70S6K isoforms play specific or redundant functional roles in the phosphorylation of their downstream target, S6. Primary lung mesenchymal cells were isolated from the lung cultures of wild-type, S6K1$^{-/-}$, and S6K2$^{-/-}$ mice, and stimulated with recombinant TGF-α. TGF-α induced an increase in phosphorylation of both Akt and S6 at both 15 and 30 minutes after incubation compared with time zero. There were no differences in the increases in S6 phosphorylation after TGF-α treatment in S6K1$^{-/-}$ cells compared with wild-type controls at all time points (Figure 2A). S6K2$^{-/-}$ cells demonstrated reduced levels of phosphorylated S6 at time zero compared with controls (Figure 2B). The addition of TGF-α to S6K2$^{-/-}$ cells increased phosphorylation of S6 compared with time zero, but values were decreased compared with TGF-α–treated wild-type cells. As the S6K can negatively regulate Akt phosphorylation, S6K inhibition could thereby release feedback inhibition of the PI3K pathway, as previously demonstrated in neoplastic cell culture lines (25). There were no differences in phosphorylated Akt expression between wild-type, S6K1$^{-/-}$, and S6K2$^{-/-}$ cells after TGF-α stimulation, signifying that the differential S6 phosphorylation was not secondary to alterations in upstream activation.

Differential Effects of p70S6K Isoforms on Proliferation and Phosphorylation of S6 in the Lungs after TGF-α Overexpression

To determine the effects of genetic S6K1 or S6K2 isoform ablation on the progression of TGF-α–induced pulmonary fibrosis, CCSP/rtTA and tetO/TGF-α transgenes were bred into either S6K1 or S6K2 KO mice to generate CCSP/TGF-α/S6K1$^{-/-}$/S6K2$^{+/+}$ (CCSP/TGF-α/S6K1$^{-/-}$) and CCSP/TGF-α/S6K1$^{+/+}$/S6K2$^{-/-}$ (CCSP/TGF-α/S6K2$^{-/-}$) mice. Fibrosing controls included litter- and age-matched CCSP/TGF-α/S6K1$^{+/+}$/S6K2$^{+/+}$ mice (CCSP/TGF-α WT). Adult mice for all experiments were then placed on Dox for 8 weeks to overexpress the TGF-α transgene and fibrosis parameters compared between the groups. Nonfibrosing controls were litter- and age-matched CCSP/TGF-α mice heterozygous for either S6K1 or S6K2 not placed on Dox (control).

TGF-α expression resulted in increased Ki67 staining in the lungs, including subpleural fibrotic regions (Figure 3). The proliferation index was determined by counting the total number of Ki67-staining cells and dividing by the total number of cells in each field (22). Consistent with our previous observations, TGF-α expression induced a twofold increase in the number of Ki67-staining cells in the lungs compared with controls (Table 1) (18). Proliferation was higher in the subpleural regions than in the interstitium. For CCSP/TGF-α/S6K1$^{-/-}$ mice, the proliferation index was significantly reduced compared with CCSP/TGF-α WT mice. The Ki67-staining index levels were attenuated, but not significantly reduced, in the CCSP/TGF-α/S6K2$^{-/-}$ mice compared with CCSP/TGF-α WT mice.

To assess *in vivo* the relative contributions of the p70S6K isoforms on activation of S6, we measured phosphorylation of signaling intermediates in the whole-lung homogenates of mice after 8 weeks of Dox. Similar to our findings in isolated lung mesenchymal cells, we observed a significant reduction in the intensity of S6 phosphorylation in the total lung lysates of CCSP/TGF-α/S6K2$^{-/-}$

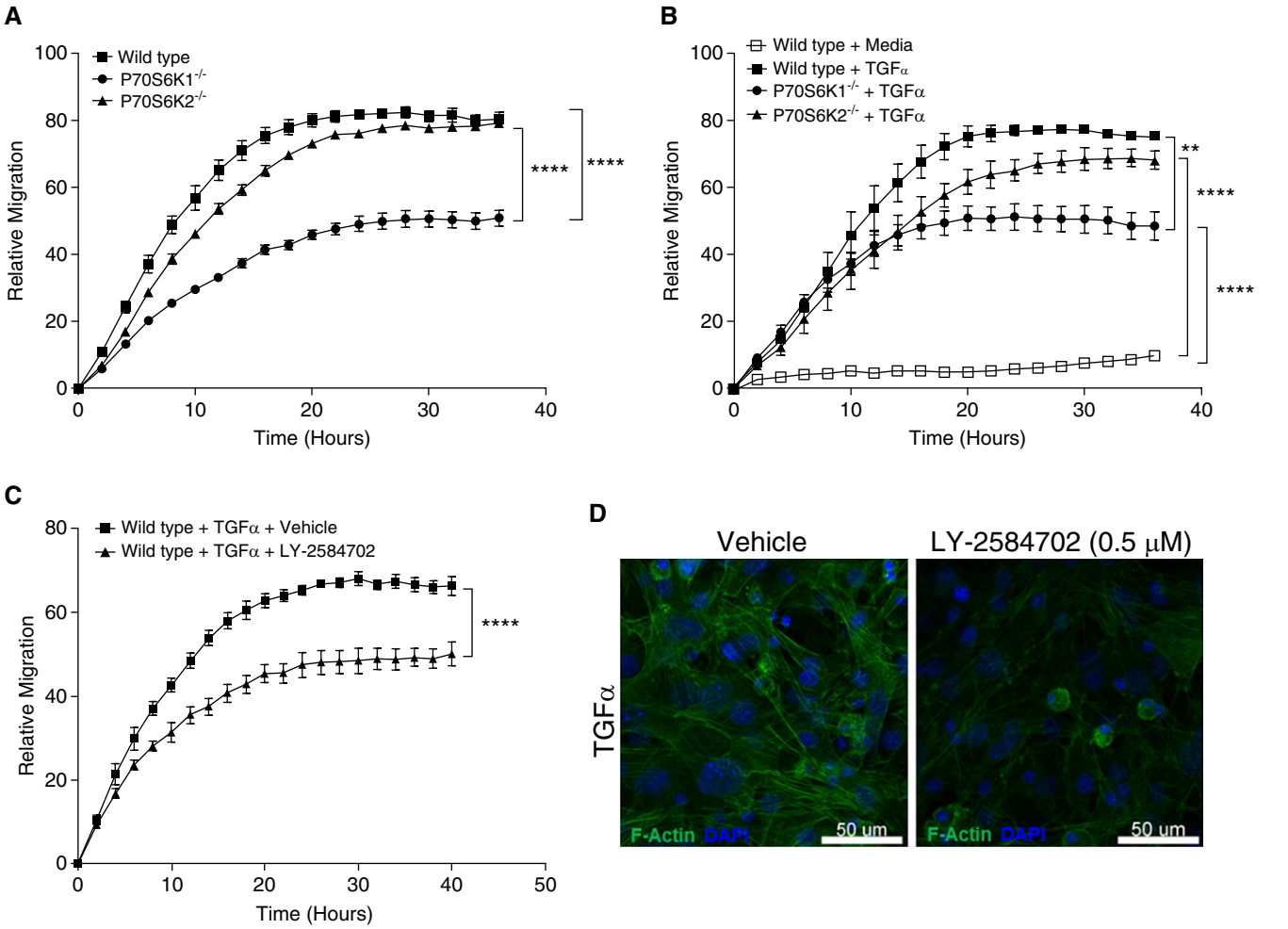

Figure 1. Knockout (KO) of p70 ribosomal S6 kinase protein (p70S6K)-1 or p70S6K2 attenuates migration of mesenchymal cells. Primary lung mesenchymal cells were isolated from wild-type, S6K1$^{-/-}$, and S6K2$^{-/-}$ mice, and changes in migration quantified in regular Iscove's Modified Dulbecco's Medium (IMDM) (*A*) and in low-serum IMDM with or without transforming growth factor (TGF)-α (20 ng/ml) (*B*). Changes in migration were assessed after TGF-α stimulation in low-FCS (0.1%) media in wild-type mesenchymal cells pretreated with or without the dual S6K1 and S6K2 inhibitor LY2584702 (*C*). F-actin polymerization was assessed using immunofluorescence in TGF-α–stimulated wild-type mesenchymal cells pretreated with and without LY2584702 for 8 hours (*D*). *Scale bars*: 50 μm. Data are means (±SEM), and statistical differences shown were performed using ANOVA. $^{**}P < 0.01$; $^{****}P < 0.0001$. Experiments were repeated in duplicate.

mice compared with control, CCSP/TGF-α WT, and CCSP/TGF-α/S6K1$^{-/-}$ mice, with no differences in phosphorylated Akt expression (Figure 4). The dampened increase in Akt and S6 phosphorylation between control and CCSP/TGF-α groups seen at 8 weeks of Dox is consistent with previous work showing diminished phosphorylation of signaling intermediates of the PI3K/mTOR pathway after continuous and prolonged TGF-α expression in the lung (23). Together, these findings show differential responses *in vivo* between the p70S6K isoforms in downstream S6 phosphorylation and proliferative responses in fibrotic lesions.

Regulation of Subpleural Thickening by p70S6K Isoforms

Masson's trichrome staining of lung sections of CCSP/TGF-α WT mice after 8 weeks of Dox revealed marked subpleural thickening with advanced perivascular and peribronchial fibrosis affecting large and small vessels and airways, consistent with our previous reports (Figure 5A) (18). Both CCSP/TGF-α/S6K1$^{-/-}$ and CCSP/TGF-α/S6K2$^{-/-}$ mice demonstrated attenuation of subpleural fibrosis compared with CCSP/TGF-α WT, whereas no changes in adventitial fibrosis were noted. Increased staining for αSMA was detected in the subpleural and adventitial fibrotic regions after TGF-α expression for all transgenic groups compared with controls. Consistent with trichrome staining, attenuated αSMA was detected in the subpleural region of CCSP/TGF-α/S6K1$^{-/-}$ and CCSP/TGF-α/S6K2$^{-/-}$ mice compared with CCSP/TGF-α WT (Figure 5A).

Morphometric measurements demonstrated a greater than sixfold increase in subpleural thickening between control and CCSP/TGF-α mice. Subpleural thickening in CCSP/TGF-α/S6K2$^{-/-}$ mice was significantly reduced compared with CCSP/TGF-α mice, and there was a trend toward reduced subpleural thickening in CCSP/TGF-α/S6K1$^{-/-}$ mice compared with CCSP/TGF-α mice, but differences were not statistically significant ($P = 0.056$;

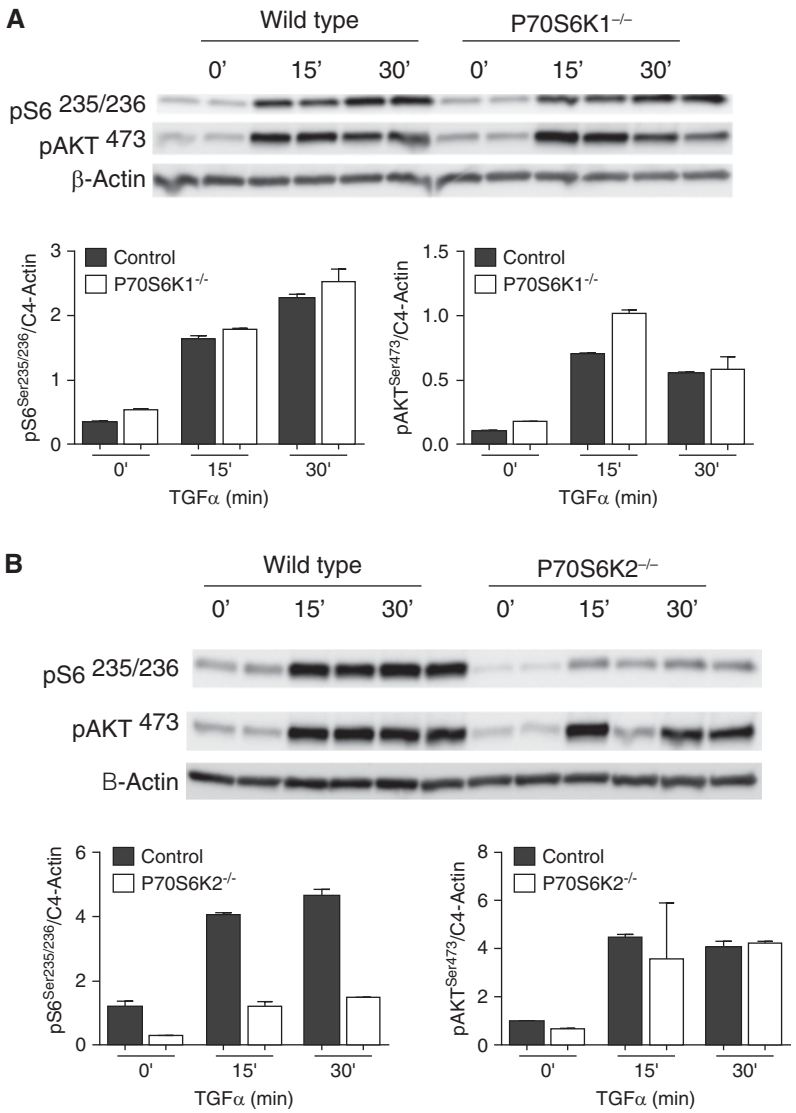

Figure 2. Reduced phosphorylated S6 signaling in lung mesenchymal cells from S6K2$^{-/-}$ mice. Primary lung mesenchymal cells were isolated from wild-type, S6K1$^{-/-}$, and S6K2$^{-/-}$ mice and stimulated with TGF-α (20 ng/ml). Western blot analysis of phosphorylated S6 (pS6) and Akt (pAKT) with quantified phosphorylated S6 compared with control for S6K1$^{-/-}$ (*A*) and S6K2$^{-/-}$ (*B*). Data are means (±SEM).

Figure 5B). Subpleural thickening for both CCSP/TGF-α/S6K1$^{-/-}$ and CCSP/TGF-α/S6K2$^{-/-}$ mice remained significantly increased compared with nonfibrosing controls.

To enumerate the fibrotic lesions of the lungs, we previously established a scoring system that was based on the distribution and severity of fibrotic lesions in the perivascular, peribronchial, alveolar, and subpleural areas of the lung (26). Compared with control mice, overexpression of TGF-α for 8 weeks caused a significant increase in the fibrosis score that was not significantly reduced in either CCSP/TGF-α/S6K1$^{-/-}$ or CCSP/TGF-α/S6K2$^{-/-}$ mice (Figure 5C).

Together, these findings support the concept that both isoforms overlap in mediating expansion of the subpleural mesenchyme after TGF-α overexpression. The isolated alterations in subpleural thickening further support our previous findings of a differential response for the p70S6K pathway in mediating TGF-α–induced mesenchymal cell expansion between the subpleural versus the adventitial region (3).

The Genetic Loss of S6K1 or S6K2 Isoforms Does Not Alter Changes in Lung Collagen or Lung Function after TGF-α Overexpression or Bleomycin Injury

Overexpression of TGF-α after 8 weeks of Dox resulted in no significant differences in total lung weight, lung hydroxyproline levels, or lung compliance between CCSP/TGF-α WT and CCSP/TGF-α/S6K1$^{-/-}$ or CCSP/TGF-α/S6K2$^{-/-}$ mice after 8 weeks of Dox (Figures 6A–6C). Hence, the attenuation of subpleural fibrosis among S6K-null mice did not lead to significant alterations in biochemical or physiologic effects of lung fibrosis.

Histologically, administration of daily intradermal bleomycin for 4 weeks caused diffuse inflammation and fibrosis in the lungs of wild-type and S6K1$^{-/-}$ and S6K2$^{-/-}$ mice compared with saline controls (Figure 7A). Total lung weight increased significantly in all bleomycin-treated mice compared with saline-treated controls, and there were no significant differences in total lung weight between bleomycin-treated wild-type, S6K1$^{-/-}$, or S6K2$^{-/-}$ mice (Figure 7B). Lung hydroxyproline levels increased significantly in all bleomycin-treated mice compared with saline-treated controls (Figure 7C); increased hydroxyproline levels were not significantly different between bleomycin-treated wild-type and S6K2$^{-/-}$ mice, but were significantly different between bleomycin-treated wild-type and S6K1$^{-/-}$ mice. Lung compliance was significantly decreased between bleomycin-treated wild-type and S6K1$^{-/-}$ mice compared with saline-treated controls, but was not significantly different between saline and bleomycin-treated S6K2$^{-/-}$ mice (Figure 7D). Overall, the bleomycin studies suggest limited or no changes in the fibrotic response after bleomycin injury in S6K1$^{-/-}$ and S6K2$^{-/-}$ mice compared with wild-type mice.

Discussion

The primary findings from this study are that the S6K1 and S6K2 isoforms regulate distinct fibroproliferative cellular processes after TGF-α/EGFR activation. Genetic deletion of the individual isoforms reduced, but did not prevent,

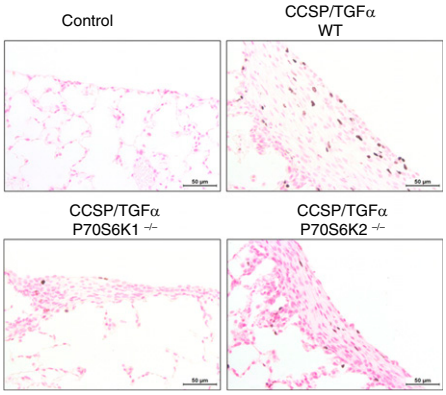

Figure 3. Differential effects of p70S6K isoforms on proliferation and phosphorylation of S6 in the lungs after TGF-α overexpression. Club cell secretory protein (CCSP)/tetracycline-responsive transactivator (rtTA) and tetracyclin-On (TetO/TGFα) transgenes were bred into S6K1 or S6K2 KO mice and administered doxycycline for 8 weeks to induce pulmonary fibrosis. Representative photomicrographs demonstrate that overexpression of TGF-α was associated with increased Ki67 staining in the mesenchymal cells in the subpleural region. *Scale bars*: 50 μm. WT, wild type.

the subpleural thickening in the TGF-α transgenic model, and did not alter fibrosis or changes in lung compliance after bleomycin injury. In aggregate, our findings support the notion that the S6K1 and S6K2 isoforms regulate overlapping, as well as unique, fibroproliferative processes in the lung. Because the p70S6K pathway controls several key fibroproliferative cellular processes and is up-regulated in the myofibroblasts of patients with IPF (12, 16), these findings are significant, as they suggest that both isoforms must be targeted to modulate p70S6K-mediated pulmonary fibrosis.

S6K1 and S6K2 share 83% identify in their highly conserved kinase and linker domains, so researchers for many years supposed they were redundant kinases with essentially overlapping functions (27). Supporting this assumption, genome-wide microarray studies revealed that S6K1 and S6K2 both regulate general transcription, and are functionally redundant, as overexpression of either isoform in double-KO cells restored changes in transcription (28). However, emerging research indicates that the two isoforms also have distinct biological regulation and function. Although S6K1 and S6K2 are both downstream of mTOR, there are differences in their upstream regulation, as each isoform reacts differently to nutrient deprivation (27, 29). S6K1 is sensitive to mTOR inhibitors, such as rapamycin, which does not alter S6K2 phosphorylation (27, 30). Our data reveal that KO of individual isoforms produces different effects on downstream phosphorylation of S6 after TGF-α overexpression. Measurement of activation of S6 by Western blot from lung homogenates *in vivo*, as well as in isolated lung fibroblasts stimulated with TGF-α, demonstrated reduced phosphorylation of S6 in S6K2$^{-/-}$ with no change in S6K1$^{-/-}$ compared with controls. These findings are consistent with two previous studies in mouse embryo fibroblasts (MEF) cells, which revealed that the extent of S6 phosphorylation in S6K1$^{-/-}$ cells was comparable to that in wild-type MEFs, but reduced in S6K2$^{-/-}$ MEFs in response to serum (17, 19). Together, these data suggest that S6K2 cannot fully compensate for the loss of S6K1 function with respect to S6 phosphorylation, and support unique upstream and downstream signaling pathways for both p70S6K isoforms.

Several studies suggest that the mTORC1–p70S6K axis promotes cell cycle progression and cell proliferation (9, 16). We previously demonstrated that pharmacological inhibition of both kinases prevented proliferation of primary mouse lung fibroblasts after TGF-α/platelet-derived growth hormone stimulation (3). *In vivo* in TGF-α overexpressing transgenic mice, dual kinase inhibition also prevented proliferation in the subpleural regions, as measured with Ki67 staining (3). However, the role of individual isoforms in controlling cell cycle progression and cell proliferation is controversial, and has not been determined in lung fibroblasts. In the current study, increases in Ki67 staining after TGF-α overexpression was inhibited in the subpleura of S6K1 KO mice, whereas it was not significantly reduced in S6K2 KOs. Our findings support S6K1 as the predominant isoform mediating cell proliferation in the subpleural mesenchyme, and are consistent with previous work in the liver, where genetic deletion of S6K1 delayed S-phase entry in hepatocytes after hepatectomy (31).

In addition to proliferation, mesenchymal cell migration is another key cellular process involved in pulmonary fibrogenesis. Fibroblasts from patients with IPF show an increased capacity for migration, which was proportional to the intensity of the fibrosis and independent of the proliferative activity (32). The direct role of either the TGF-α or the p70S6K pathway in pulmonary fibroblast migration has not been previously studied. We demonstrate markedly enhanced migration of primary lung fibroblasts after TGF-α stimulation. This increased migration was significantly reduced in primary lung fibroblasts from S6K1 KO mice, but not in S6K2 KO or wild-type cells. A recent study showed that S6K1 inhibition prevents the migration of breast tumor cells, supporting our findings that S6K1 is the dominant kinase in fibroblast migration (33). However, the precise mechanism for reduced S6K1-mediated migration is unclear. One mechanism for the reduced migration in S6K1 KO may be reduced proliferation that we observed *in vivo*. Reduced migration for the S6K1 KO cells occurred relatively early, especially in the 5% Iscove's Modified Dulbecco's Medium media conditions in Figure 1A, suggesting that differences in

Table 1. Proliferation Index as Assessed by Ki67 Staining in Transgenic Mice

	Average Total Lung	Average Interstitium	Average Subpleural
Control	3.6 ± 0.5	3.7 ± 0 0.5	3.5 ± 0 0.6
CCSP/TGF-α	7.2 ± 1.1*	5.8 ± 0 0.8	10.1 ± 1.8*
CCSP/TGF-α S6K1$^{-/-}$	2.2 ± 0.7†	1.9 ± 0 0.6†	2.8 ± 1.0†
CCSP/TGF-α S6K2$^{-/-}$	5.9 ± 0 0.5	4.9 ± 0.5	7.7 ± 1.0

Definition of abbreviations: CCSP, club cell secretory protein; S6K, S6 kinase protein; TGF-α, transforming growth factor-α.
Data are means ± SEM ($n = 16$ mice in controls, 24 in CCSP/TGF-α wild type, 11 in CCSP/TGF-α/S6K1$^{-/-}$, and 15 in CCSP/TGF-α/S6K2$^{-/-}$). Statistical analysis performed using ANOVA.
*$P < 0.05$ compared with control.
†$P < 0.05$ compared with CCCP/TGF-α.

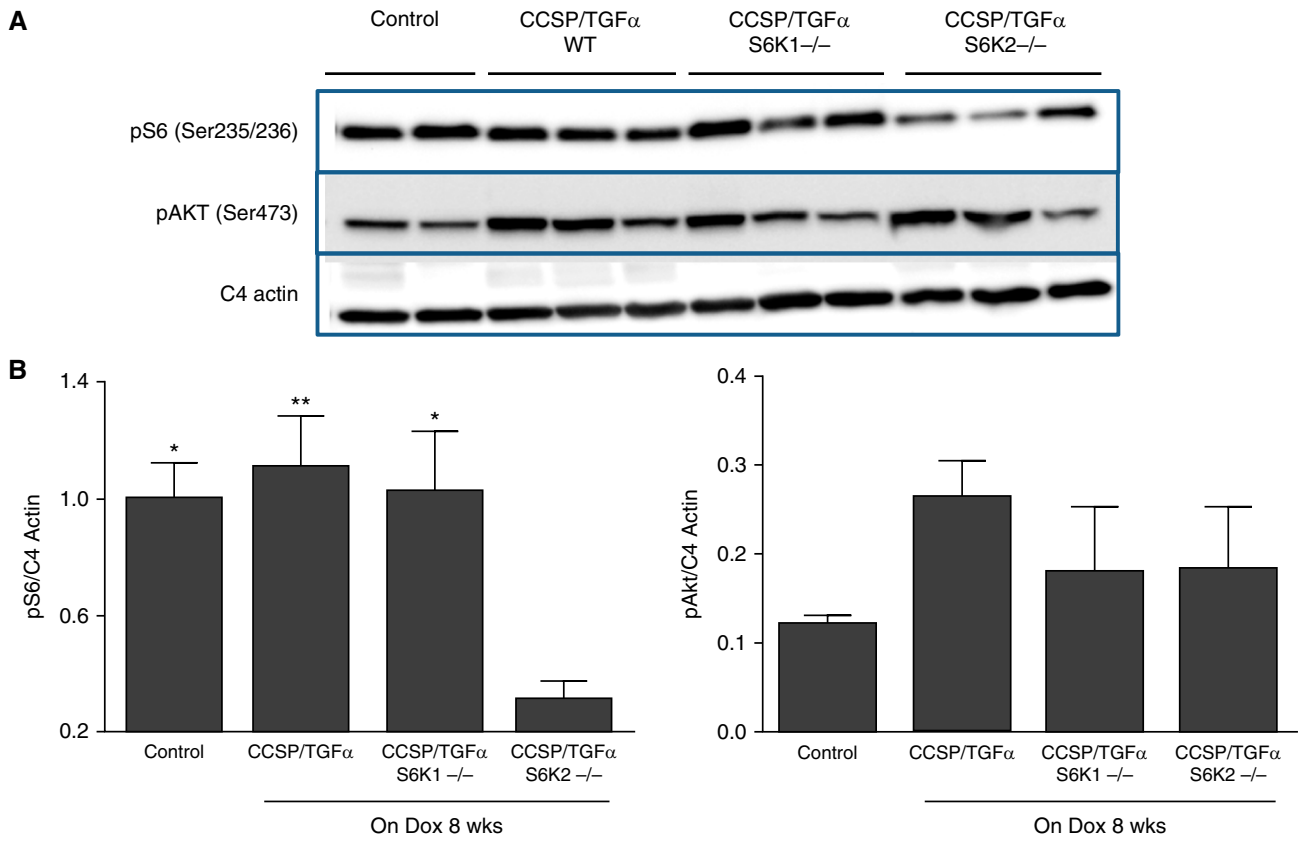

Figure 4. Deletion of S6K2, but not S6K1, reduces downstream S6 phosphorylation. CCSP/rtTA and tetO/TGF-α transgenes were bred into S6K1 or S6K2 KO mice and administered doxycycline (Dox) for 8 weeks to induce pulmonary fibrosis. (*A*) Western blot analysis of phosphorylated S6 and protein kinase B (AKT) from lung homogenates of mice. (*B*) Quantification of ratio of the intensity of phosphorylated S6 and Akt over C4 actin. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with controls by unpaired *t* test.

migration are not solely due to changes in proliferation. Because incubation of mesenchymal cells with the dual S6K1 and S6K2 inhibitor, LY2584702, prevented the increase in migration after TGF-α stimulation similar to that seen in the S6K1 KO cells, we also examined F-actin polymerization, which is a crucial step in the migration of cells. We detected reduced F-actin when cells were pretreated with LY2584702 before TGF-α administration. Together, these findings suggest changes in proliferation and alterations in actin polymerization are primary underlying mechanisms explaining S6K1-mediated alterations in migration.

Although we demonstrate a primary role for S6K1 in proliferation and migration and S6K2 for S6 phosphorylation, we did not detect differences in lung fibrosis parameters *in vivo* between S6K1- and S6K2-null mice when TGF-α was overexpressed. One explanation for this finding is that S6K2 may be the primary isoform mediating other cellular processes mediating fibrogenesis. A number of studies support a role for S6K2, but not S6K1, in cell survival (27, 34, 35). Overexpression of a kinase-active mutant for S6K2, but not S6K1, up-regulated the translation of antiapoptotic proteins, B-cell lymphoma-extra large and X-linked inhibitor of apoptosis protein, promoted baseline cell survival, and induced drug resistance in lung cancer cells. S6K2 is expressed in the majority of cancer samples investigated (88%), and overexpression of S6K2 was more common than that of S6K1 in most cancers (27, 34, 36–39). S6K2 expression is also linked to cancer progression, and increased expression of S6K2 correlates with drug resistance in small-cell lung cancer (34). Another explanation is that there is redundancy in fibrogenic process, and targeting just one of these processes, such as proliferation or migration, is not sufficient to adequately inhibit fibrosis progression after TGF-α/EGFR expression. Other alternative explanations include activation of nonkinase functions of p70S6K or isoform functions in nonmesenchymal cells. In sum, our findings support the need to target both p70S6K isoforms in subpleural fibrosis, and further investigations will be necessary to identify clearer mechanisms.

The progressive fibrotic lesions in the inducible TGF-α model develop primarily in the subpleural and adventitial compartments of the lung. Whereas KO of individual p70S6K isoforms attenuated the degree of pleural fibrosis, we did not detect any reduction in adventitial fibrosis by histology. This finding suggests either a complete redundancy in the S6K isoforms in mediating adventitial fibrosis in the TGF-α transgenic model, or, rather, that the adventitial mesenchyme does not require p70S6K signaling to expand. The latter hypothesis is supported by our previous study, in which we demonstrated that mesenchymal cells in the subpleural region exhibited strong immunostaining for phosphorylated S6K compared with only rare immunostaining in the adventitia (3). Previous reports describe lung fibroblast

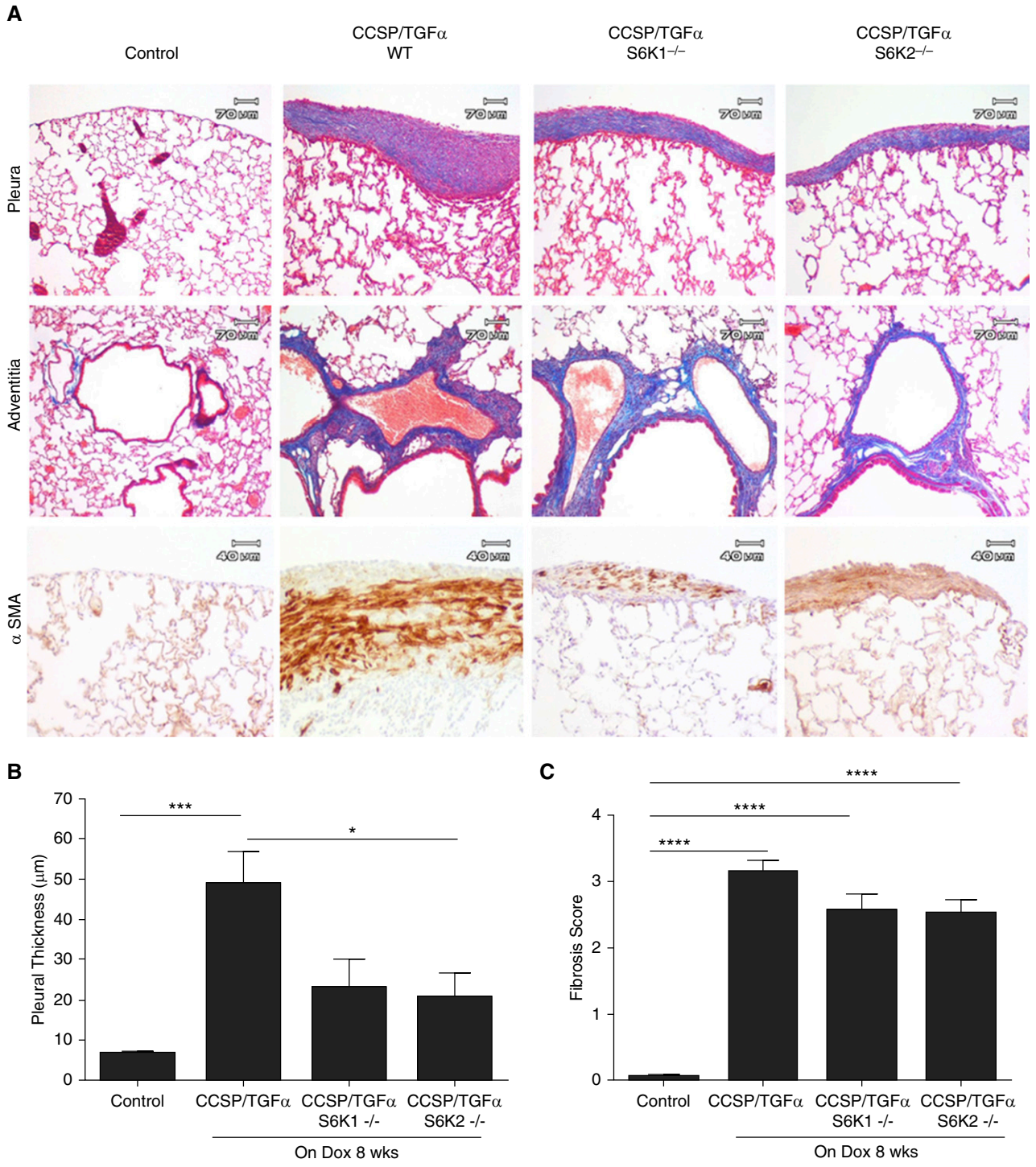

Figure 5. Deletion of S6K1 or S6K2 attenuates TGF-α–induced subpleural fibrosis. CCSP/rtTA and tetO/TGF-α transgenes were bred into S6K1 or S6K2 KO mice and administered Dox for 8 weeks to induce pulmonary fibrosis. Representative photomicrographs of lung tissues stained with Masson trichrome staining and α-smooth muscle actin (αSMA) comparing no-Dox controls to CCSP/TGF-α wild-type mice and CCSP/TGF-α mice with either the S6K1 or S6K2 KO (*A*). *Upper panels* center on the subpleural regions of the lung (*scale bars*: 70 μm), *middle panels* on the peribronchial and perivascular areas (*scale bars*: 70 μm), and *lower panels* on higher-power αSMA staining of the subpleural region (*scale bars*: 40 μm). Morphometric quantification of the subpleural thickness measured from lung sections demonstrates that increased subpleural thickness was significantly reduced in CCSP/TGF-α/S6K1$^{+/+}$/S6K2$^{-/-}$ (CCSP/TGF-α/S6K2$^{-/-}$) mice and attenuated in CCSP/TGF-α/S6K1$^{-/-}$/S6K2$^{+/+}$ (CCSP/TGF-α/S6K1$^{-/-}$) mice compared with CCSP/TGF-α wild-type mice (*B*). The lung fibrosis score was significantly increased in CCSP/TGF-α wild-type mice compared with controls but was unchanged between CCSP/TGF-α wild-type and CCSP/TGF-α/S6K1$^{-/-}$ or CCSP/TGF-α/S6K2$^{-/-}$ mice (*C*). Data are means (±SEM) ($n = 16$ mice in controls, 24 in CCSP/TGF-α wild type, 11 in CCSP/TGF-α S6K1$^{-/-}$, and 15 in CCSP/TGF-α S6K2$^{-/-}$). Statistical analysis was performed using ANOVA. *$P < 0.05$; ***$P < 0.001$; ****$P < 0.0001$.

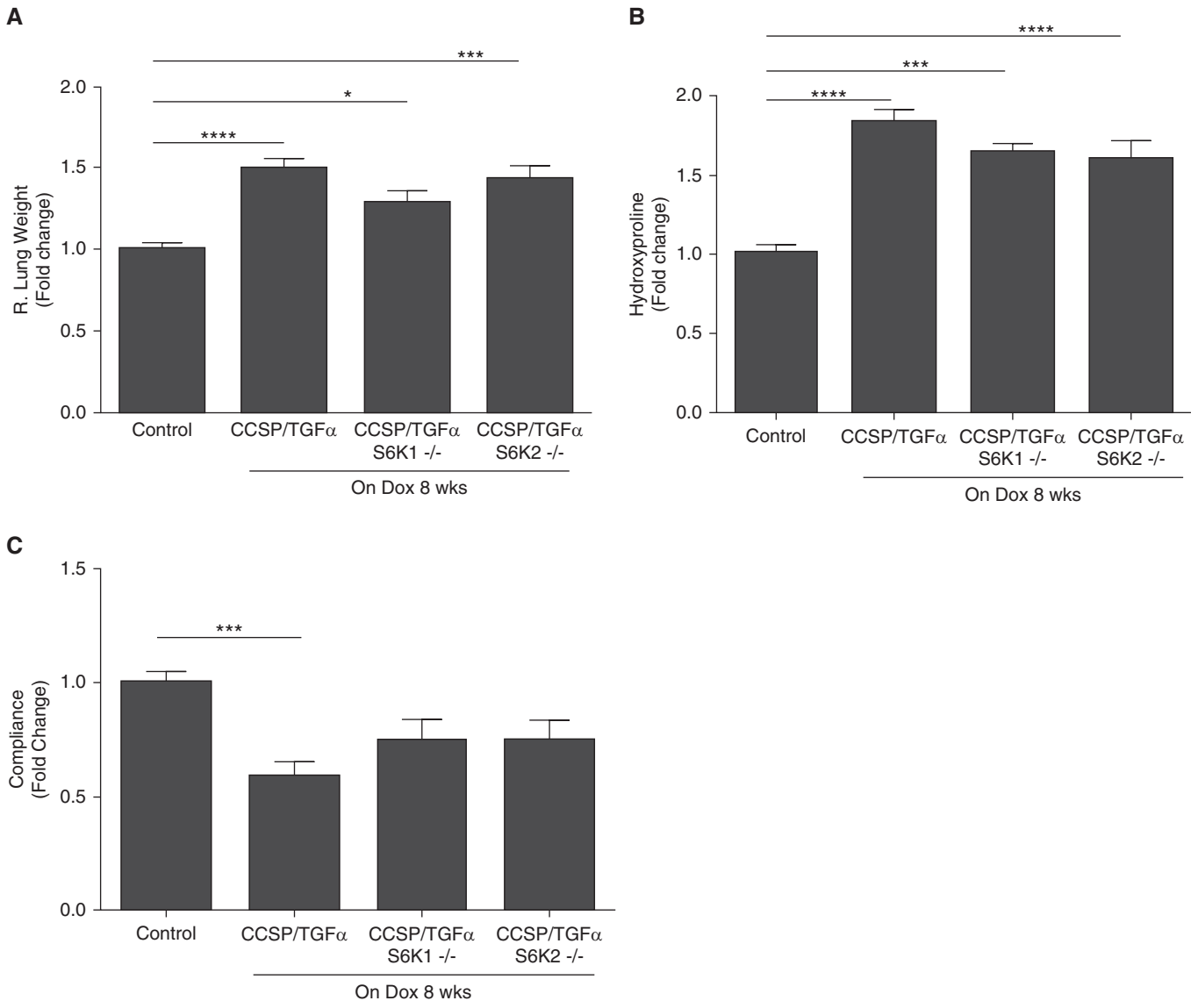

Figure 6. Deletion of neither S6K1 nor S6K2 alters changes in lung weight, lung hydroxyproline, or lung compliance after TGF-α overexpression. CCSP/rtTA and tetO/TGF-α transgenes were bred into S6K1 or S6K2 KO mice and administered Dox for 8 weeks to induce pulmonary fibrosis. Alterations in total right lung weight (*A*), lung hydroxyproline content (*B*), and lung compliance (*C*) induced by TGF-α overexpression were not significantly altered in CCSP/TGF-α S6K1$^{-/-}$ or CCSP/TGF-α S6K2$^{-/-}$mice. Data are means (±SEM) ($n = 16$ mice in controls, 24 in CCSP/TGF-α wild type, 11 in CCSP/TGF-α S6K1$^{-/-}$, and 15 in CCSP/TGF-α S6K2$^{-/-}$). Statistical analysis was performed using ANOVA. *$P < 0.05$, ***$P < 0.0005$, ****$P < 0.00005$.

heterogeneity with regard to changes in morphology, proliferation, surface marker expression, collagen type and production, intracellular metabolic pathways, and cytokine production after various stimuli (40–42). Fibroblastic foci in IPF represent an interconnected matrix that is comprised of polyclonal fibroblasts (43). Our data further support the premise that persistent local pathologic stimuli selectively activate fibroblast subpopulations through both unique and overlapping cell signaling pathways that ultimately contribute to fibroproliferation in lung compartments. In the current study, overexpression of TGF-α caused the expansion of mesenchymal cells in both subpleural and adventitial regions, but likely through distinctive signaling pathways, with subpleural lesions demonstrating sensitivity to inhibition of the p70S6K pathway. Together, these findings further support the heterogeneity of fibrotic lesions in the lung.

Pleural fibrosis is a significant contributor to morbidity and mortality in several disease processes including asbestos-associated diffuse pleural thickening, pleural infection, and drug reactions (44). Subpleural-predominant interstitial fibrosis is found in IPF, and is exclusive in pleuroparenchymal fibroelastosis, where there is an abrupt transition from subpleural fibrosis to normal lung parenchyma (45, 46). The mechanisms and signaling pathways mediating pleural fibrosis in human disease remain unknown, and future studies identifying up-regulated signaling

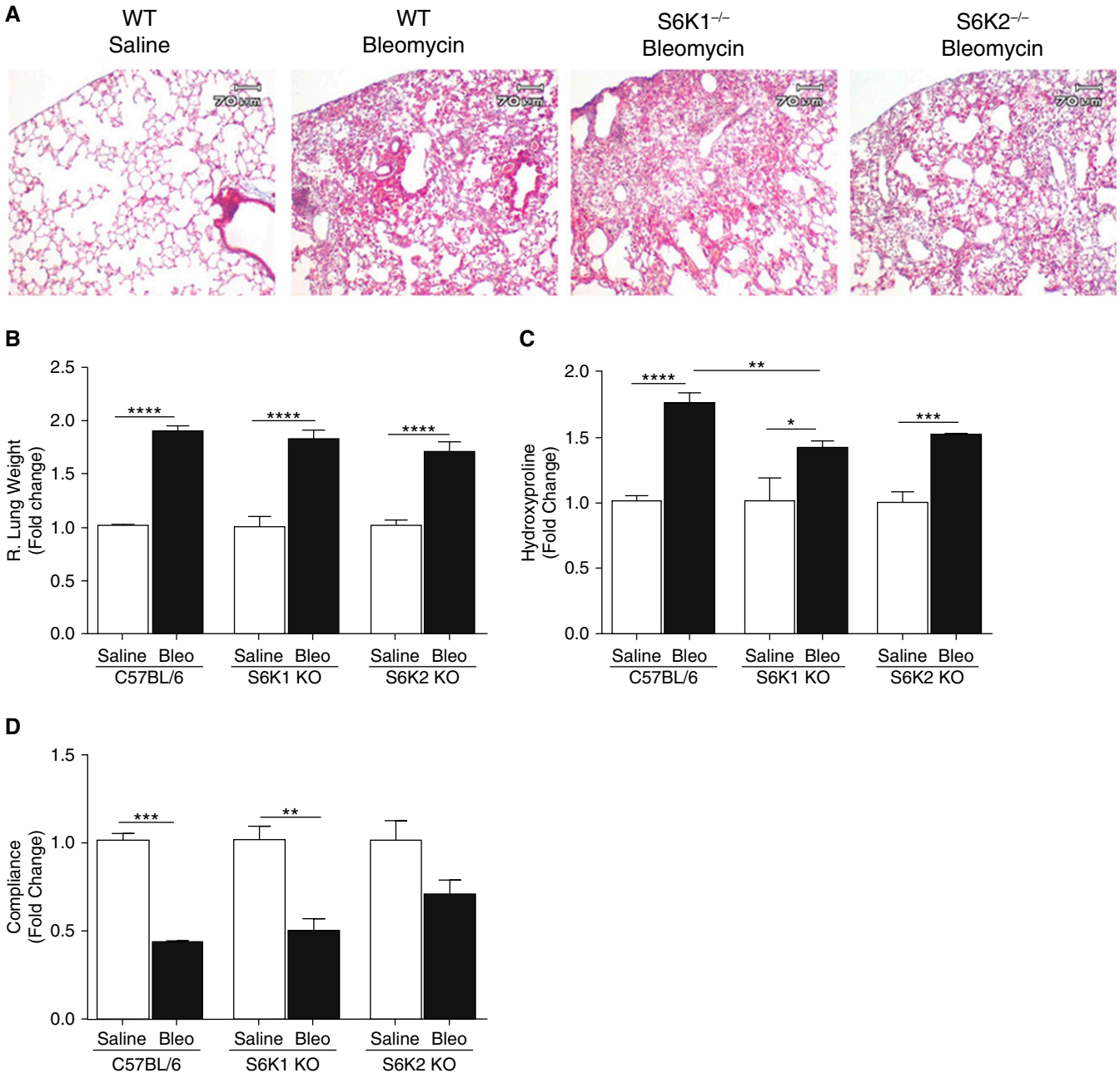

Figure 7. Deletion of neither S6K1 nor S6K2 alters changes in lung weight, lung hydroxyproline, or lung compliance after bleomycin (Bleo) injury. Wild-type and S6K1$^{-/-}$ and S6K2$^{-/-}$ mice were administered daily intradermal bleomycin or saline for 4 weeks. Bleomycin injury induced diffuse injury and inflammation in all groups of mice compared with saline controls, as demonstrated by low-power trichrome staining of lung tissue sections (*A*). Alterations in total right lung weight (*B*), lung hydroxyproline content (*C*), and lung compliance (*D*) induced by bleomycin injury versus saline were compared between wild-type and S6K1$^{-/-}$ and S6K2$^{-/-}$ mice. Data are means (±SEM) ($n = 7$–8 mice in each group). Statistical analysis was performed using ANOVA. *Scale bars*: 70 μm. *$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, ****$P < 0.00005$.

pathways in the advancing pleural lesions could lead to novel treatment strategies. Our previous studies examining the progression of subpleural fibrosis in the TGF-α transgenic model confirmed that selective pharmacologic inhibition of the mTORC1, mitogen-activated protein kinase (MAPK), or PI3K pathways reversed or prevented the progression of the subpleural lesions (14, 15, 22, 23). Pharmacologically targeting the p70S6K is a logical target, as it may represent a crucial converging downstream pathway of these known fibroproliferative signaling pathways that regulate subpleural fibroproliferative disease. The findings from this study and our previous work indicate that both S6K1 and S6K2 must be targeted to prevent subpleural fibrosis progression, likely from both overlapping and unique cellular properties controlled

by the isoforms. A number of p70S6K inhibitors are currently in preclinical development or in clinical trials for oncology, with some of these inhibitors only targeting the S6K1 isoform (16). Our data indicate that future pharmacologic interventions targeting p70S6K-mediatied subpleural pulmonary fibroproliferation must include targeting both isoforms.

Several cell signaling pathways have been identified in human pulmonary fibrotic disease; however, the role of these individual pathways in mediating progression is likely heterogeneous between and within fibrotic disorders, and may change after treatment with cell signaling inhibitors (1). As selective cell signaling pathway inhibitors continue to emerge for clinical use, identifying specific and feasible biomarkers of fibrogenic signaling pathways is an important future research direction that will be important not only to understand the role of these pathways in lung fibrosis progression, but to effectively target their activation for individual patients. ■

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

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