RESEARCH REPORT

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Inactivation of sestrin 2 induces TGF-β signaling and partially rescues pulmonary emphysema in a mouse model of COPD

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SUMMARY

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide. Cigarette smoking has been identified as one of the major risk factors and several predisposing genetic factors have been implicated in the pathogenesis of COPD, including a single nucleotide polymorphism (SNP) in the latent transforming growth factor (TGF)-β binding protein 4 (Ltbp4)-encoding gene. Consistent with this finding, mice with a null mutation of the short splice variant of Ltbp4 (Ltbp4S) develop pulmonary emphysema that is reminiscent of COPD. Here, we report that the mutational inactivation of the antioxidant protein sestrin 2 (sesn2) partially rescues the emphysema phenotype of Ltbp4S mice and is associated with activation of the TGF-β and mammalian target of rapamycin (mTOR) signal transduction pathways. The results suggest that sesn2 could be clinically relevant to patients with COPD who might benefit from antagonists of sestrin function.

INTRODUCTION

Transforming growth factor-βs (TGF-βs) belong to a protein superfamily whose members control cell growth and differentiation in a variety of tissues, and are involved in a wide range of immune and inflammatory responses. Most cells secrete TGF-β as a large latent complex containing one of the three latent TGF-β binding proteins (Ltbp1, Ltbp3 and Ltbp4). The Ltbps belong to the large protein family of fibrillins and, like the fibrillins, are an integral part of the extracellular matrix (ECM). By targeting TGF-β to the ECM, Ltbps create local deposits of TGF-β that can be rapidly mobilized in a tissue-specific manner (reviewed in Rifkin, 2005). We have previously shown that the mutational inactivation of the short splice variant of Ltbp4 (Ltbp4S) causes pulmonary emphysema in transgenic mice (Ltbp4S−/−), which is associated with defective TGF-β signaling (Sterner-Kock et al., 2002). However, the abnormalities in Ltbp4S−/− mice are also associated with defective elastic fiber structure, an anomaly that may or may not be TGF-β dependent. Although TGF-β controls ECM molecule expression, including elastin, the structural resemblance of Ltbp4 to the fibrillins suggests that certain phenotypes of Ltbp4S−/− mice may be TGF-β independent.

To distinguish between the TGF-β-dependent and the TGF-β-independent phenotypes induced by the Ltbp4S mutation, we crossed Ltbp4S−/− mice to mice that were originally assumed to carry a gene trap mutation in the *Smad7* gene. Since smad7 inhibits TGF-β signaling (reviewed in Massague et al., 2005), its inactivation in Ltbp4S−/− mice was expected to selectively rescue TGF-β-dependent phenotypes. Although early results in double mutant offspring supported this assumption, a more detailed genotyping protocol revealed that the gene trap insertion was not in the *Smad7* gene but in the gene encoding sestrin 2 (sesn2).

Sestrin 2 belongs to a family of highly conserved antioxidant proteins that were initially discovered as p53-inducible proteins. Mammalian cells express three isoforms referred to as sestrin 1 (sesn1; also known as PA26), sestrin 2 (sesn2; also known as Hi95) and sestrin 3 (sesn3) (Budanov et al., 2004; Velasco-Miguel et al., 1999). Sesn2 regulates intracellular peroxide [reactive oxygen species (ROS)] levels, presumably by regenerating over-oxidized peroxiredoxins (Prdx) that cannot be reduced by typical cellular reductants such as thioredoxin or glutathione (Budanov et al., 2004). Besides protecting against oxidative stress in mammalian cells, Prdxs are thought to be involved in cell signaling by controlling the local availability of second messenger ROS (reviewed in Rhee et al., 2005; Wood et al., 2003).

In addition to their catalytic function, sesn1 and sesn2 were recently reported to negatively regulate the activity of mammalian target of rapamycin (mTOR) by a redox-independent mechanism (Budanov and Karin, 2008).

mTOR is a serine/threonine protein kinase found in two distinct multiprotein complexes: mTORC1 and mTORC2. mTORC1 primarily regulates translation and is susceptible to inhibition by the antibiotic rapamycin. The best-characterized downstream effectors of mTOR within the rapamycin-sensitive mTORC1 complex are the ribosomal protein S6 kinase (S6K) and the eukaryotic initiation factor 4E-binding protein 1 (4E-BP1). Phosphorylation of S6K by mTOR enhances translation by phosphorylating the S6 ribosomal protein (rbS6) within the ribosomal translation initiation complex. Phosphorylation of 4E-BP1 by mTOR enhances cap-dependent mRNA translation (reviewed in Wullschleger et al., 2006). mTORC1 is tightly

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controlled by multiple layers of upstream regulators (reviewed in Corradetti and Guan, 2006), including sesn2 and TGF-β. More specifically, TGF-β and sesn2 have been recently identified as positive and negative regulators of mTORC1, respectively (Budanov and Karin, 2008; Lamouille and Derynck, 2007). Both have a direct impact on the activity of the hamartin (TSC1)/tuberin (TSC2) protein complex [also known as the tuberous sclerosis complex (TSC1:TSC2)], which is a strong suppressor of mTOR. TGF-β inactivates this complex through Akt/protein kinase B (PKB)-mediated site-specific phosphorylation (Lamouille and Derynck, 2007). By contrast, sestrin activates the TSC1:TSC2 repressor complex by recruiting the AMP-dependent kinase (AMPK) to TSC2 (Budanov and Karin, 2008).

Here, we show that the mutational inactivation of sesn2 in Ltbp4S knockout (KO) mice results in a dramatic recovery of the pulmonary emphysema phenotype that is associated with an induction of the TGF-β and mTOR signal transduction pathways.

RESULTS

Characterization of sesn2 knockout mice

Sesn2 knockout mice (sesn2–/–) were derived from an embryonic stem (ES) cell clone with a pT1βgeo gene trap insertion in the last intron of the *Sesn2* gene (supplementary material Fig. S1) (Hansen et al., 2003; Wiles et al., 2000).

Since sesn2–/– mice developed normally after birth and were indistinguishable from their wild-type and heterozygous littermates until 2 years of age, we established mouse lung fibroblasts (MLFs) in culture to test whether these cells would replicate the previously described sesn2 knockdown phenotype that was induced in cultured cells by RNA interference (RNAi) (Budanov et al., 2004). Consistent with that phenotype, the sesn2–/– MLFs (–/– MLFs) accumulated increased levels of ROS after exposure to hydrogen peroxide (H_2O_2) or antimycin (supplementary material Fig. S2A), an antibiotic that enhances mitochondrial ROS production (Dröse and Brandt, 2008). Similar to the sesn2 knockdown cells, the –/– MLFs proliferated more slowly and exhibited a higher rate of apoptosis than the wild-type MLFs (supplementary material Fig. S2B,C). Moreover, in line with the more recent data implicating sesn2 in the regulation of mTORC1 (Budanov and Karin, 2008), the –/– MLFs expressed increased levels of phosphorylated S6K (P-S6K) (supplementary material Fig. S2D).

Overall, the phenotype of the –/– MLFs suggests that the pT1βgeo gene trap insertion had induced a null mutation.

Sesn2 null alleles partially rescue the pulmonary emphysema of Ltbp4S–/– mice

We and others have previously reported that mice with an inactivating mutation of the small splice variant of the *Ltbp4* gene (Ltbp4S–/–) are born with alveolar septation defects that deteriorate with age (Dabovic et al., 2009; Koli et al., 2004; Sterner-Kock et al., 2002). By the age of 4-5 months, Ltbp4S–/– lungs develop symptoms reminiscent of the centrilobular emphysema that is associated with the late stages of chronic obstructive pulmonary disease (COPD) in humans (Snider et al., 1985). Accordingly, Ltbp4S–/– lungs display a massive enlargement of airspaces distal to the terminal bronchioles accompanied by the destruction of the septal walls without obvious fibrosis (Fig. 1A, upper middle panel).

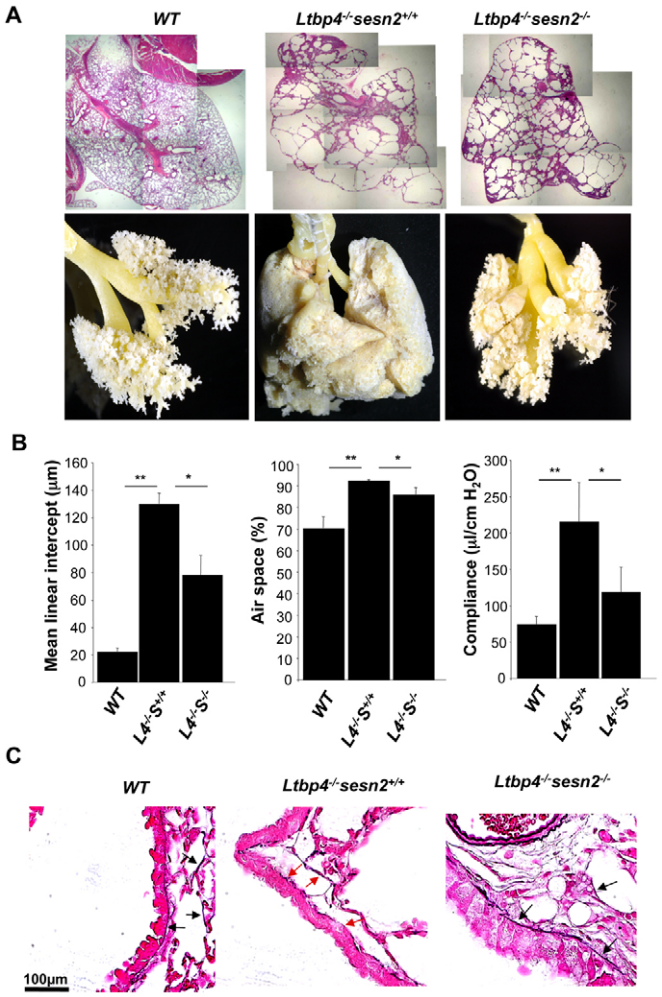

Fig. 1. Pulmonary emphysema rescue in Ltbp4S–/–sesn2–/– mice. (A) Upper panels: hematoxylin and eosin (H&E)-stained lung sections photographed at ×2.5 magnification. Between six and eight photographs were taken from each section and assembled to cover the entire section. Lower panels: tracheobronchial silicone casts of 5-month-old wild-type, single mutant (Ltbp4S–/–) and double mutant (Ltbp4S–/–sesn2–/–) mice (see Methods). The casts shown are representative for three sets of littermates. (B) Mean linear intercept, total airspace and dynamic lung compliance of 5-month-old wild-type, single mutant (Ltbp4S–/–) and double mutant (Ltbp4S–/–sesn2–/–) mice (see Methods). The results are the means ± S.D. of three or four mice per genotype. (C) Elastic fibers in wild-type, single mutant (Ltbp4S–/–) and double mutant (Ltbp4S–/–sesn2–/–) lungs photographed at ×40 magnification. The black and red arrows indicate intact and fragmented fibers, respectively. Statistical significance values (*) were obtained by a Bonferroni multiple comparison test. *P<0.05, **P<0.001.

To assess the damage in the bronchial compartment of the Ltbp4S–/– lungs, we used plastination to visualize the tracheobronchial tree. Plastination is an anatomical method that involves the instillation of a silicone polymer into the trachea of isolated lungs. By diffusing through bronchial ramifications towards the terminal bronchioles, the solidified polymer accurately reflects the three-dimensional architecture of the tracheobronchial tree (Perry et al., 2000). As shown in Fig. 1A, the normal vast and delicate

arborization of a tracheobronchial tree (lower left panel) was profoundly altered in the Ltbp4S–/– lungs, where the vast majority of the airway ramifications are replaced by excessively enlarged airspaces (middle panels).

This lung pathology recovered considerably in Ltbp4S–/– mice harboring two *Sesn2* null alleles (Ltbp4S–/–sesn2–/–). As shown in Fig. 1A, the tracheobronchial architecture of the double mutant Ltbp4S–/–sesn2–/– mice appeared nearly normal (lower right panel) and the parenchymal lesions were less severe than in the single mutant Ltbp4S–/– lungs (upper right panel). The recovery of the parenchymal lesions included a decrease in alveolar size and total lung enlargement (Fig. 1A, upper right panel), which was confirmed by quantitative alveolar morphometry. Fig. 1B shows that the increased mean linear intercept and airspace of the Ltbp4S–/– lungs were reduced significantly in the double mutant lungs. Consistent with this structural improvement, the compliance of Ltbp4S–/–sesn2–/– lungs was ~40% below the value obtained from the Ltbp4S–/– lungs, indicating a functionally meaningful recovery of pulmonary elasticity (Fig. 1B, right panel). This recovery was associated with a reduced elastic fiber fragmentation in both airways and alveolar walls (Fig. 1C).

TGF-β signaling is induced in mice harboring *Sesn2* null alleles

Several previous studies, in addition to our own, have shown that genetically modified mouse models with impaired TGF-β signaling develop pulmonary emphysema (Chen et al., 2005; Morris et al., 2003; Sterner-Kock et al., 2002). A similar correlation between TGF-β signaling and emphysema was observed recently in patients with COPD. More specifically, when compared with age-matched healthy individuals, phosphorylated smad2 protein (P-smad2), which is indicative of TGF-β signaling, was reduced in the lungs of patients with advanced COPD (Leppäranta et al., 2009). Therefore, we hypothesized that the emphysema rescue phenotype induced by the *Sesn2* null alleles in the Ltbp4S knockout mice may be associated with improved TGF-β signaling.

To test this, we quantified the P-smad2-positive cells in the lungs of wild-type (WT), Ltbp4S–/– and Ltbp4S–/–sesn2–/– mice by immunohistochemistry. Because the levels of P-smad2 that were expressed by the bronchial walls of normal and mutant mice were about three times higher than the levels expressed by the septal walls, we assessed the two compartments separately (Fig. 2A, upper and middle panels). As expected from previous data (Sterner-Kock et al., 2002), the numbers of P-smad2-positive cells in the Ltbp4S–/– lungs were below normal, particularly in the septal walls (Fig. 2A, lower left panel). The bronchial walls also contained fewer P-smad2-positive cells, but the difference compared with the wild-type lungs was not statistically significant (Fig. 2A, lower right panel). Fig. 2A (lower left panel) shows that the number of P-smad2-positive cells returned to normal in the septal walls of the double mutant lungs, suggesting a compartment-restricted reactivation of TGF-β signaling in these lungs.

To test whether the enhanced TGF-β signaling in the double mutant lungs was associated with the upregulation of TGF-β target genes that are implicated in lung remodeling and regeneration, we quantified the expression of alpha smooth muscle actin (α-SMA), collagen type I, III and IV, and tropoelastin I in WT, Ltbp4S–/– and Ltbp4S–/–sesn2–/– lungs by quantitative reverse transcription PCR (qRT-PCR). Similar to P-smad2, the expression of α-SMA and collagen type IV was repressed and derepressed in Ltbp4S–/– and Ltbp4S–/–sesn2–/– lungs, respectively (Fig. 2B). However, tropoelastin, although strongly induced in the double mutant lungs, was already slightly induced in the single mutant lungs, suggesting that its regulation may also involve TGF-β-independent factors (Fig. 2B). Surprisingly, profibrotic collagen type I and III expression was not affected by either the Ltbp4S or the sesn2 mutations. Accordingly, transcript levels of collagen type I and III were similar in all lungs regardless of their genotype (data not shown). In line with previous observations, these results imply that

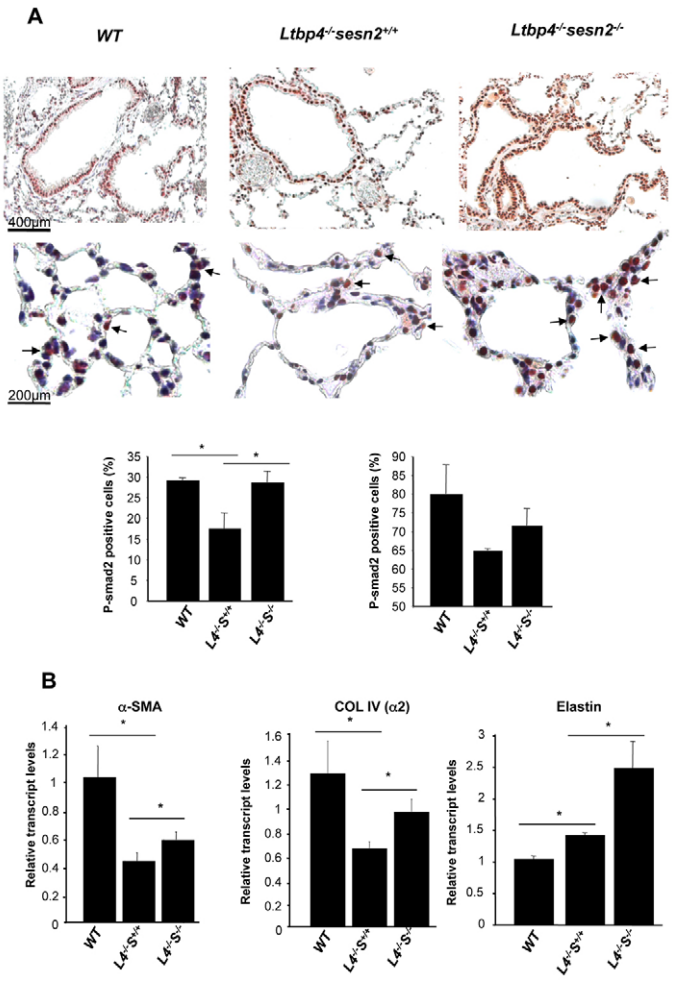

Fig. 2. Activation of TGF-β signaling in Ltbp4S–/–sesn2–/– lungs. (A) P-smad2 levels in the lungs of 5-month-old wild-type, single mutant (Ltbp4S–/–) and double mutant (Ltbp4S–/–sesn2–/–) mice. Upper and middle panels: representative lung sections stained with a monoclonal anti-P-smad2 antibody and photographed at ×10 (upper panels) and ×20 (middle panels) magnifications. P-smad2-positive cells (arrows) have brown nuclei. Lower panels: quantification of P-smad2-positive cells in septal (left) and airway (right) walls. Differential counts of P-smad2-positive cells were performed on lung sections derived from three or four mice per genotype. All cells within an area of 1 mm^2 were counted at ×20 magnification, from at least four randomly chosen sections per lung, using an eyepiece with a built-in grid. *P<0.05. (B) Quantification of TGF-β target gene expression by qRT-PCR. The results are the means ± S.D. from two or three lungs per genotype. Statistical significance values (*) were obtained by a Bonferroni multiple comparison test. *P<0.05.

TGF-β target genes can be selectively affected by the Ltbp4S mutation (Dabovic et al., 2009). Moreover, the regional P-smad2 activation pattern (Fig. 2A) suggests that the induction of TGF-β target genes is cell-context dependent and thus precludes its detection by qRT-PCR.

Sesn2 inactivation enhances TGF-β signaling in mouse lung fibroblasts (MLFs)

To directly test whether sesn2 affects TGF-β signaling, we first investigated the levels of phosphorylated smad2/3 proteins in wild-type and –/– MLFs. Consistent with TGF-β activation in –/– MLFs, P-smad2 and P-smad3 were elevated above normal levels by two- to threefold (Fig. 3A). To further validate this observation, we analyzed the expression of several TGF-β target genes, including those encoding α-SMA, connective tissue growth factor (CTGF) and matrix metalloprotease-2 (MMP-2). All three proteins were induced in –/– MLFs (Fig. 3A,B) and, like P-smad2/3, could be readily reversed to wild-type levels by overexpressing sesn2 (Fig. 3A), indicating that their elevation is related to the sesn2 mutation. The TGF-β pathway activation was further supported by the upregulation of TGF-β-responsive luciferase reporter plasmids expressed in the –/– MLFs. Thus, luciferase expression from the P-smad3-responsive $(CAGA)_{12}$-luc reporter plasmid (Dennler et al., 1998), transiently transfected into –/– MLFs, exceeded the luciferase expression in similarly treated wild-type MLFs by eightfold (Fig. 3C, left panel). Significant, but less prominent, luciferase induction was also observed in –/– MLFs transfected with the P-smad2/Fast-1 responsive ARE-luc reporter plasmid (Fig. 3C, right panel) (Yakymovych et al., 2001). As was the case with the TGF-β-induced proteins, stable overexpression of sesn2 in the –/– MLFs reversed the activation of the luciferase reporter plasmids (Fig. 3C). Overall, the results suggest that the loss of sesn2 function induces TGF-β signaling in mouse lung fibroblasts.

The increased TGF-β signaling in sesn2–/– MLFs is ROS independent

Previous studies showed that ROS interfere with TGF-β activation at different levels of the signaling cascade. For example, ROS have been implicated in (1) the activation of latent TGF-β in vitro and in vivo (reviewed in Annes et al., 2003); (2) the phosphorylation of smad2/3 proteins (Cucoranu et al., 2005); and (3) the transactivation of various of target genes such as those encoding CTGF and plasminogen activator inhibitor-1 (Jiang et al., 2003; Park et al., 2001).

To test whether the excess intracellular ROS in –/– MLFs (supplementary material Fig. S2A) are responsible for TGF-β activation, we treated the –/– MLFs with the antioxidant N-acetyl-cysteine (NAC). As shown in supplementary material Fig. S3A, NAC reduced the P-smad2/3 levels in a dose-dependent manner. Conversely, and consistent with a previous report (Cucoranu et al., 2005), addition of H_2O_2 to the MLFs induced smad2 phosphorylation (data not shown). However, an equivalent reduction of ROS by the alternative antioxidants ebselen or trolox failed to replicate the NAC effect (supplementary material Fig. S3B and data not shown), suggesting that the reduction of P-smad2/3 by NAC is caused primarily by its previously described ability to reduce the biologically active TGF-β dimer to a biologically inactive monomer (Lichtenberger et al., 2006; Meurer et al., 2005). Although the mechanisms responsible for the enhanced TGF-β signaling in –/– MLFs remain to be established, these results support the conclusion that the sesn2 effect on TGF-β is most likely to be ROS independent.

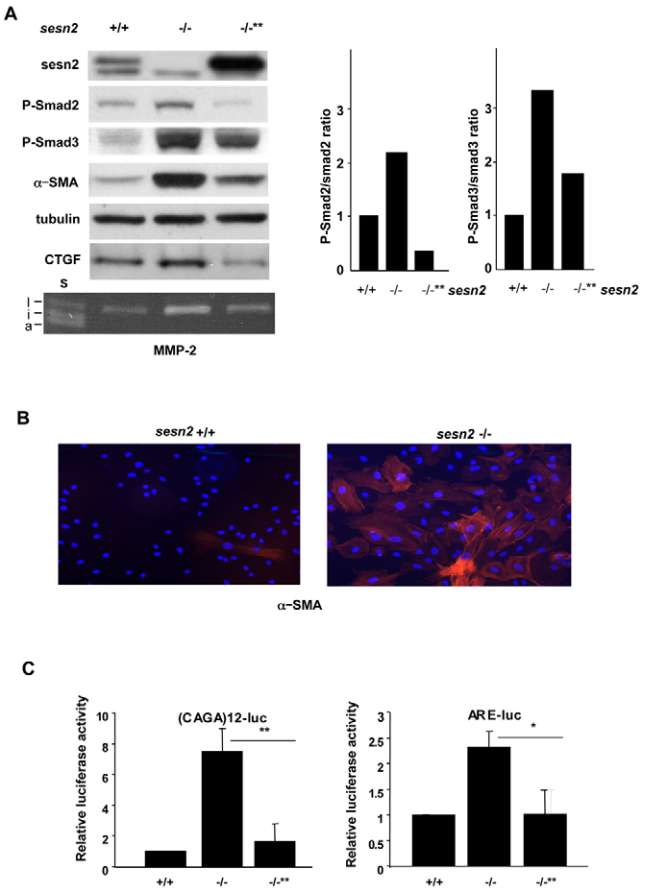

Fig. 3. TGF-β signaling in MLFs. (A) Increased smad2/3 phosphorylation and induction of TGF-β target genes in sesn2–/– MLFs. Phosphorylated (P-) smad2/3, sesn2 and α-SMA expression were estimated in cell lysates by western blotting. P-smad2/smad2 and P-smad-3/smad3 ratios were estimated by densitometry. The relative intensity of the bands was evaluated on dedicated western blots using the Quantity One 4.2.1 (Bio-Rad) software. The ratios of the intensity of P-smad versus smad samples were normalized to the ratio calculated for the WT samples. CTGF and MMP-2 levels were estimated in concentrated serum-free conditioned media by western blotting and MMP-2 gelatin zymography, respectively. The MMP-2 standard (S) shows the migration of latent (l), inactive (i) and active (a) forms of MMP-2. –/–, sesn2–/– MLFs; –/–**, sesn2-overexpressing –/– MLFs. (B) α-SMA protein (red) levels were estimated by immunofluorescence staining of cultured MLFs. (C) Activation of TGF-β-responsive reporter plasmids in –/– MLFs. MLFs were transiently transfected with the P-smad3-specific $(CAGA)_{12}$-luc or the P-smad2-specific ARE-luc reporter plasmids. The results are expressed as the mean relative luciferase activitiy ± S.D. (wild-type MLFs=1) from three independent experiments. Statistical significance values (*) were obtained by Student's *t*-test. $^{**}P<0.002$; $^{*}P<0.015$.

The induction of mTOR by the sesn2 mutation is dependent on TGF-β signaling

Fig. 4A shows that the phosphorylated S6K (P-S6K) levels were significantly above normal in the –/– MLFs and could be readily

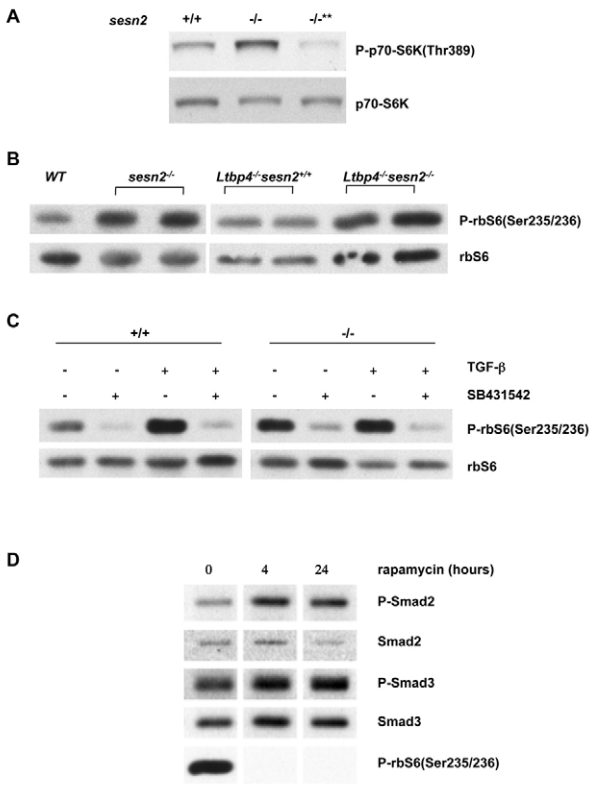

Fig. 4. Induction of the mTOR pathway by *Sesn2* null alleles. (A) P-S6K and S6K protein expression in MLFs estimated by western blotting. –/–, sesn2–/– MLFs; –/–**, sesn2-overexpressing –/– MLFs. (B) P-rbS6 and rbS6 protein levels in whole lung tissue lysates estimated by western blotting. (C) P-rbS6 levels in serum-starved MLFs exposed to 1 ng/ml of recombinant TGF-β1±10 μM of SB431542 inhibitor, analyzed by western blotting. (D) P-rbS6 levels in serum-starved –/– MLFs treated with 1 μM of rapamycin.

reversed by overexpressing sesn2, thus confirming previous data describing sesn2 as a repressor of mTOR (Budanov and Karin, 2008). Moreover, mTOR activation was also evident in vivo; the lungs of mice with *Sesn2* null alleles expressed about three times as much P-rbS6 compared with their wild-type littermates (Fig. 4B).

To test whether the induction of mTOR by the sens2 mutation involves the TGF-β pathway, we used the small molecule inhibitor SB431542 to block the TGF-β type I receptor kinase (TβR1). TβR1 initiates TGF-β signaling after binding to the TGF-β type II receptor (TβR2)/TGF-β ligand complex. Fig. 4C shows that SB431542 completely abrogated basal and TGF-β1-stimulated P-rbS6 phosphorylation in both WT and –/– MLFs, suggesting that TGF-β signaling is required for mTOR pathway activation in these cells.

Interestingly, the inhibition of mTORC1 in the –/– MLFs by rapamycin induced smad2 and, to a lesser extent, smad3 phosphorylation, which is consistent with a previously described negative feedback loop between the TGF-β and mTOR pathways (Fig. 4D) (Song et al., 2006).

DISCUSSION

Mice with a loss-of-function mutation of the short splice variant of the *Ltbp4* gene (Ltbp4S) develop severe pulmonary emphysema associated with impaired TGF-β signaling and defects in elastic fiber structure (Sterner-Kock et al., 2002). Similar to other mouse models with impaired TGF-β signaling, Ltbp4S–/– mice are born with alveolar septation defects that are the congenital antecedent of the COPD-like centrilobular emphysema developing in the adult (Chen et al., 2005; Dabovic et al., 2009; Morris et al., 2003). The present experiments showed that the inactivation of the antioxidant protein sesn2 partially rescues the emphysema phenotype in Ltbp4S–/– mice, thereby improving lung function. The emphysema rescue correlated with the upregulation of the TGF-β and mTOR pathways, both of which are involved in tissue remodeling and regeneration.

Cell culture experiments using MLFs with mutationally inactivated sesn2 identified sesn2 as an upstream repressor of TGF-β and mTOR signaling. Accordingly, sesn2–/– MLFs upregulated P-smad2/3 and P-S6K, which are the direct downstream targets of TβR1 and mTORC1, respectively. This upregulation could be blocked by the TβR1 inhibitor SB431542, but not by antioxidants, indicating that the effect of the sesn2 mutation on mTORC1 is TGF-β dependent. Further experiments will determine whether TGF-β signaling itself prevents the previously described ROS-independent, mTORC1-inhibitory association between sesn2, TSC1:TCS2 and AMPK (Budanov and Karin, 2008).

Activation of the TGF-β pathway in the Ltbp4S–/–sesn2–/– double mutant lungs upregulated the expression of several TGF-β target genes including that encoding tropoelastin. Because lack of elastin, or accelerated degradation of elastin, are common causes of emphysema (Wendel et al., 2000), we believe that the induction of tropoelastin by the sesn2 mutation is a crucial factor in the functional recovery of the Ltbp4S mutant lung. A second crucial factor is the lack of profibrotic gene induction despite increased TGF-β signaling. Although TGF-β has been directly implicated in the pathogenesis of tissue fibrosis (reviewed in Gauldie et al., 2006; Kisseleva and Brenner, 2008), its most well-characterized profibrotic target genes – those encoding collagen type I and III – were not affected by the sesn2 mutation. This finding seems initially surprising but may be explained by the high variability in TGF-β signal interpretation by the transcriptome, depending on signal strength, tissue type and cell context (reviewed in Feng and Derynck, 2005; Massague et al., 2005). Accordingly, only some of the TGF-β target genes were upregulated in the lungs from 7-day-old Ltbp4S knockout mice, which, unlike the adults, exhibit slightly increased TGF-β signaling (Dabovic et al., 2009). Thus, although the mechanisms of differential TGF-β target gene regulation in Ltbp4S–/–sesn2–/– lungs remain to be established, they are quite likely to be based on a fine tuning of TGF-β signals through cross signal transduction pathway feedback loops, similar to those described here between smad2/3 and mTORC1.

COPD is a leading cause of morbidity and mortality worldwide. Cigarette smoking has been identified as one of the major risk factors, and several predisposing genetic factors have been implicated in the pathogenesis of COPD. These include highly significant SNPs in the genes encoding TGF-β1 or Ltpb4 (Celedon et al., 2004; Hersh et al., 2006) that support the Ltbp4S knockout mouse as a factual animal model of COPD. The emphysema rescue achieved in this model through inactivation of sesn2 suggests that sesn2 could be clinically relevant to patients with COPD because such patients might benefit from antagonists of sestrin function.

METHODS

Antibodies, growth factors and reagents

The following antibodies used for western blotting were obtained from Cell Signaling/NEB: anti-P-smad2 (S465/467; cat. no. 3101), anti-P-smad3 (S423/425; cat. nos. 9514 and 9520), anti-smad3 (cat. no. 9523), anti-p-P70-S6K (T389; cat. no. 9206), anti-P70-S6K (cat. no. 9202), anti-p-rbS6 (S235/236; cat. no. 2211), anti-rbS6 (cat. no. 2217), and anti-caspase3-cleaved poly(ADP-ribose) polymerase (PARP) (cat. no. 9544). The anti-smad2 monoclonal antibody was purchased from Transduction Laboratories (cat. no. 66220). The anti-α-SMA (cat. no. CBL171) and anti-CTGF (cat. no. sc14939) antibodies were from Chemicon and Santa Cruz, respectively. The anti-sesn2 antibody was purchased from ProteinTech Group/PTG (cat. no. 10795-1-AP) and the anti-α-tubulin antibody was from Neomarkers (cat. no. MS-581-9). The anti-P-smad2 antibody (S465/467) (cat. no. AB3849) was purchased from Chemicon.

Recombinant TGF-β1 was obtained from R&D Systems (cat. no. AB-100-NA) and N-acetyl-cysteine (NAC) was obtained from Sigma (cat. no. A9165). Ebselen and trolox (cat. nos. CAY-70530-10 and CAY-10011659-250) were purchased from Biozol.

SB431542 and rapamycin were purchased from Sigma (cat. no. S4317) and Calbiochem (cat. no. 553210), respectively. Finally, carboxy-H_2DCFDA was from Molecular Probes/Invitrogen (cat. no. C400) and the H_2O_2 was from Roth (cat. no. 8072.2).

ES cell injections, breeding and genotyping

The W077E06 (TBV-2; 129SvPas) gene trap ES cell line obtained from the German Gene Trap Consortium (GGTC) was converted into mice by blastocyst injection, as described previously (Hansen et al., 2003). Mice heterozygous for the gene trap insertion were backcrossed to C57BL/6J mice for at least six generations. Genotyping was performed by genomic PCR using mouse tail DNA and primers that were complementary to the sequences flanking the gene trap insertion sites. All primers are available on request.

Plasmids

A full-length *Sesn2* cDNA was obtained from the Resource Center of the German National Genome network (RZPD) and cloned into the *Eco*RI/*Not*I site of the pEF-IRES-P vector (Hobbs et al., 1998). The $(CAGA)_{12}$-luc, ARE-luc and pCI-neo-Fast-1 plasmids were obtained from Peter ten Dijke (the Netherlands Cancer Institute, Amsterdam).

Cell cultures and transfections

Mouse lung fibroblasts (MLFs) were established in tissue culture, as described previously (Koli et al., 2004). Cells were grown in DMEM (Gibco) supplemented with 20% fetal calf serum (FCS) (Hyclone), 100 IU/ml penicillin and 50 μg/ml streptomycin.

Sesn2-overexpressing cells were obtained by transfecting sesn2–/– MLFs with the sesn2/pEF-IRES-puro expression plasmid and selecting in puromycin, as described previously (Koli et al., 2004).

The TGF-β activity reporter assays, including those using the $(CAGA)_{12}$-luc or ARE-luc/Fast-1 reporter constructs, were performed as described previously (Koli et al., 2004). Luciferase activities were measured after 24 hours using the dual luciferase kit (Promega) and the Digene DCR-1 luminometer (MGM Instruments), and normalized to the activity of co-transfected Renilla luciferase (Promega).

Preparation of conditioned media and gelatin zymography

MLF cultures were serum starved overnight and re-incubated with fresh serum-free medium for 24 hours. The harvested conditioned media (CM) were clarified by centrifugation and equalized according to cell number. Where indicated, CMs were concentrated tenfold using Microcon YM-10 centrifugal filters (Millipore).

Aliquots of MLF-conditioned media were run under nonreducing conditions in 10% SDS-polyacrylamide gels containing 1 mg/ml of gelatin (BioRad). The gels were washed twice with 50 mM Tris-HCl buffer, pH 7.6, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 2.5% Triton X-100 for 15 minutes to remove SDS, followed by a brief rinse in washing buffer without Triton X-100. Next, the gels were incubated at 37°C overnight in 50 mM Tris-HCl developing buffer, pH 7.6, containing 5 mM $CaCl_2$, 1 μM $ZnCl_2$ and 1% Triton X-100. The gels were then stained with Coomassie Blue for 2 hours and destained with a 10% methanol plus 10% acetic acid solution.

Determination of intracellular ROS

ROS levels were determined under serum-free conditions by exposing the cell cultures to 10 μM carboxy-H_2DCFDA at 37°C for 20 minutes. Cells were then trypsinized, washed once in PBS and subjected to flow cytometry using a FACScan machine (Becton Dickinson).

Nucleic acids and protein analyses

Total cellular RNA was isolated using an RNeasy mini kit (Qiagen) according to the manufacturer's instructions. Real-time reverse transcription (RT)-PCR analysis of gene expression was performed using SYBR Green chemistry (ABgene, Epsom, UK) and an Opticon 2 qPCR machine (MJ Research). cDNA was synthesized from total RNA using random priming and Superscript II (Invitrogen) reverse transcriptase. PCR reactions were run as triplicates on 96-well plates, with each reaction containing cDNA derived from 7.5-15 ng of total RNA, 1× Absolute SYBR fluorescein mix (ABGene) and 5 pmol of gene-specific primers in a total volume of 25 μl. All gene-specific primers are available on request. Reactions were normalized by simultaneously carrying out RT-PCR reactions for RNA pol II using the primers: 5′-ATG AGC TGG AAC GGG AAT TTG A-3′ and 5′-ACC ACT TTG ATG GGA TGC AGG T-3′. The temperature profile was 10 minutes at 94°C followed by 40 cycles of 94°C for 15 seconds, 61°C for 30 seconds, 72°C for 30 seconds.

Western blotting was performed as described previously (Lutz et al., 2006) using the specific antibodies listed above.

Immunofluorescence analysis of -SMA expression

MLFs were grown on glass coverslips for 3 days, fixed in ice-cold methanol and stained with α-SMA and Alexa Fluor 594-conjugated secondary antibodies (Molecular Probes), as described previously (Koli et al., 2006). The coverslips were finally washed in water, mounted on glass slides using Vectashield anti-fading reagent (Vector Laboratories), and examined under the imaging microscope (model Axioplan 2; Carl Zeiss MicroImaging, Inc.) using a 40× objective. Images were acquired with an AxioCamHRc camera (Carl Zeiss MicroImaging, Inc.) assisted by AxioVision4.6 software (Carl Zeiss MicroImaging, Inc.).

Histology, histochemistry and immunohistochemistry

Paraffin sections of mouse tissues were prepared and stained using standard histology procedures. To visualize elastic fibers, microscopic slides were stained with Weigert's resorcin-fuchsin solution, as described previously (Sterner-Kock et al., 2002). For immunostainings (immunoperoxidase), the 5 µm deparaffinized and rehydrated tissue slides were first boiled for 15 minutes in a microwave with citrate buffer pH 6.0 (LabVision), and then cooled for 30 minutes. After rinsing in ddH_2O and PBS-Tween (PBS-T) for 5 minutes, the slides were treated for 10 minutes with H_2O_2 block (UltraVision) and for 5 minutes with Ultra V block (UltraVision) to inactivate the endogenous peroxidases. After rinsing in ddH_2O and soaking in PBS for 5-10 minutes, the slides were treated with 2% (wt/vol) BSA in PBS to saturate nonspecific protein-binding sites. The slides were then exposed to the specific antibodies with 0.2% (w/v) BSA in PBS at 4°C overnight. After removing excess antibody, the slides were treated with appropriate biotin-labeled secondary antibodies (UltraVision) at room temperature for 10 minutes, and finally with streptavidin peroxidase (UltraVision) at room temperature for 10 minutes. After washing, the slides were incubated in AEC chromogen substrate mixture (UltraVision) at room temperature for 15 minutes. The slides were finally counterstained with hemalaun and mounted in Mowiol.

Tracheobronchial silicone casts

The heart, esophagus and any other mediastinal tissue and fat were separated from the lungs and tracheobronchial tree. An 18-gauge intratracheal catheter was ligated to the trachea and attached to a source of water. Lungs were gently flushed 6-10 times with water to remove the blood and the secretions from the lung and airways. The surface of the lungs was kept moist during the flushing period. After flushing, lungs were dried by channeling air into the trachea. The dried lungs were suspended in a vertical position and instilled with Rhodorsil RTV II polymer (Rhone-Poulenc, Specialty Plastics Division, Princeton, USA; cat. no. 1547), which was prepared according to the manufacturer's instructions. After allowing the silicone to dry overnight, the lungs were placed in boiling water for several hours until most of the soft tissue was macerated away from the casts. Finally, tissue remnants were removed by submerging the casts in 10% sodium hydroxide.

Lung processing and morphometric analysis

All animals were anesthetized with ketamine and xylazine, and treated with heparin (1000 U/kg). Lungs were artificially perfused, as described previously, and ventilated with negative pressures of 2 cm H_2O and 12 cm H_2O in an artificial thorax (Weissmann et al., 2004). The end-expiratory pressure was kept constant at 2 cm H_2O. The dynamic lung compliance was calculated using the HSE Pulmodyn software (Hugo Sachs Elektronik, March Hugstetten, Germany) (Held and Uhlig, 2000).

For alveolar morphometry, lungs were fixed with 4.5% paraformaldehyde in PBS (pH 7.0) via the trachea at a pressure of 22 cm H_2O, and the mean linear intercept and the airspace were assessed in lung sections stained with hematoxylin and eosin, as described previously (Woyda et al., 2009).

TRANSLATIONAL IMPACT

Clinical issue

Chronic obstructive pulmonary disease (COPD) is a leading cause of morbidity and mortality worldwide. Cigarette smoking is a major risk factor and several predisposing genetic factors have been implicated in COPD pathogenesis. Although a variety of drugs are available for symptomatic relief, the complicated biology of COPD has so far eluded researchers' hopes of finding a cure. This work describes a mouse model of COPD in which the antioxidant protein sestrin 2 is identified as a possible drug target for the treatment of COPD. The inactivation of sestrin 2 in this COPD mouse model significantly improves both pulmonary morphology and function. This implies that patients with COPD might benefit from treatment with sestrin antagonists.

Results

Mice lacking the short splice variant of the latent transforming growth factor beta (TGF-β) binding protein 4 (Ltbp4S) develop pulmonary emphysema reminiscent of COPD. Here, mutational inactivation of the antioxidant protein sestrin 2 (sesn2) in Ltbp4S knockout mice partially rescues the emphysema phenotype. This phenotypic rescue is associated with an improvement of pulmonary elastic fiber structure and the activation of the TGF-β and mammalian target of rapamycin (mTOR) signal transduction pathways. The rescue could be blocked by the TGF-β type I receptor (TβR1) inhibitor SB431542, but was not blocked by antioxidants, indicating that the effect of the sesn2 mutation on mTOR is TGF-β dependent. The induction of the TGF-β pathway in the lungs of Ltbp4S–/–sesn2–/– double mutant mice induced the expression of several TGF-β target genes, including that encoding tropoelastin. Lack of elastin, or accelerated degradation of elastin, contributes to emphysema and the authors suggest that the induction of tropoelastin by the sesn2 mutation is a crucial factor in the functional recovery of the Ltbp4S mutant lung. Mutants also maintain normal levels of profibrotic gene expression despite increased TGF-β signaling, which should help reduce pathology.

Implications and future directions

The results imply that sesn2 plays an important role in the pathogenesis of COPD by affecting several signal transduction pathways that are involved in tissue repair and regeneration. Future studies will determine whether the sesn2 mutation can protect mice from developing pulmonary emphysema after chronic exposure to tobacco smoke – an animal model that closely mimics human COPD. Additionally, sesn2 expression may be altered in the lungs of patients with COPD and could thus serve as a diagnostic marker.

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Statistical analysis

Data are expressed as means ± S.D. Comparisons between groups were performed using the Bonferroni multiple comparison test or the Student´s *t*-test where appropriate. *P* values less than 0.05 were considered significant.

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COMPETING INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

F.W., S.D.-Z., K.K., T.B., N.W., J.K.-O. and H.v.M. conceived and designed the experiments; F.W., S.D.-Z., K.K., T.B., N.P., R.D. and A.S.-K. performed the experiments; F.W., S.D.-Z., K.K., T.B., N.W., J.K.-O. and H.v.M. analyzed the data; F.W., T.B., N.W. and H.v.M. wrote the paper.

SUPPLEMENTARY MATERIAL

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