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Dual Functions for LTBP in Lung Development: LTBP-4 Independently Modulates Elastogenesis and TGF-β Activity

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Abstract

The latent TGF-β binding proteins (LTBP) −1, −3, and −4 are extracellular proteins that assist in the secretion and localization of latent TGF-β. The null mutation of LTBP-4S in mice causes defects in the differentiation of terminal air-sacs, fragmented elastin, and colon carcinomas. We investigated lung development from embryonic day 14.5 (E14.5) to day 7 after birth (P7) in order to determine when the defects in elastin organization initiate and to further examine the relation of TGF-β signaling levels and air-sac septation in *Ltbp4S*$^{-/-}$ lungs. We found that defects in elastogenesis are visible as early as E14.5 and are maintained in the alveolar walls, in blood vessel media, and subjacent air–way epithelium. The air-sac septation defect was associated with excessive TGF-β signaling and was reversed by lowering TGF-β2 levels. Thus, the phenotype is not directly reflective of a change in TGF-β1, the only TGF-β isoform known to complex with LTBP-4. Reversal of the air-sac septation defect was not associated with normalization of the elastogenesis indicating two separate functions of LTBP-4 as a regulator of elastic fiber assembly and TGF-β levels in lungs.

Keywords

TGF-β; LTBP-4; elastogenesis; air-sac septation; TGF-β activation

Introduction

The latent transforming growth factor-β (TGF-β) binding proteins (LTBPs) comprise a family of four extracellular matrix proteins, LTBPs −1 to −4 that are structurally similar to the fibrillins (Rifkin, 2005). Both the LTBPs and fibrillins contain multiple calcium-binding epidermal growth factor-like (CB-EGF) domains and signature domains with eight

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intramolecularly bound cysteine residues (8-Cys domain) (Rifkin, 2005). LTBP-1, −3, and −4, but not LTBP-2 nor the fibrillins, form covalent bonds with latent TGF-β (Rifkin, 2005).

TGF-βs 1, 2, and 3 are all synthesized as homodimeric proproteins with three intermolecular disulfide bonds (Annes et al., 2003). The TGF-β propeptide dimer is cleaved from the mature cytokine in the trans-Golgi, but the propeptide remains tightly bound to the cytokine by non-covalent interactions. This interaction of TGF-β and its propeptide prevents the growth factor from binding to its receptor. Therefore, the propeptide is referred to as the latency associated protein (LAP) and the TGF-β-LAP complex as the small latent complex (SLC). The release of TGF-β from its interaction with LAP, known as latent TGF-β activation, is a crucial step in the regulation of TGF-β activity (Annes et al., 2003). *In vivo*, the SLC is often bound to a LTBP by disulfide bonds between two cysteines from the LAP dimer and a pair of cysteines from the third 8-Cys LTBP domain (Chen et al., 2005; Gleizes et al., 1996; Munger et al., 1998; Saharinen et al., 1996). This SLC-LTBP complex is known as the large latent complex (LLC).

The LTBPs may direct and facilitate TGF-β action through several complimentary mechanisms. LTBPs enhance TGF-β secretion, as the formation of disulfide bonds with LAP engages what otherwise would be reactive cysteine residues and promotes proper LAP folding (Miyazono et al., 1991). In the extracellular environment, LTBPs interact with several matrix molecules, including fibronectin and fibrillin, thereby targeting latent TGF-β to specific locations for subsequent activation (Isogai et al., 2003; Taipale et al., 1996). LTBP-1 also directly participates in the activation of latent TGF-β by the integrin αvβ6 presumably by anchoring the LLC to the matrix, and allowing the integrin to apply force to the complex (Annes et al., 2004). This force is believed to distort the LAP, promoting release of the TGF-β.

The three TGF-β-binding LTBPs, LTBP-1, −3, and −4, display differences both in their matrix distribution and TGF-β binding. For example, LTBP-1 and −3 effectively bind all three isoforms of TGF-β, whereas LTBP-4 binds only TGF-β1 (Saharinen et al., 1996). LTBP-1 and −4 bind to fibrillin 1, but LTBP-3 does not (Isogai et al., 2003). The kinetics of LTBP assembly into the matrix differ with LTBP-1 incorporated most rapidly and LTBP-3 most slowly (Koli et al., 2005). In addition LTBP-1 and LTBP-4 exist as both long (LTBP-1L and LTBP-4L) and short (LTBP-1S and LTBP-4S) forms initiated from separate promoters ((Koski et al., 1999) and H. von Melchner – unpublished observations). It has been shown that LTBP-1L incorporates into extracellular matrix (ECM) more efficiently than LTBP-1S (Olofsson et al., 1995). However, biological significance of these different forms of LTBP-1 and LTBP-4 is not fully understood.

Phenotypes of mice with null or hypomorphic mutations in LTBP genes (*Ltbp*) have been interpreted as being consistent with decreased TGF-β activity. Thus, the heart outflow tract abnormalities in *Ltbp1L*$^{-/-}$ mice (Todorovic et al., 2007), the skeletal abnormalities in *Ltbp3*$^{-/-}$ (Dabovic et al., 2002) and *Ltbp1*$^{-/-}$ mice (Drews et al., 2008), and the pulmonary emphysema and the colorectal tumors in *Ltbp4S*$^{-/-}$ mice ((Sterner-Kock et al., 2002), and H. von Melchner, unpublished observations) are consistent with previously published data on genetically modified mouse models and human tumors with impaired TGF-β signaling (Choudhary et al., 2006; Erlebacher and Derynck, 1996; Filvaroff et al., 1999; Markowitz et al., 1995; Riggins et al., 1997; Yang et al., 2001). However, because of their incorporation into the matrix and their structural resemblance to the fibrillins, certain phenotypes in *Ltbp* mutant mice may represent the loss of a TGF-β-independent function. Indeed, not all effects of blocking LTBP in cell culture can be replicated by blocking TGF-β (Dallas et al., 1995). Moreover, LTBP-2 does not bind TGF-β, yet the *Ltbp2*$^{-/-}$ mutation is embryonic lethal, indicating an essential non-TGF-β-related role for this protein (Shipley et al., 2000).

Developmental abnormalities *in Ltbp4S*$^{-/-}$ mice are twofold: 1) a defective elastic fiber structure and 2) a strong impairment of terminal air-sac septation, first evident at the saccular stage of lung development (Sterner-Kock et al., 2002). Properly organized elastin at the tips of the growing alveolar septae is required for alveolar differentiation (Wendel et al., 2000) and this requirement may account for the defect in terminal lung septation in *Ltbp4S*$^{-/-}$ animals. However, as TGF-β is a regulator of matrix molecule expression, the elastin anomaly may be TGF-β-dependent. In addition, it has been reported that LTBP-4 binds only TGF-β1 (Saharinen et al., 1996), yet *Tgfb1*$^{-/-}$ mice have no obvious lung abnormalities (Kulkarni et al., 1993; Shull et al., 1992). This raises an apparent contradiction in interpreting the *Ltbp4S*$^{-/-}$ lung defects as a consequence of decreased TGF-β.

To clarify the cause of the alveolar septation and elastogenesis defects in *Ltbp4S*$^{-/-}$ lungs, we examined elastogenesis in lungs from wild type (WT) and *Ltbp4S*$^{-/-}$ mice at embryonic day (E) 14.5 to postnatal day (P) 7. We found that there was a defect in elastogenesis as early as E14.5–16.5 in the mutant animals in the lung alveolar walls, large airways and blood vessels. Contrary to what we expected, we found increased TGF-β signaling in *Ltbp4S*$^{-/-}$ lungs. Decreasing TGF-β improved septation of terminal air-sacs but did not reverse the defects in elastogenesis suggesting that alterations in TGF-β signaling and abnormal elastogenesis represent two separable functions of LTBP-4.

Materials and Methods

Drugs and antibodies

TβR1 inhibitor SB431542 was purchased from Sigma-Aldrich, (St. Louis, MO). Antibodies to P-Smad2 and Smad2/3 were purchased from Cell Signaling Technology (Danvers, MA). Anti TGF-β1 was purchased from R&D Systems (Minneapolis, MN).

Mice

The *Ltbp4S*$^{-/-}$ mice were previously described by Sterner-Kock et al. (Sterner-Kock et al., 2002). *Tgfb2*$^{+/-}$ mice were purchased from Jackson Labs (Bar Harbor, Maine). All mice were maintained on normal lab diet. For staged embryos, female and male mice were housed together overnight. Noon of the day of vaginal plug appearance was considered 0.5 dpc (days post coitum) or P0.5. Pregnant females were killed by asphyxiation by CO_2 and cervical dislocation and the embryos were collected and placed immediately in 10% buffered formalin at room temperature. All procedures were conducted according to the regulations of the NYU Langone Medical Center IACUC.

Genotyping

Mice from *Ltbp4S*$^{+/-}$ × Ltbp4S$^{+/-}$ crosses were genotyped by PCR using reverse primers 3C7Wt: GGCTCATGCTTGAATGTTCAG and 3C7Tg: ATCATGCAAGCTGGTGGCTG specific for the mutated and the WT allele, respectively, and a common forward primer P3: CCAATCTTGCTTCTTTGCTG AGC. Mice from Tgfb2$^{+/-}$ × Tgfb2$^{+/-}$ crosses were genotyped using forward allele –specific primers for the WT, B2–6F: AATGTGCAGGATAATTGCTGC and the mutant, Neo-1L: CGACCACCAAGCGAAACATCGC, and a common reverse primer B2–6R: AACTCCATAGATATGGGGATGC.

Quantitative real time RT-PCR

RNA was extracted from freshly dissected lungs using Trizol (Invitrogen). Reverse transcription (RT) reactions were performed using 1 μg of RNA and Superscript III Reverse Transcriptase (Invitrogen) at 50° C for 60 minutes. The cDNA produced was used for quantitative real-time RT-PCR (Q-RT-PCR) analysis (Wang et al., 2006). Q-RT-PCR

reactions were carried out with specific primers and the Quanti Fast SYBR Green PCR Kit (Qiagen) using an iCycler Thermal Cycler (Bio-Rad). The transcript expression for each target was quantified by comparing the threshold cycle (TC) with that of hypoxanthine guanine phosphoribosyl transferase using the comparative TC method. The primers used are shown in Supplemental Table III.

Histology and Immunohistochemistry

Mouse lungs were inflated with 10% buffered formalin (Sigma-Aldrich) at room temperature through the cannulated trachea under water pressure of 25 cm for day 7 and 15 cm for new-born and E 18.5 lungs. The tissues were fixed in 10% buffered formalin, processed and embedded in paraffin. Five-micrometer sections were used in all studies. For histological and histomorphometric analysis the sections were stained with hematoxilin and eosin (H&E)(Sigma). Elastin was stained using orcinol – new fuchsin technique (Sheehan and Hrapchak, 1980).

Immunohistochemistry with P-Smad2 antibody was performed following the manufacturers protocol. The staining was revealed using ABC Vector Elite Kit (Vector Laboratories, Burlingame, CA).

Western Blot Analysis

Western blot analysis was performed on lung extracts from P7 mice. Tissue was snap-frozen in liquid nitrogen, pulverized using mortar and pestle and resuspended in lysis buffer (10 mM HEPES pH 7.9, 10 mM KCl, 0.1 mM EDTA, 1% TritonX-100, 1 mM glycerophosphate, 2.5 mM sodium pyrophosphate, 1 mM sodium orthovanadate) containing protease inhibitor cocktail (Roche, Indianapolis, IN). After 10 min incubation on ice, lysates were passed at least 5 times through a 22-gauge needle fitted to a syringe and thereafter centrifuged for 10 minutes at full speed in a micro centrifuge at 4°C. The supernatants were collected and protein concentrations were determined using Pierce BCA kit (Thermo Scientific, Rockford, IL). Equivalent amounts of protein from each sample, corresponding to 50–100 μl of lysate, were used for further analysis. Western blotting with P-Smad2 and Smad2/3 antibodies was performed according to manufacturer's protocol. Immunoreactive bands were revealed using Pierce ECL Western Blotting Substrate (Thermo Scientific). Relative intensity of the bands was evaluated using Kodak 1D 3.5.4 software (Kodak Scientific Imaging System, Rockville, MD). The ratio of the intensity of P-Smad2 versus Smad2/3 bands in *Ltbp4S*$^{-/-}$ samples was normalized to the ratio calculated for the WT samples.

TβR1 inhibitor treatment

On 16.5 and 18.5 dpc pregnant *Ltbp4S*$^{+/-}$ females from *Ltbp4S*$^{+/-}$× *Ltbp4S*$^{+/-}$ matings were injected intraperitoneally with 2 mg/kg of SB431542 dissolved in PBS. Control females were injected with PBS. The pups were sacrificed the first day after birth (P0.5), and the lungs were processed as described above.

Histomorphometric analysis

For the assessment of mean terminal sac diameter five lung sections were stained with H&E and 10–12 random fields were photographed under 20X magnification. 2–3 horizontal lines were drawn across each photographed field in areas without large airways or vessels and each intercept of the lines and terminal air-sac walls was counted. The number of lines was multiplied by 580 (which corresponds to the length of the line connecting opposite vertices in a 20x objective microscope field in μm) and divided by the number of intercepts to obtain the mean terminal air-sac diameter.

Transmission electron microscopy

For transmission electron microscopy of lung elastic fibers, lungs were perfused with ice-cold 3% gluteraldehyde in 0.1 M cacodylate buffer (pH 7.4). The left lobe was removed and placed in fresh fixative overnight. Samples were trimmed to 1.5 mm^3 pieces and sequentially stained *en bloc* with 1% osmium tetroxide, 2% tannic acid and 2% uranyl acetate prior to dehydration and Epon embedding as previously described (Davis, 1993). Thin sections (60 nm) were placed on formvar-coated grids and counterstained with 7% methanolic uranyl acetate followed by lead citrate. Sections were viewed using a Tecnai 12 transmission electron microscope at 120 kV and images were digitally captured.

Results

Defective elastogenesis in *Ltbp4S*$^{-/-}$ lungs

In the initial report on *Ltbp4S*$^{-/-}$ mice, Sterner-Kock et al. (Sterner-Kock et al., 2002) described abnormal elastic fibrils in the lungs and intestines of 12-week-old mice. As it was not clear whether fragmented elastin was a result of degradation of preformed fibrils or a result of defective elastic fiber formation, we first characterized elastogenesis in the WT and *Ltbp4S*$^{-/-}$ lungs at P7 and P0.5.

At P7, the impairment of terminal septation in *Ltbp4S*$^{-/-}$ lungs was obvious with a patch-like distribution of regions undergoing alveolarization interspersed with regions with large terminal air-sacs (Fig. 1A, upper panel). In contrast, alveolarization was uniform throughout WT lungs at this time. In the WT lung at P7, the elastin was assembled into fibrils in the alveolar walls and under the airway epithelium (Fig. 1A, lower panel). In the mutant lung, elastin was not organized into fibrils but rather appeared globular or fragmented (Fig.1A, lower panel). At P0.5, we observed uniform severe enlargement of terminal air-sacs in *Ltbp4S*$^{-/-}$ compared to WT lungs (Fig. 1B, upper panel). The elastic fibers in WT lungs at P0.5 were thinner, but the overall elastin distribution in the WT and *Ltbp4S*$^{-/-}$ lungs was similar to that observed at P7 (Fig. 1B, lower panel). Since the defects in lung development and elastin organization were already obvious in newborn *Ltbp4S*$^{-/-}$ mice, we also analyzed lungs during embryogenesis. At E18.5, air-sac enlargement was already apparent in *Ltbp4S*$^{-/-}$ compared to WT lungs (Fig. 1C, upper panel). The elastin organization resembled that seen in P0.5 lungs (Fig. 1C, lower panel). At E16.5, *Ltbp4S*$^{-/-}$ lungs could not be distinguished from WT lungs by histological analysis (Fig. 1D, upper panel). However, in WT lungs, we detected elastic fibers around the airways, including the bronchioles (Fig. 1D, lower panel), whereas at this time the elastin fiber ultrastructure in *Ltbp4S*$^{-/-}$ lungs was already abnormal with the elastin appearing as globules (Fig. 1D, lower panel). At E14.5, no elastin was detectable in the WT lungs using orcinol-new fucsin, which is probably indicative of fibrils too thin to be visualized by histological staining, since we did detect granules of elastin surrounding the bronchi in the *Ltbp4S*$^{-/-}$ lungs (Fig. 1E, lower panel). Together, our results indicate that *Ltbp4S*$^{-/-}$ lung septation is defective starting from E16.5–18.5 and suggest that the abnormality in elastin organization coincides with the beginning of elastogenesis in the lung.

We next examined the elastin fibers in WT and mutant lungs using electron microscopy (EM) at P0.5 (Fig. 2). Differences were apparent in both the alveolae and the airways. In the mutant lung (Fig. 2A), the usual small collection of elastic fibers normally seen in the WT alveolar tips appeared abnormally large and often formed single, fused aggregates of elastin. Similar abnormalities in elastin fiber ultrastructure were observed subjacent to the airway epithelium (EP) (Fig. 2B). Defects in elastin organization were also obvious in the walls of pulmonary blood vessels (Fig. 2C). In WT vessels, elastin was organized into nearly

continuous lamellae between the smooth muscle cells (SMCs), whereas in *Ltbp4S*$^{-/-}$ lungs, the lamellae were not well formed and appeared fragmented.

To determine if the defect in elastin organization persists in older animals, we also examined lung and skin from 1-month-old mice by EM. At one month of age, after the elastic fibers are fully developed, the defective assembly of elastin with respect to the microfibrils in *Ltbp4S*$^{-/-}$ lung was still obvious. In the WT mice, microfibrils are enmeshed in the elastin to form a lace-like structure (Fig. 3A), whereas in *Ltbp4S*$^{-/-}$ lung, large globules of elastin were localized next to the microfibrils with only a small amount of elastin integrated within the microfibril bundle. A similar abnormality in elastic fiber ultrastructure was observed in the dermis of *Ltbp4S*$^{-/-}$ mice demonstrating that the elastic fiber defect is not restricted to the lung (Fig. 3B).

We quantified the expression levels of a number of key proteins known to be involved with elastogenesis using Q-RT-PCR. We examined the mRNA levels for tropoelastin, fibrillin-1 and 2, fibulin-4 and 5, Lox and Loxl-1 (Supp. Table 2). None of the assayed RNA transcripts showed a significant difference between WT and *Ltbp4S*$^{-/-}$ lung samples at P7. At P0.5, the level of Loxl-1 was considerably higher in the mutant samples compared to WT, whereas other transcripts were expressed at similar levels. As differences in lung maturation are seen at day P7 as well as P0.5, the significance of the Loxl-1 difference is unclear. Therefore, the observed differences in *Ltbp4S*$^{-/-}$ lung elastin organization cannot be explained by decreased expression of major proteins known to be involved in elastic fiber assembly.

TGF-β levels in Ltbp4S$^{-/-}$ lungs

We next examined the level of TGF-β signaling in the WT and *Ltbp4S*$^{-/-}$ lungs. To assess the levels of TGF-β signaling in *Ltbp4S*$^{-/-}$ lungs, we characterized the tissue for the TGF-β signaling transducer P-Smad2 by immunohistochemical (IHC) and Western blot analysis. Upon TGF-β binding to its receptors, Smad2 is phosphorylated and is transported to the nucleus (Derynck and Miyazono, 2008). Therefore, both the ratio of P-Smad2 versus total Smad2 and the cellular distribution (cytosolic versus nuclear) of P-Smad2 are indicators of TGF-β signaling. Our IHC analysis using a P-Smad2 antibody on lung sections from 7-day-old mice indicated increased levels of P-Smad2 in *Ltbp4S*$^{-/-}$ compared to WT lungs, as the intensity of the stain in individual nuclei was greater in the mutant tissue as was the total number of stained nuclei (Fig 4A). To confirm and quantify the IHC data, Western blot analysis of proteins extracted from P7 *Ltbp4S*$^{-/-}$ and WT lungs was carried out. The blot was first probed with a P-Smad2 antibody and then, after stripping, with a Smad2/3 antibody. The bands were scanned, and after densitometry, we calculated the ratio of P-Smad2 to Smad2/3 (Fig. 4B). Our results showed that the level of TGF-β signaling in *Ltbp4S*$^{-/-}$ lungs, as indicated by the ratio of P-Smad2/Smad2/3, is higher than in the WT lungs, demonstrating increased levels of active TGF-β.

We also examined the transcript levels of three TGF-β-responsive genes: *Pai-1*, *Ctgf-1* and *C-Myc* (Supp. Table 3), whose expression levels are often assayed as indicators of TGF-β signaling (Coffey et al., 1988; Duncan et al., 1999; Flaumenhaft and Rifkin, 1991; Wu et al., 2007). At P0.5 a small decrease in expression of *C-Myc* was observed in *Ltbp4S*$^{-/-}$ compared to WT lungs, which is consistent with enhanced TGF-β signaling. However, there was a high degree of variability in expression levels of the TGF-β responsive gene *Ctgf-1* in different lung samples of the same genotype, and the overall differences between *Ltbp4S*$^{-/-}$ and WT lungs were not significant. The expression of *Pai-1* was decreased in *Ltbp4S*$^{-/-}$ lungs, which is consistent with decreased TGF-β signaling. Taken together Q-RT-PCR studies did not give a clear indication of a change in TGF-β levels in P0.5 *Ltbp4S*$^{-/-}$ lungs.

At P7, expression levels of *Pai-1*, *C-Myc*, and *Ctgf-1* were similar in WT and *Ltbp4S*$^{-/-}$ lungs (Supp. Table 3).

The transcription factors TTF-1 and HNF3β, which are expressed during lung development, are positively regulated by TGF-β1 (Zeng et al., 2001). We observed increased levels of both of these transcription factors in *Ltbp4S*$^{-/-}$ lungs at P0.5. At P7, *TTF-1* expression still remained much higher in mutant lungs compared to WT, whereas the expression of *HNF3β* was a little less than 2 fold higher in *Ltbp4S*$^{-/-}$ than in WT lungs (Fig. 4C). These data are consistent with continued enhanced TGF-β signaling in *Ltbp4S*$^{-/-}$ lungs.

Our results suggested that the LTBP-4 deficit resulted in increased levels of active TGF-β in the lung. We reasoned that if excess TGF-β signaling caused the defect in septation of terminal air-sacs in *Ltbp4S*$^{-/-}$ lungs, we could normalize the mutant phenotype by lowering TGF-β levels. In order to decrease TGF-β activity in the developing mouse lung, we treated pregnant females from *LtbpS4*$^{+/-}$ × *Ltbp4S*$^{+/-}$ crosses with SB431542, a small molecule inhibitor of the TGF-β receptor I (TβRI / Alk 5). We examined the effects of SB431542 on lungs from newborn animals (P0.5), as the *Ltbp4S*$^{-/-}$ lung phenotype becomes heterogeneous as the animals age, making it difficult to quantify differences at later times. Morphometric studies assessing mean terminal air-sac diameter indicated a small but statistically significant increase ($p<0.057$) in terminal air-sac septation in the treated *Ltbp4S*$^{-/-}$ lungs, suggesting that an increase in TGF-β signaling contributed to the *Ltbp4S*$^{-/-}$ lung developmental defect (Supp. Fig. 1A). There was no difference between SB431542-treated and non-treated WT lungs indicating that decreasing TGF-β signaling with a short treatment of the TβR1 inhibitor did not affect normal lung development. The relatively small decrease in mean diameter of terminal air-sacs in *Ltbp4S*$^{-/-}$ lungs induced by SB431542 treatment prompted us to investigate whether a further reduction of TGF-β signaling would further improve septation.

A more effective method to decrease TGF-β levels or signaling earlier during development and to avoid the potential toxicity of the chemical inhibitor is to cross *Ltbp4S*$^{-/-}$ mice with *Tgfb*$^{-/-}$ *or Tgfr*$^{-/-}$ animals. TβR1 is not specific for TGF-β, and both *Tgfr1* and *Tgfr2* null mutations are lethal early in development. *Tgfb1* and *Ltbp4* both map to chromosome 7, only 1.1 Mb apart, which makes the generation of *Ltbp4S*$^{-/-}$*;Tgfb1*$^{-/-}$ mice by simple crossing of *Ltbp4S*$^{+/-}$ *and Tgfb1*$^{+/-}$ animals difficult. *Tgfb3* expression in mouse embryonic lungs decreases in later stages of development and the transcript is not detectable by E 16.5 (Schmid et al., 1991), the stage of development that precedes the lung morphogenesis defect observed in the *Ltbp4S*$^{-/-}$ embryos. However, *Tgfb2* is expressed at high levels in developing mouse lungs and its expression is increased at later stages of development (Schmid et al., 1991). In addition, several studies have indicated an important role of TGF-β2 in lung morphogenesis (Liu et al., 2000; Sanford et al., 1997). Therefore, we reasoned that in order to decrease overall TGF-β levels in lungs at the appropriate time, attenuating TGF-β2 would be the most effective genetic approach. Therefore, we crossed *Ltbp4S*$^{+/-}$ mice with *Tgfb2*$^{+/-}$ mice, and we examined the lungs from *Ltbp4S*$^{-/-}$*;Tgfb2*$^{+/-}$ and *Ltbp4S*$^{-/-}$*;Tgfb2*$^{-/-}$ animals. Visual examination of stained lung sections suggested that the loss of a single *Tgfb2* allele had a small effect on lung septation (Supp. Fig. 1B). However, when we performed histomorphometric analysis of mean terminal air-sac diameter, we observed no significant differences between *Ltbp4S*$^{-/-}$*;Tgfb2*$^{+/-}$ and *Ltbp4S*$^{-/-}$*;Tgfb2*$^{+/+}$ lungs. As ablation of one *Tgfb2* allele might have been insufficient to produce a biologically significant decrease of TGF-β levels in *Ltbp4S*$^{-/-}$ lungs, we also examined *Ltbp4S*$^{-/-}$*;Tgfb2*$^{-/-}$ lungs. *Tgfb2*$^{-/-}$ animals die at birth from multiple organ defects (Sanford et al., 1997). Therefore, we characterized *Ltbp4S*$^{-/-}$*;Tgfb2*$^{-/-}$ lungs before birth, at a late stage of development, E18.5. At E18.5 there was an obvious defect in *Ltbp4S*$^{-/-}$ lung morphogenesis and elastogenesis (Fig. 5), however histological analysis of E18.5

Ltbp4S$^{-/-}$;*Tgfb2*$^{-/-}$ lungs revealed a significant improvement in lung septation compared to *Ltbp4S*$^{-/-}$ lungs (Fig. 5A). Quantitation by histomorphometric analysis revealed a complete (100%) rescue of terminal air-sac development (Fig. 5B). These results imply that increased, rather than decreased, TGF-β is responsible for the impairment of lung development *Ltbp4S*$^{-/-}$ mice.

Increased TGF-β signaling may result from either an increase in TGF-β synthesis or an increase in latent TGF-β activation. It has been reported that cultured *Ltbp4S*$^{-/-}$ lung fibroblasts express elevated levels of TGF-β2 and TGF-β3 (Koli et al., 2004). To assess TGF-β expression *in vivo*, we analyzed RNA extracted from WT and *Ltbp4S*$^{-/-}$ lungs by Q-RT-PCR. At P0.5 a small increase in expression of all three TGF-β isoforms was observed in the mutant lungs compared to control, but by P7 the differences were very small (Supp. Table 3).

Elastin Organization in *Ltbp4S*$^{-/-}$;*Tgfb2*$^{-/-}$ Lungs

Finally, we examined whether the increased TGF-β levels associated with decreased alveologenesis might also be the cause for defective elastogenesis in *Ltbp4S*$^{-/-}$ mice and whether the reduction of TGF-β2 levels would improve elastin organization. We found that the elastic fiber organization in E18.5 *Ltbp4S*$^{-/-}$;*Tgfb2*$^{-/-}$ lungs resembled that in *Ltbp4S*$^{-/-}$;*Tgfb2*$^{+/+}$ lungs (Fig. 6). Thus elastogenesis still appeared to be defective regardless of improved alveolar septation and presumably lower active TGF β levels. Therefore, we suggest that Ltbp-4 plays an important function in elastogenesis distinguishable from its role in the regulation of TGF-β tissue levels.

Discussion

The experiments presented indicate that the loss of LTBP-4 synthesis in *Ltbp4S*$^{-/-}$ mouse lungs results in increased TGF-β signaling and an impairment of terminal air-sac development. Decreasing TGF-β expression or signaling *in vivo* either by genetic or by pharmacological intervention meliorated *Ltbp4S*$^{-/-}$ lung septation. *Ltbp4S*$^{-/-}$ mice also display an abnormality in lung elastogenesis apparent as early as E14.5–16.5, which appeared to be independent of TGF-β signaling, as normalization of terminal air-sac septation by decreasing TGF-β levels did not normalize the defects in elastic fiber structure.

Impairment of LTBP function either through null mutations or biochemical inhibition is believed to result in decreased TGF-β action because of faulty secretion, impaired localization, or lack of latent TGF-β activation (Annes et al., 2004; Dabovic et al., 2002; Koli et al., 2005; Miyazono et al., 1991; Sterner-Kock et al., 2002; Taipale et al., 1996; Todorovic et al., 2007). Although blockade of LTBP function may result in decreased signaling in some circumstances (Dabovic et al., 2002; Sterner-Kock et al., 2002; Todorovic et al., 2007), this might not always be the case. It is clear that the SLC can be activated *in vivo* in the absence of an LTBP, and if sufficient SLC is secreted, it can be activated to provide TGF-β signaling (Mazzieri et al., 2005). Moreover, in several systems, the pathological outcomes of interference with TGF-β localization have been attributed to excessive TGF-β signaling (Cohn et al., 2007; Habashi et al., 2006; Mazzieri et al., 2005; Neptune et al., 2003). The clearest example of this are transgenic mice that produce in the epidermis a truncated LTBP-1, which binds to SLC, but cannot localize to the ECM (Mazzieri et al., 2005). These animals have an early onset of the catagen stage of the hair cycle, concordant with increased TGF-β signaling (Mazzieri et al., 2005). A similar explanation has been proposed for the lung (Neptune et al., 2003), vascular (Habashi et al., 2006) and muscle (Cohn et al., 2007) abnormalities in patients and mice with Marfan syndrome, which is caused by mutations in fibrillin-1 (Dietz et al., 1991; Pereira et al., 1997). In this case, LLCs generate abnormally high levels of active TGF-β, perhaps because

of improper targeting of the LLC to defective microfibrils (Mazzieri et al., 2005; Neptune et al., 2003). Decreasing total TGF-β by the administration of either neutralizing antibodies to TGF-β or drugs that decrease TGF-β signaling prevents the development of pathological changes in the affected tissues (Habashi et al., 2006; Mazzieri et al., 2005; Neptune et al., 2003). Therefore, excessive and/or ectopic activation of improperly localized latent TGF-β complexes can result in pathological processes (Habashi et al., 2006; Mazzieri et al., 2005; Neptune et al., 2003).

By decreasing TGF-β with pharmacological intervention using a small molecule inhibitor of TβR1, we improved terminal air-sac septation in *Ltbp4S*$^{-/-}$ lungs by a small degree, and with complete ablation of TGF-β2, we normalized lung morphology. Thus, for normalization of lung development in *Ltbp4S*$^{-/-}$ mice, TGF-β may have to be decreased early in embryogenesis and by a significant amount. The initiating events affecting *Ltbp4S*$^{-/-}$ lung development probably occur before we commenced inhibitor treatment at E16.5, and we may not have maintained sufficiently high levels of inhibitor using our protocols.

Our results indicate complexity in the regulation of TGF-β expression and action. Studies on cultured cells showed that LTBP-4 binds only TGF-β1 (Saharinen et al., 1996). Thus in the absence of LTBP-4, we expected to find a TGF-β1-dependent effect. We were unable to generate mice with compound mutations of TGF-β1 and LTBP-4 in order to specifically decrease TGF-β1 levels in *Ltbp4S*$^{-/-}$ tissues because *Ltbp4* and *Tgfb1* are only 1.1 Mb apart on the same chromosome. However, we found that *Ltbp4S*$^{-/-}$ lung development was rescued by decreasing TGF-β2. The loss of extracellular TGF-β1 and a decrease in the level of active TGF-β1 might stimulate the expression of TGF-β2 in lungs, as has been described for *Ltbp4S*$^{-/-}$ cells in culture (Koli et al., 2004). Indeed, we did observe small increases in TGF-β2 and TGF-β3 expression in the lungs of P0.5 mice. Many studies have shown that increased synthesis of TGF-β does not necessarily result in increased active TGF-β. As we observed an increase in TGF-β signaling in P7 *Ltbp4S*$^{-/-}$ lungs, we hypothesize that the apparent increase in TGF-β activity in *Ltbp4S*$^{-/-}$ lungs is a result of improper latent TGF-β activation, rather than increased TGF-β synthesis. However our results did indicate increased TGF-β signaling in *Ltbp4S*$^{-/-}$ *lung*. It is also possible that the TGF-β isoform elevated in *Ltbp4S*$^{-/-}$ lungs is TGF-β1 and that by eliminating TGF-β2 we normalized the overall levels of TGF-β1, 2, and 3 and consequently restored lung development. Another possibility is that LTBP-4 *in vivo* or in some cell types can bind not only TGF-β1 but also TGF-β2 and 3. Therefore, additional studies are required to elucidate the complex mechanism of elevated TGF-β signaling in the absence of LTBP-4.

Our results also indicate a critical function of LTBP-4 in elastic fiber assembly. The EM data show that in the absence of LTBP-4, the proper deposition of elastin within the microfibril bundles is impaired. The defect in elastic fiber structure in *Ltbp4S*$^{-/-}$ lungs resembles that observed in fibulin-5 (Fib-5) null mice and in patients with mutations in the Fib-5 gene (Hu et al., 2006; Yanagisawa et al., 2002). Fib-5 interacts with fibrillin-1 and with tropoelastin and is essential for elastic fiber formation (Wachi et al., 2008; Zheng et al., 2007). In cell culture LTBP-2, which cannot bind TGF-β, can interact with Fib-5 and regulate the deposition of Fib-5 on microfibrils (Hirai et al., 2007). The significance of this interaction *in vivo* could not be addressed as *Ltbp2*$^{-/-}$ *mice* die at an early stage of development, preceding the beginning of elastogenesis (Shipley et al., 2000). LTBP-4 interacts with both fibrillin-1 and −2 and is deposited on microfibrils (Isogai et al., 2003). It is not known whether LTBP-4 interacts with fibulins and elastin, but we propose that this may be an important function of LTBP-4. Further experiments are required to reveal the molecular interactions of LTBP-4 with proteins involved in elastogenesis and to elucidate LTBP-4 function in elastic-fiber formation.

In summary, our results indicate that LTBP-4 has a dual role in lung development by regulating TGF-β activity and elastic fiber formation. Our genetic and EM data strongly imply that LTBP-4 plays a fundamental role in elastogenesis, independent of its function in regulating TGF-β bioavailability. Whether this is influenced by secondary matrix turnover abnormalities induced by TGF-β remains to be established.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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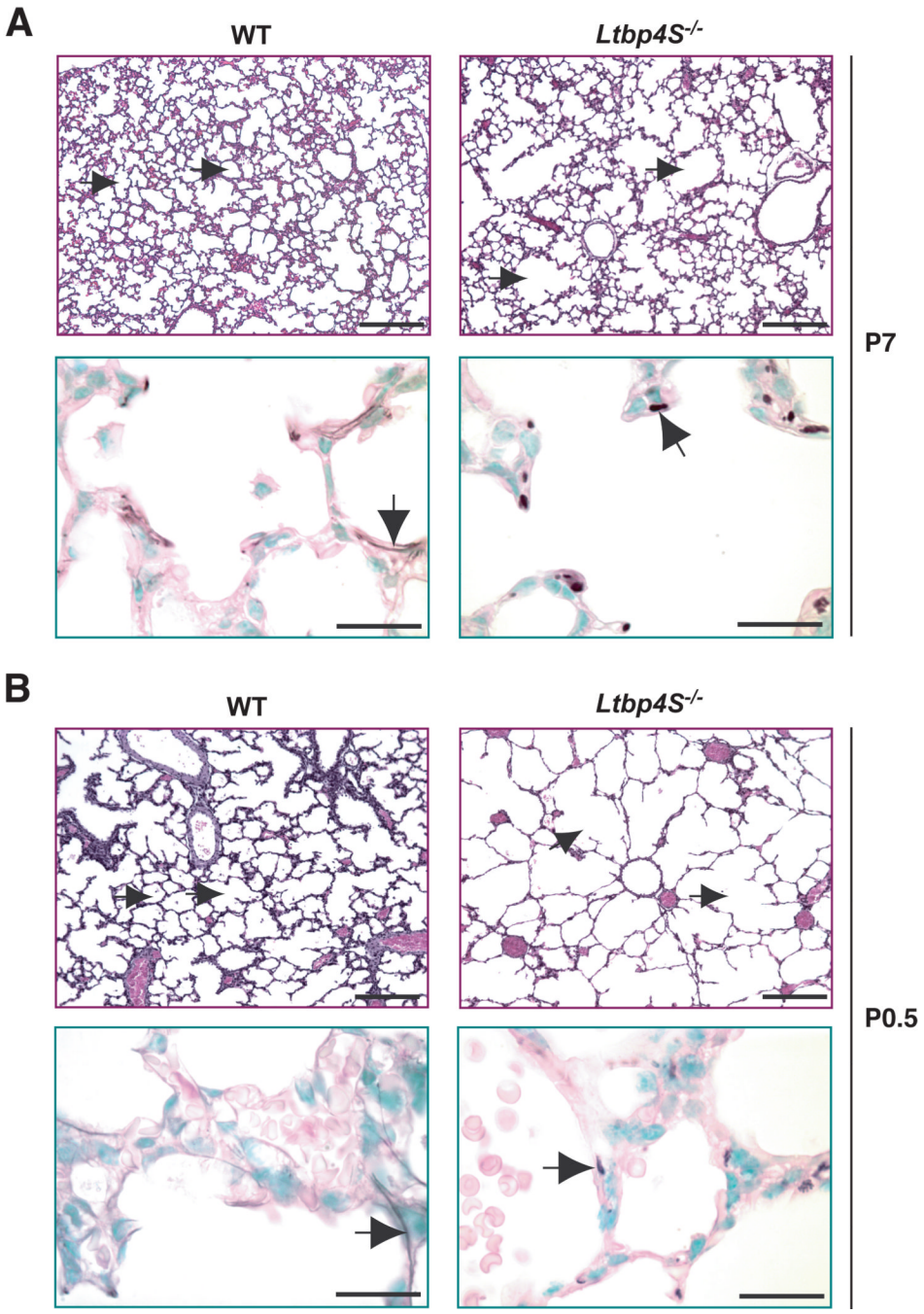

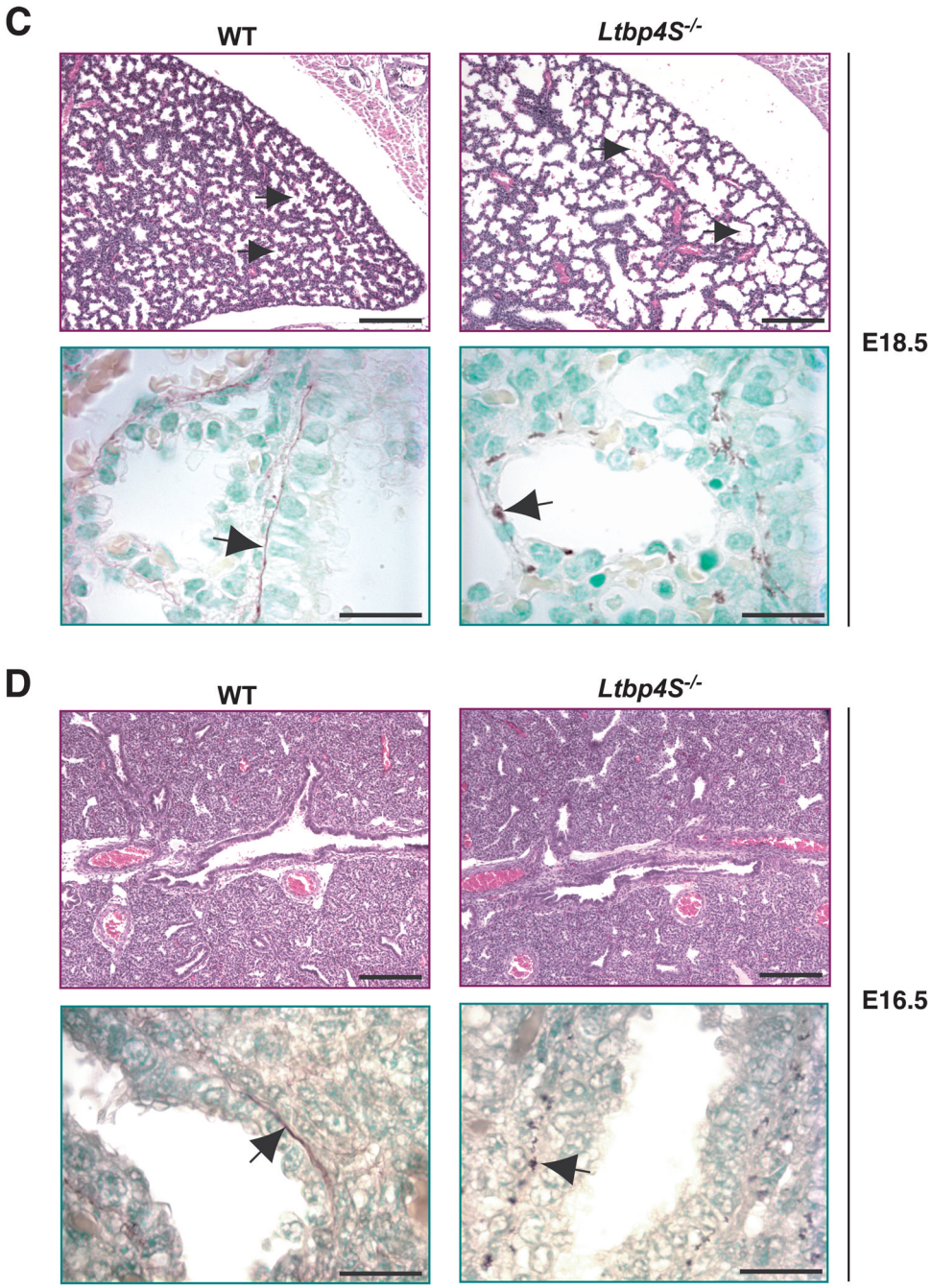

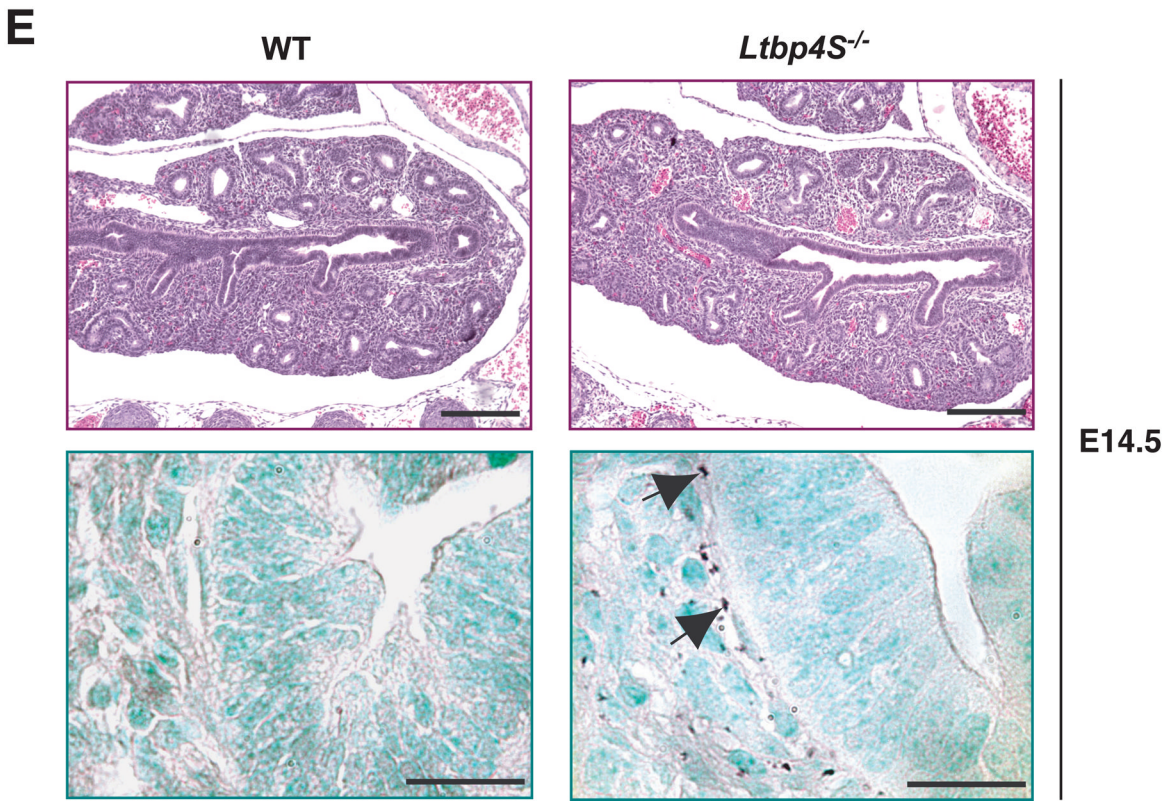

Figure 1.

Defective terminal air-sac septation and elastic fiber formation in *Ltbp4S*$^{-/-}$ lungs. Upper panels show lung sections stained with H&E, lower panels present orcinol-new fucsin staining of elastin. The arrows in upper panels point to terminal air-sacs; the arrows in the lower panels point to elastin. **A. P7** In WT lungs at P7 terminal air-sacs are divided into small units by the process of alveolarization, whereas in *Ltbp4S*$^{-/-}$ lungs alveolarization is not uniform yielding regions with large terminal air-sacs. Elastin in the WT alveolar walls appears fibrillar, whereas in the *Ltbp4S*$^{-/-}$ alveolar walls only globules of elastin were observed. **B. P0.5** At P0.5, the terminal air-sacs in *Ltbp4S*$^{-/-}$ lungs are much larger than in WT lungs. The differences in elastin organization between WT and mutant lungs are similar to those illustrated in A. **C. E18.5** The difference in terminal air-sac septation and in elastin organization between WT and *Ltbp4S*$^{-/-}$ lungs was already obvious at E18.5. **D and E. E16.5 and E14.5** No differences in WT and *Ltbp4S*$^{-/-}$ lung histology were observed both at E16.5 and E14.5. However the differences in elastin organization were observed at E16.5 (**D**) and E14.5 (**E**). Bars: upper panels − 200 μm, lower panels − 20 μm.

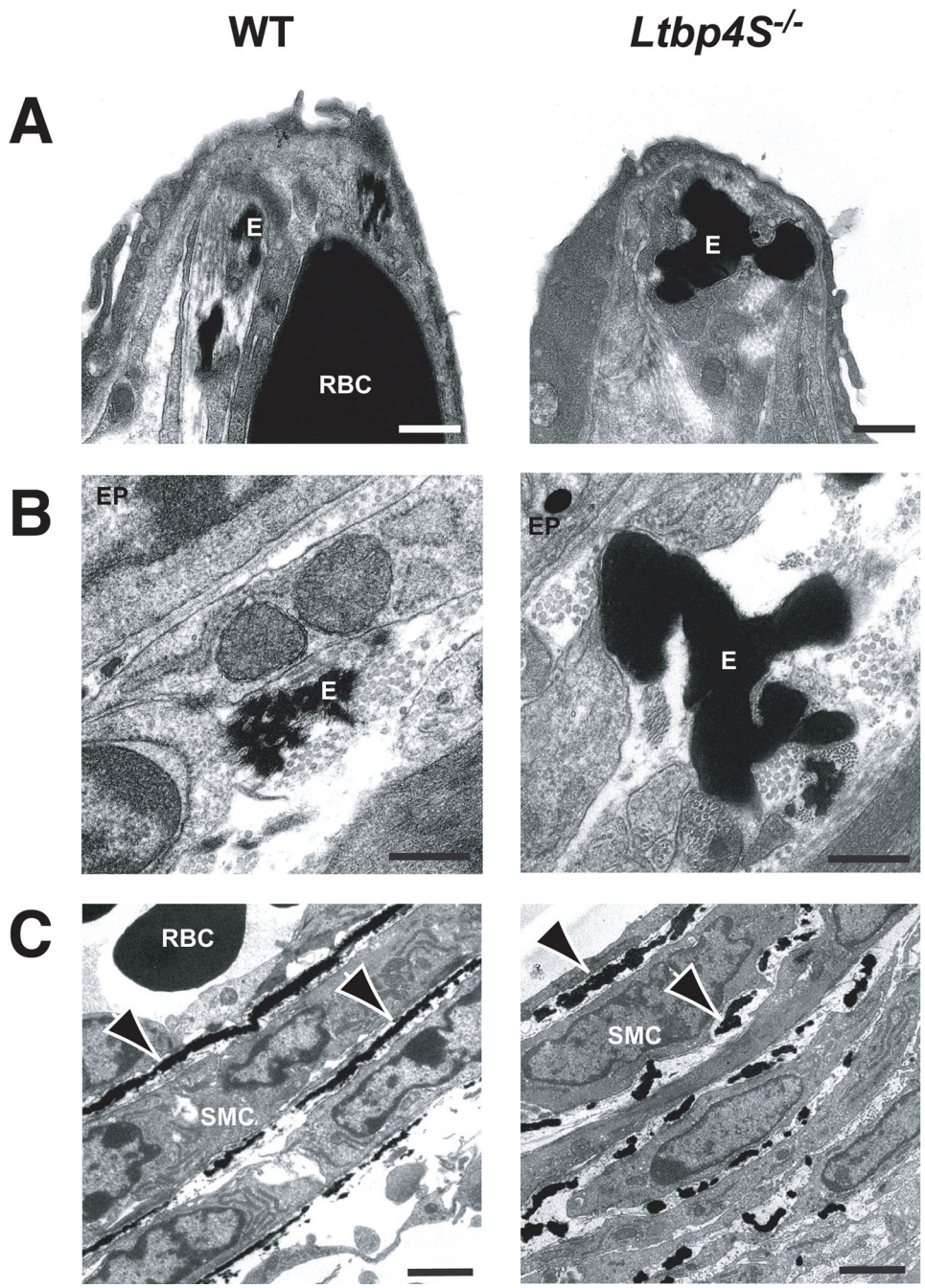

Figure 2.
Electron micrography of P0.5 WT and *Ltbp4S*$^{-/-}$ lungs. **A** Alveolar tips. **B** Airways. **C** Blood vessels. The lack of continuous lamellae in the mutant blood vessels is clear in C where there are essentially no ordered lamellae. E – elastin, RBC – red blood cell, SMC - smooth muscle cell, EP – epithelial cell. Arrows point to elastic lamellae in the walls of blood vessels. Bars: A −0.5 m, B - 0.2 μm, C - 2 μm.

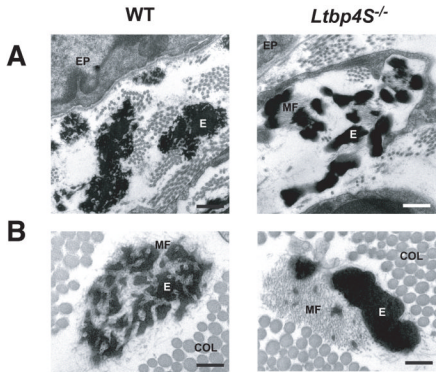

Figure 3.

Ultrastructure of elastic fibers in 4-week-old WT *and Ltbp4S*$^{-/-}$ mice. **A** - airways, **B**- dermis. In WT tissues the microfibrils are enmeshed in the elastin, whereas in *Ltbp4S*$^{-/-}$ tissues large globules of elastin localize next to the microfibrils. COL- Collagen, EP – epithelial cell, E- elastin, MF- microfibrils. Bars: A - 0.5 μm, B – 0.2 μm.

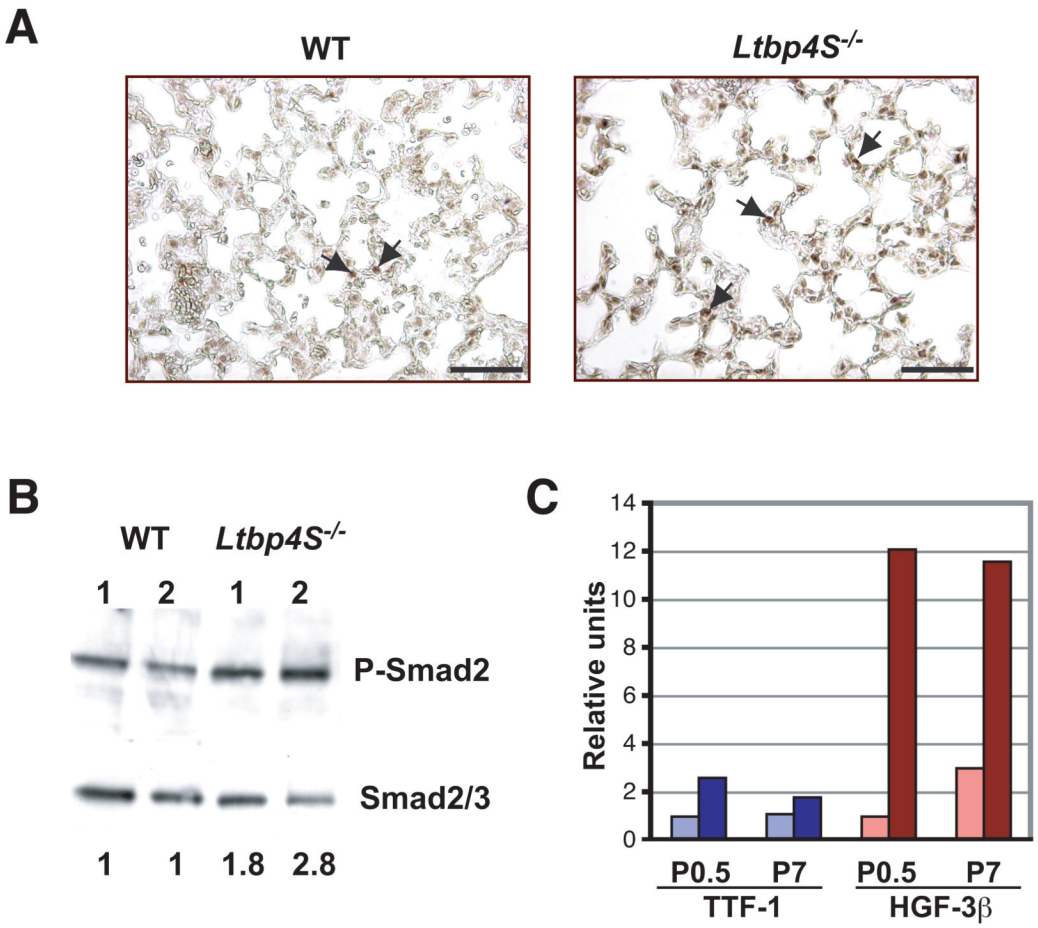

Figure 4.

Increased TGF-β signaling in *Ltbp4S*$^{-/-}$ lungs. **A** Immunohistochemistry with P-Smad2 antibody revealed a higher number of positive cell nuclei in *Ltbp4S*$^{-/-}$ lungs. The arrows point to the P-Smad2 positive nuclei. **B** Quantitative Western Blot analysis of P-Smad2 in the lungs from 2 WT *and 2 Ltbp4S*$^{-/-}$ P7 mice. The numbers at the bottom indicate the ratio of the intensity of P-Smad2 vs. Smad2/3 in the *Ltbp4S*$^{-/-}$ *samples normalized* to the P-Smad2 to Smad2/3 ratio in the WT samples. The ratio of the intensity of the P-Smad2 and the Smad2/3 bands was equivalent in both WT samples. The result shown is representative of four experiments using different animals. **C** Graphic representation of expression of *TTF* and *HNF-3h* in WT and mutant lungs. The expression of both genes was enhanced in *Ltbp4S*$^{-/-}$ mice indicating increased TGF-β levels. Bar: 5 μm.

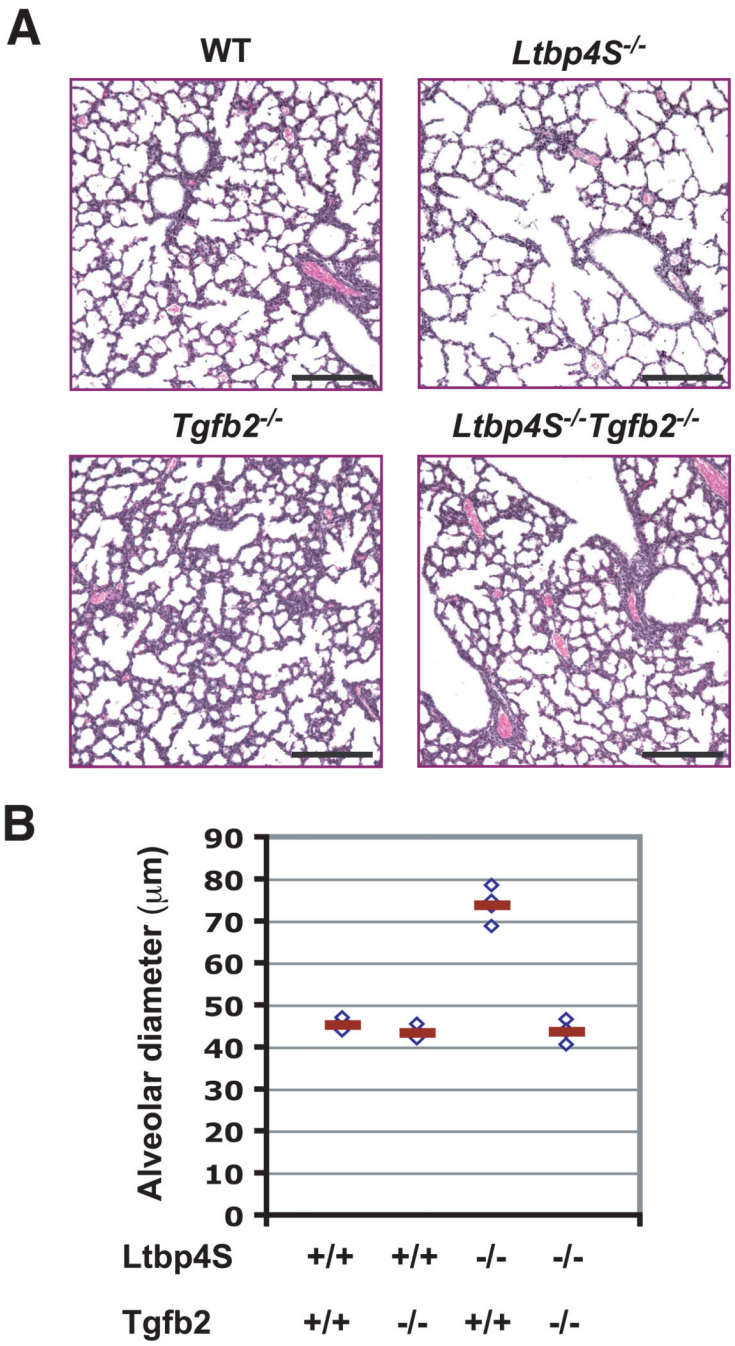

Figure 5.

Rescued lung development in *Ltbp4S*$^{-/-}$;*Tgfb2*$^{-/-}$ *lungs*.**A** Histological analysis of WT, *Ltbp4S*$^{-/-}$, *Tgfb2*$^{-/-}$ and *Ltbp4S*$^{-/-}$;*Tgfb2*$^{-/-}$ indicated improved terminal air-sac septation in *Ltbp4S*$^{-/-}$;*Tgfb*$^{-/-}$ compared to *Ltbp4S*$^{-/-}$ lungs. Bars: 200 μm **B** Histomorphometric studies showed a complete rescue of lung development in *Ltbp4S*$^{-/-}$;*Tgfb2*$^{-/-}$ lungs, as measured by average terminal air-sac diameter. Four animals of each genotype were analyzed in this study. Bar: 200 μm.

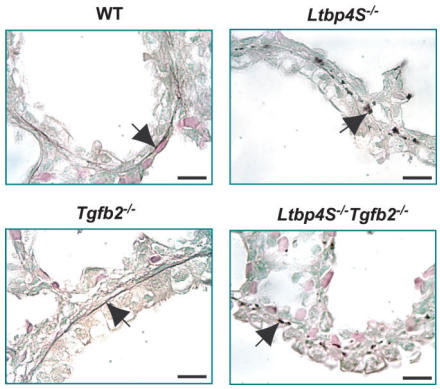

Figure 6.

Decreasing TGF-β2 levels in *Ltbp4S*$^{-/-}$ lungs does not rescue elastic fiber formation. Under conditions in which terminal air-sac septation was normalized (Fig. 5A and B), elastogenesis was still defective as indicated by the absence of elastic lamellae. Elastin in sections from P0.5 lungs was stained with orcinol - new fuchsin. The arrows point to the elastic fibers in WT and *Tgfb2*$^{-/-}$ lungs and to the globular elastin in *Ltbp4S*$^{-/-}$ and *Ltbp4S*$^{-/-}$;*Tgfb2*$^{-/-}$ lungs. Bar: 10 μm.