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Mechanisms of TGFβ inhibition of LUNG endodermal morphogenesis: The role of TβRII, Smads, *Nkx2.1* and *Pten*

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ABSTRACT

Transforming growth factor-beta is a multifunctional growth factor with roles in normal development and disease pathogenesis. One such role is in inhibition of lung branching morphogenesis, although the precise mechanism remains unknown. In an explant model, all three TGFβ isoforms inhibited FGF10-induced morphogenesis of mesenchyme-free embryonic lung endoderm. Inhibition of budding by TGFβ was partially abrogated in endodermal explants from Smad3$^{-/-}$ or conditional endodermal-specific *Smad4*$^{\Delta/\Delta}$ embryonic lungs. Endodermal explants from conditional TGFβ receptor II knockout lungs were entirely refractive to TGFβ-induced inhibition. Inhibition of morphogenesis was associated with dedifferentiation of endodermal cells as documented by a decrease in key transcriptional factor, NKX2.1 protein, and its downstream target, surfactant protein C (*SpC*). TGFβ reduced the proliferation of wild-type endodermal cells within the explants as assessed by BrdU labeling. Gene expression analysis showed increased levels of mRNA for *Pten*, a key regulator of cell proliferation. Conditional, endodermal-specific deletion of *Pten* overcame TGFβ's inhibitory effect on cell proliferation, but did not restore morphogenesis. Thus, the mechanisms by which TGFβ inhibits FGF10-induced lung endodermal morphogenesis may entail both inhibition of cell proliferation, through increased *Pten*, as well as inhibition or interference with morphogenetic mediators such as *Nkx2.1*. Both of the latter are dependent on signaling through TβRII.

Introduction

The mammalian lung is a complex structure that is derived by branching morphogenesis from at least two separate tissues; the foregut endoderm and the splanchnic mesenchyme. Whereas some molecules promote endodermal branching, others are known to be inhibitory. On the positive side, members of the fibroblast growth factor (FGFs) and in particular FGF10 that originate from the mesenchyme are potent chemotactic signaling molecules. FGF10 acts on the pulmonary endoderm to elicit budding and branching morphogenesis (De Moerlooze et al., 2000). Disruption of *Fgf10* blocks pulmonary endodermal morphogenesis resulting in a complete lack of lung parenchyma distal to the trachea (Min et al., 1998; Sekine et al., 1999). In vitro, FGF10 alone is both necessary and sufficient for morphogenesis in mesenchyme-free endodermal explants (Bellusci et al., 1997).

In contrast to FGFs, the overall role of TGFβ is thought to be inhibitory. TGFβ is a key regulator of cell migration, differentiation, proliferation and apoptosis (Roberts, 1998). In the lung, two aspects of TGFβ function have been addressed. First, pathological effects of TGFβ and in particular fibrosis have been extensively studied in various lung injury models including bleomycin and hyperoxia (Westergren-Thorsson et al., 1993; Santana et al., 1995; Coker et al., 1997). Physiological role(s) of endogenous TGFβ in lung development and regulation of gene expression have also been addressed via deletion of specific genes encoding the three TGFβ isoforms (for a review please see Shi and Massagué, 2003).

The binding of TGFβ to its receptors, TβRII and TβRI initiates a cascade of phosphorylation events that eventually result in nuclear translocation of Smad2 and Smad3. Smads interact with several transcriptional activators or repressors, and modify gene expression (Eickelberg, 2001; Shi and Massagué, 2003; ten Dijke et al., 2004). Gene knockout studies suggest that the function of each Smad is distinct. Whereas *Smad3*$^{-/-}$ animals are viable (Datto et al., 1999), *Smad2*$^{-/-}$ dies during early embryogenesis (Heyer et al., 1999). Lung morphogenesis occurs normally in the *Smad3*$^{-/-}$ mice, indicating that the role of endogenous TGFβ in lung development is not dependent on SMAD3 activity (Datto et al., 1999). In contrast, pathological impact of TGFβ on lung morphogenesis may be distinct (Zhou et al., 1996; Serra et al.,

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1994). For example, in the *Smad3*$^{-/-}$ mice *bleomycin*-induced pulmonary fibrosis is attenuated indicating at least a partial role for SMAD3 (Zhao et al., 2002). Also, TGFβ inhibition of cell differentiation and gene expression appears to be in large part due to the activity of SMAD3 (Alliston et al., 2001; Liu et al., 2001; Li et al., 2005). Whether the impact of TGFβ on lung morphogenesis is dependent on the activity of SMADs remains unknown.

A central pathway of growth factor signaling occurs through the activity of phosphotidylinositol-3-kinase, or PI3K. *Pten* a cancer suppressor gene localized to 10q23 encodes a protein tyrosine phosphatase that counters the activity of PI3K, therefore affecting cell proliferation, size, migration and death (Andrés-Pons et al., 2007; Stiles et al., 2004). Absence of *Pten* results in over-activation of several key signaling molecules including AKT/PKB (reviewed in Stiles et al., 2004). Thus, the PTEN/AKT pathway is a major participant in regulation of cell cycle progression and cell differentiation. In transformed cell lines, *Pten* mRNA is rapidly reduced in response to TGFβ (Chow et al., 2007). *Pten* is also expressed at high levels in embryonic stem cells (Takahashi et al., 2005). Loss of *Pten* in the intestinal stem cells initiates polyposis, a condition characterized by precancerous neoplastic increase in the number of crypts, which contain intestinal stem cells (He et al., 2007). Therefore, *Pten* governs the proliferation rate and number of intestinal stem cells and its loss results in an excess of such cells. The in vivo relationship between TGFβ signaling, *Pten* and cell proliferation remains unknown. However, an indirect link between diminished *Pten* and TGFβ has been noted in human idiopathic pulmonary fibrosis (Waite and Eng, 2003).

In the current study we assessed the direct impact of TGFβ on isolated mesenchyme-free embryonic lung endodermal explants. The results demonstrate that all three isoforms of TGFβ inhibit lung endodermal morphogenesis. Inhibitory impact of TGFβ is only partly dependent on Smads, but requires functional TβRII activity. TGFβ-induced inhibition of endodermal morphogenesis is associated with inhibition of cell proliferation, which is in large part due to increased expression of *Pten*.

Materials and methods

Mouse lines

Smad3$^{+/-}$ mice were generated as previously described and were a gift from Dr. Datto (Datto et al., 1999). Generation of TGFβ Receptor II, TβRII $^{flox/flox}$ mice has been previously described (Chytil et al., 2002). These mice were a gift from Dr. Chai (USC Dental School). Generation of *Nkx2.1-cre* mice has been reported (Xu et al., 2008). *Bmp4*$^{LacZ/+}$ mice were a gift from Dr. Brigid Hogan (Duke University, Durham, NC). *Smad4*$^{flox/flox}$ mice were kindly provided by Dr. Deng (Yang et al., 2002). *Pten*$^{flox/flox}$ mice (Freeman et al., 2006) were purchased from Jackson Labs (Maine, USA). All of the mice are C57BL/6 background. The DNA sequences of the Primers were obtained from the respective publications for each of the strains of mice listed above.

Lung culture and lung endodermal explant culture

Whole embryonic lungs were dissected at gestational stage E11.5. In each Grobstein Falcon dish, two to three lungs were placed on filters (Millipore, Bedford, MA) that were placed on top of a stainless steel grid. The filters were in close contact with BGJb (GIBCO) growth medium supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml), with 10 or 50 ng/ml recombinant human TGFβ1 peptide (R&D systems) or same amount of Bovine Serum Albumin (BSA) as control. Heparin beads (Sigma) were incubated with recombinant human TGFβ1 (10 μl of a 50 ng/μl solution; R&D Systems, MN) or in BSA (control) at 37 °C for 2 h, grafted onto lung explants and cultured for 48 h or 96 h. The lungs were incubated under optimal humidity in 95% air/5% CO_2. At indicated times, the lung explants were either homogenized in Trizol (GIBCO) for RNA isolation or fixed in 4% Paraformaldehyde (PFA).

For mesenchyme-free endodremal lung explant culture, distal lung tips were isolated as previously described (Bellusci et al., 1997). Briefly, lungs from mutant or control embryos were dissected at E12.5 and treated with Dispase (50 U/ml, BD Biosciences) at 4 °C for 20 min. Epithelial buds of distal lung tips were then isolated by removing mesenchyme with tungsten needles and embedded into growth factor-reduced Matrigel (Fisher Scientific), diluted 1:1 in culture medium (50% DMEM: 50% Ham's F12, 0.05 U/ml penicillin, 0.05 mg/ml streptomycin). After polymerization of the Matrigel at 37 °C, explants were covered with culture medium with or without FGF and TGFβs and cultured at 37 °C at 5% CO_2 for various lengths of time as indicated.

Proliferation assay

Subsequent to culturing, endodermal explants were treated with bromodeoxyuridine (BrdU) reagent at 1 μl per 400 μl medium for 3 h. The explants were fixed, dehydrated and paraffin embedded. The sections were then re-hydrated and labeled by BrdU staining kit (Zymed Laboratories Inc.) and Hematoxylin/Eosin staining. The sections were then photographed. The *BrdU* positive cells as well as the total number of cells (Hematoxylin/Eosin positive cells) in the explants per section were counted manually using photomicrograph of tissue taken at 40~ on a Zeiss Microscope. The percent of labeled cells was calculated. To compare the proliferation in FGF10 or FGF10+TGFβ1 treated endodermal explants, 678 cells/5 sections from FGF10 treatment and 399 cells/4 sections from FGF10+TGFβ1 treatment were analyzed. To compare the proliferation in wild-type and *Pten*$^{\Delta/\Delta}$ endodermal explants after FGF10 or FGF10+TGFβ1 treatments, 2651 cells/7 sections and 734 cells/7 sections from wild-type explants and 1343 cells/8 sections and 1035 cells/8 sections from *Pten*$^{\Delta/\Delta}$ explants were analyzed.

Cell death analysis

The cultured endodermal explants were fixed, dehydrated and embedded in paraffin and 5-μm sections were prepared. The "In Situ Cell Death Detection Kit, Fluoresein" kit (Roche Allied Science) was used for detection and quantification of cell apoptosis at single cell level, based on labeling of DNA strand breaks (TUNEL technology). The sizes of labeled areas and whole sections were calculated for the percentage of cell death. 5 sections from each treatment were analyzed.

Immunohistochemistry

5 μm tissue sections were prepared. Subsequent to deparaffinization, the sections were hydrated, heated in 10 mM citrate buffer (pH 6.0) and treated with 10% H_2O_2 in methanol for 20 min and blocked with 10% of nonimmune serum. Sections were then incubated with primary antibodies at 4 °C overnight. Biotinylated secondary antibody and streptavidin-peroxidase conjugate (Vector Laboratories, Inc.) were used to detect the bound antibodies. The sections were developed with diaminobenzidine. NKX2.1 primary antibody was purchased from R&D Systems.

RNA extraction and RT-PCR

Total RNA was isolated from lungs or endodermal explants using Trizol (GIBCO). The cDNA was synthesized from 1 μg total RNA by following the protocol of the SuperScript™ First-Strand Synthesis Kit (Invitrogen, Carlsbad, USA). Primer sets for following genes were used for RT-PCR.

β-*actin*: 5′-GTCGTACCACAGGCATTGTGATGG-3′ and 5′-GCAATGCCTGGGTACATGG TGG-3′; *Sp-C*: 5′-GGACATGAGTAGCAAAGAGG-3′ and 5′-TAGAGTGGTAGCTCTCCAC; *Nkx2.1*: 5′-AACCTGGGCAACATGAGCGAGCTG-3′ and 5′-ATCTTGACCTGCGTGGGTGT CAGG-3′; CK19: 5′-GCAGAACCGGAAGGATGCT-3′ and 5′-TCCGTTTCTGCCAGTGTGT C -3′; *N-myc*: 5′-CAATGGTGCTTAAGTTCCAG-3′ and 5′-AGTGCTCATAAGGTGCAGCA -3′; *Shh*: 5′-GTGGCCGAGAAGACCCTAG-3′ and 5′-AGACGTGGTGATGTCCACTG-3′; *Sox9*: 5′-TGAAGAAGGAGAGCGAGGAAGATAA-3′ and 5′-GGTGGCAAGTATTGGTCAA ACTCA-3′; *Tff3*: 5′-ATGGAGACCAGAGCCCTCTG-3′ and 5′-CAAAATGTGCATTCTGTC TCC-3′; *Bmp4*: 5′-ACAATGTGACACGGTGGGAAAC-3′ and 5′-TGTGGGTGATGCTTGG GACTAC-3′; *Pten*: 5′-CCACAAACAGAACAAGATGCTC-3′ and 5′-CCATTTTCCACTTTT TCTGAGG-3′; Primer sets for following genes were used for Real-time RT-PCR: *Pten*: 5′-CCACAAACAGAA-CAACAAGATGCTC-3′ and 5′-TTCCATTTTCCACTTTTTC TGAG-3′; *Egr1*: 5′-GACGAGT-TATCCCAGCCAAA-3′ and 5′-GGCAGAGGAAGACGA TGAAG-3′. Real-time RT-PCR was performed by a LightCycler System (Roche).

Results

TGFβ1 does not inhibit branching morphogenesis in embryonic lung explant culture

As a first step toward analyzing the impact of TGFβ on lung morphogenesis, we attempted to recapitulate the previously reported findings, using embryonic whole lung explants (Zhao et al., 1999a; Serra et al., 1994). E11.5 embryonic mouse lungs were excised from mouse embryos and cultured in a serum-free medium (Minoo et al., 1995) in presence or absence of 10 or 50 ng/ml TGFβ1 ligand (Fig. 1, Panels A to C). At 48 and 96 h, cultures were terminated and the individual lung explants removed and analyzed for overall branching morphogenesis and gene expression. Contrary to previous findings (Serra et al., 1994; Zhao et al., 1999a), exposure to TGFβ for 48 h had little if any discernible impact on branching morphogenesis of E11.5 embryonic lungs. The measured average number of terminal branches in two set of lungs, one consisting of 8 and the other 10 embryonic lungs treated respectively with 10 ng/ml or 50 ng/ml of TGFβ1 was 13

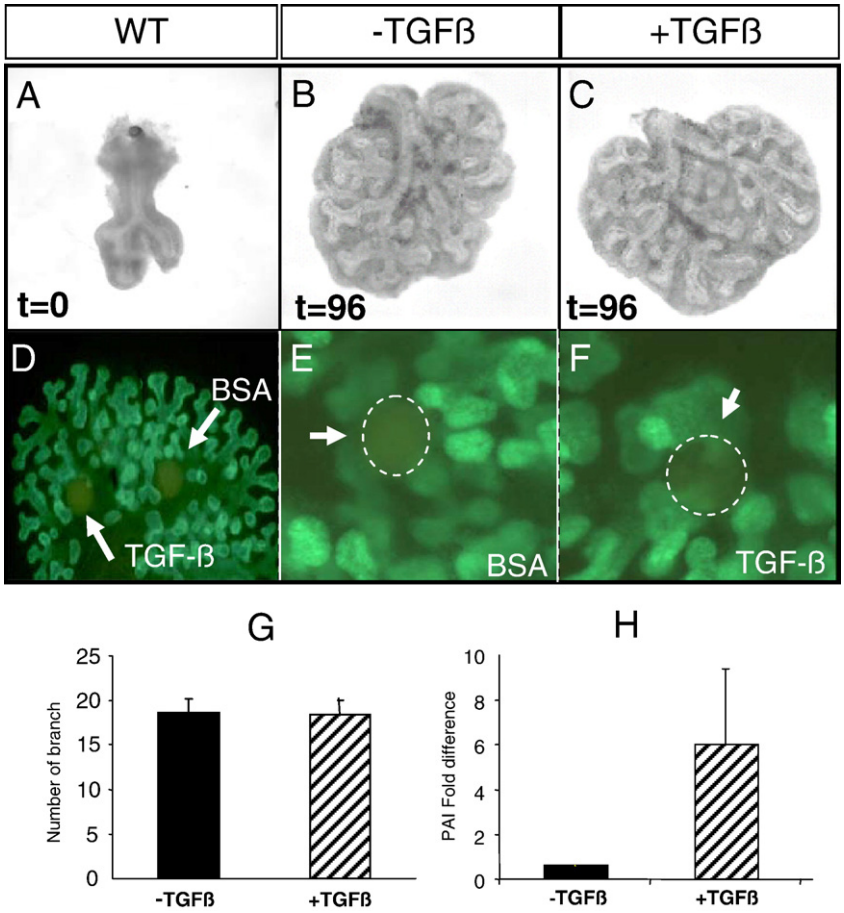

Fig. 1. TGFβ does not inhibit branching morphogenesis of lung explants. E11.5 mouse embryonic lungs (Panel A) underwent morphogenesis after 96 h in explant culture in defined serum-free medium (Panel B). TGFβ had little, if any impact on branching morphogenesis (Panel C). No differences in branching were observed after 48 h between *SpC-Gfp* lungs embedded with TGFβ- or BSA-soaked beads (Panels D–F). Panels E and F are high magnification of Panel D. Arrows show the location of the beads. Branching morphogenesis was quantified by determination of the number of terminal branch points (Panel G). Real-time PCR analysis of Plasminogen Activator Inhibitor 1 (PAI-1) in presence or absence of 50 ng/ml TGFβ ligand (Panel H).

and 14, respectively. In comparison, 6 embryonic lung explants in the control group without TGFβ1 treatment showed an average of 14 branches (data not show). Similarly, exposure of E11.5 embryonic lungs for 4 days to TGFβ1 had little effect on branching morphogenesis. The average number of terminal branches in the 3 lungs treated for 4 days was measured to be 19. An exactly identical average number of terminal branch points was measured for the lungs in the control group (Fig. 1, Panel G).

To make certain that the TGFβ ligand added to the cultured lungs was functional we measured mRNA for Plasminogen Activator Inhibitor 1, PAI-1, a well-established downstream target of TGFβ (Liu et al., 1997). Competitive real-time PCR quantification revealed that PAI-1 mRNA was increased by nearly 10 fold in lungs treated with TGFβ, compared with untreated controls (Fig. 1, Panel H). The validity of the explant culture results was further tested by implantation of TGFβ-soaked (Bovine Serum Albumin as control) heparin beads in lung explants. To refine the visualization of the endodermal branches we used E11.5 embryonic lungs from transgenic *SpC-Gfp* (Green Fluorescent Protein) mice. As before, no significant differences in branching morphogenesis were detectable between the surrounding lung tissue containing TGFβ1 beads (Fig. 1, Panel F) versus BSA beads (Fig. 1, Panel E).

TGFβ inhibits FGF10-induced morphogenesis of mesenchyme-free lung endoderm in explant culture

Because contrary to what has been reported (Serra et al., 1994; Zhao et al., 1999a) TGFβ did not inhibit branching morphogenesis in whole embryonic lung explants, we examined the response of mesenchyme-free endoderm to all three isoforms of TGFβ. Mesenchyme-free nascent endodremal tissue can be isolated from whole mouse embryos on day 12.5 of gestation (E12.5) and explanted in Matrigel for various studies (Bellusci et al., 1997). This model offers a number of advantages over the whole lung explant. For example, as the endoderm in whole lung explants is surrounded by mesenchyme, molecules under investigation (e.g. growth factors) may never penetrate through this layer to reach the endodermal cells to elicit their physiologic effects. In addition, this model allows for direct visualization of the behavior of the endoderm in response to various experimental factors. Culturing of endodermal explants requires the presence of FGF10, which is necessary and sufficient to direct morphogenesis (Bellusci et al., 1997b). In the absence of exogenous FGF10, the endodermal explants fail to proliferate and rapidly undergo necrosis. Thus, in the following studies what is precisely examined is the interaction between FGF10 and TGFβ isoforms in isolated lung endodermal explants. Overall, a total of 44 mesenchyme-free endodermal explants were cultured in Matrigel in groups of 4 in presence or absence of 10 ng/ml of each of the three TGFβ ligands. All explant cultures contained 400 ng/ml of FGF10 ligand for survival and induction of morphogenesis as described previously (Bellusci et al., 1997). Fig. 2 shows the results of these studies at times 0 and 48 h. As expected, FGF10 had a robust impact on induction of morphogenesis in E12.5 wild-type lung endoderm (Fig. 2, compare Panels A and E). Without exception, presence of any of the three TGFβ isoforms at 10 ng/ml strongly inhibited FGF10-induced morphogenesis of the endodermal explants (Fig. 2, compare Panels E to F–H). Doses as

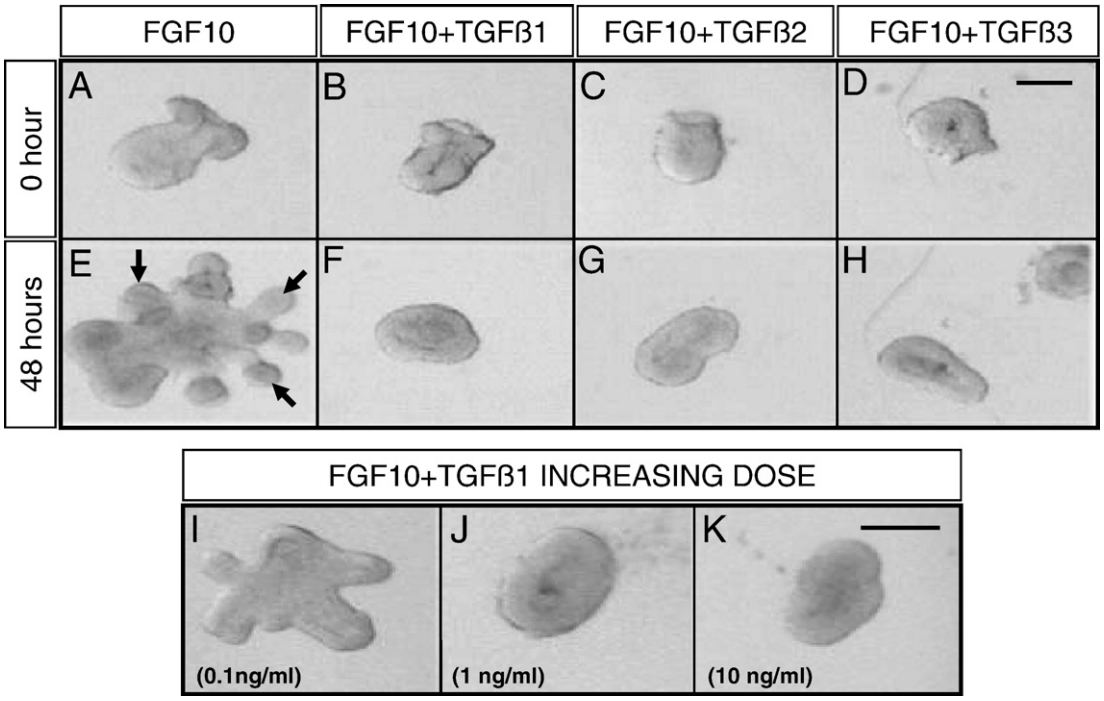

Fig. 2. All three TGFβ ligands inhibit FGF10-induced morphogenesis of mesenchyme-free lung endodermal explants. Isolated endodermal explants from E12.5 mouse embryos were cultured in presence or absence of 10 ng/ml of each of the three TGFβs. Panels A–D show representative explants at time zero. Panels E–H show the same explants after 48 h of culturing. FGF10 induced endodermal morphogenesis in the absence of TGFβ (Panel E, arrows). Doses as low as 1 ng/ml or as high as 10 ng/ml of TGFβ profoundly inhibited FGF10-induced morphogenesis (Panels J and K). A dose of 0.1 ng/ml of TGFβ showed partial inhibition (Panel I).

low as 1 ng/ml of TGFβ1 effectively inhibited endodermal budding in Matrigel cultures (Fig. 2, Panels J to K).

TGFβ inhibition of FGF10-induced endodermal morphogenesis is rescued by TβRII deficiency

To determine the mechanisms of TGFβ-induced inhibition of endodermal morphogenesis, we analyzed the cell surface and intracellular components of the TGFβ pathway using genetically modified endodermal tissues. At the cell surface, TGFβ ligand binding stabilizes a complex consisting of type I and type II receptors. The role of type II receptor, TβRII is pivotal as it mediates the phosphorylation and activation of SMAD2 and SMAD3, which can then bind to SMAD4, enter the nucleus and activate or repress target genes. We therefore, examined the role of SMAD3, SMAD4 and TβRII in the TGFβ inhibition of FGF10-induced endodermal morphogenesis. For this purpose, lung endodermal explants were isolated from E12.5 *Smad3*$^{-/-}$ embryos or embryos conditionally deleted for *Smad4* or TβRII. Conditional deletion of *Smad4* [referred to here as *Smad4*$^{\Delta/\Delta}$] or TβRII [TβRII$^{\Delta/\Delta}$] was achieved by the use of an *Nkx2.1*-cre/loxP system. Recombination by *Nkx2.1*-cre was verified by PCR genotyping as described previously for each of the Floxed genes used in this study (Please see Materials and methods and Fig. 3).

Mesenchyme-free lung endodermal explants from *Smad3*$^{-/-}$, *Smad4*$^{\Delta/\Delta}$ or TβRII$^{\Delta/\Delta}$ embryos were then treated with FGF10 in presence or absence of TGFβ In presence of FGF10 alone, morphogenesis of *Smad3*$^{-/-}$ endoderm was somewhat, but not significantly reduced compared to the wild-type controls (Fig. 4. compare Panels I and K). Whereas little to no bud formation could be detected in the wild-type endoderm exposed to a combination of FGF10 plus TGFβ (Fig. 4, Panel J), those from the *Smad3*$^{-/-}$ embryos showed definitive evidence of morphogenesis (Fig. 4, Panel L). However, deletion of *Smad3* did not completely restore budding to the extent found in the wild-type endoderm. Similarly, *Smad4*$^{\Delta/\Delta}$ endodermal explants branched in the absence of TGFβ (Fig. 4, Panel M), but were only partly resistant to TGFβ inhibition (Fig. 4, Panel N). Therefore, TGFβ inhibition is only partly mediated through the activity of SMAD3 or SMAD4. We did not deem it necessary to examine the role of SMAD2, primarily due to the partial

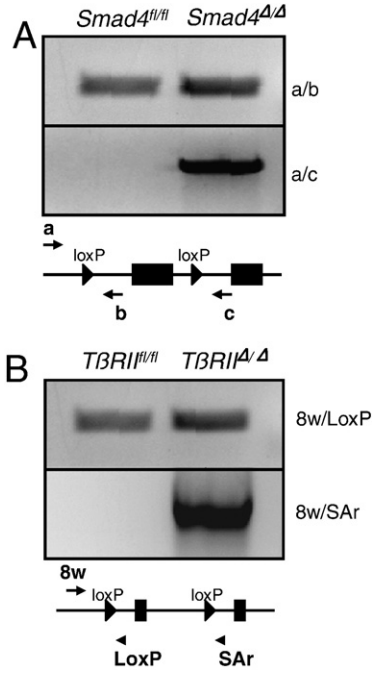

Fig. 3. Generation and validation of Smad4 and TβRII conditional knock-out alleles. Lung endodermal-specific inactivation of *Smad4* (Panel A) and TβRII (Panel B) via recombination by *Nkx2.1-cre* was detected by PCR analysis as described in the Materials and methods. DNA isolated from control and mutant lungs was analyzed using primers that were previously described (Yang et al., 2002; Chytil et al., 2002). The PCR products and the identity of the genotypes are as follows: a/b = *Smad4*$^{fl/fl}$, a/c = *Smad4*$^{\Delta/\Delta}$, 8w/LoxP = TβRII $^{fl/fl}$ and 8w/SAr = TβRII $^{\Delta/\Delta}$.

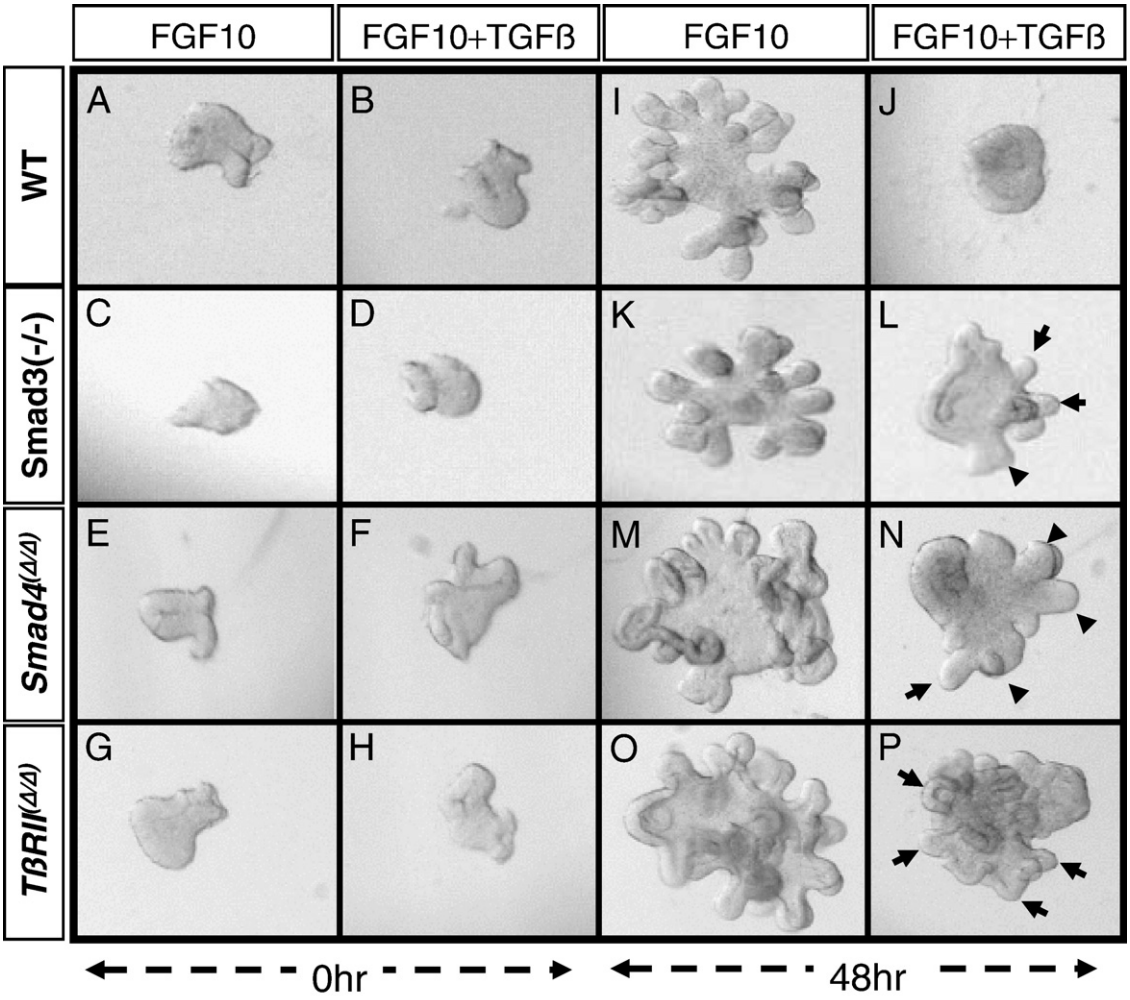

Fig. 4. Analysis of the role of TGFβ pathway components in TGFβ-induced inhibition of endodermal morphogenesis. Endodermal explants from E12.5 wild type, Smad3$^{-/-}$, Smad4$^{\Delta/\Delta}$ and TβRII$^{\Delta/\Delta}$ embryos were cultured in presence of 400 ng/ml FGF10, plus or minus 10 ng/ml of TGFβ. Panels A–H show the explants at time zero. After 48 h in FGF10 alone, all endodermal explants underwent robust morphogenesis (Panels I, K, M and O). Whereas TGFβ had a profound inhibitory impact on wild-type endodermal branching (Panel J), endoderm from Smad3$^{-/-}$ and Smad4$^{\Delta/\Delta}$ lungs showed partial resistance to inhibition (Panels L and N). TβRII $^{\Delta/\Delta}$ endoderm was refractive to TGFβ inhibition (Panel P). Arrows show the sites of budding.

rescue results obtained in *Smad4*$^{\Delta/\Delta}$ endoderm. Finally, we sought to interrupt TGFβ signaling by conditional, endodermal-specific deletion of TβRII. Mesenchyme-free lung endodremal explants from TβRII$^{\Delta/\Delta}$ E12.5 embryos showed robust endodermal morphogenesis when exposed to FGF10 (Fig. 4, Panel O) and were almost entirely refractive to the inhibitory effect of TGFβ (Fig. 4, Panel P).

TGFβ1 inhibition of FGF10-induced budding is associated with repression of Nkx2.1

To ascertain the impact of TGFβ treatment on cell differentiation we used immunohistochemistry to examine the distribution of NKX2.1, a homeodomain transcription factor that is an early marker of lung endodermal progenitor cell specification. Robust expression of NKX2.1 could be detected in endodermal cells explanted in presence of FGF10 alone (Fig. 5, Panel B). Addition of TGFβ for 48 h reduced NKX2.1 protein in endodermal cells (Fig. 5, Panel C). A downstream target of NKX2.1 in embryonic lung endoderm is *SpC*, which encodes pulmonary surfactant protein C. *SpC* expression in the lung is strictly dependent on NKX2.1 (Minoo et al., 1999). Therefore, we speculated that repression of NKX2.1 should also result in reduced or absent SPC in the endodermal explants treated with TGFβ. Using endodermal explants from *SpC-Gfp* transgenic embryos, we examined by fluorescence microscopy the activity of the *SpC* promoter in response to TGFβ. GFP levels were readily detected throughout the lung endoderm, explanted in presence of FGF10 alone (Fig. 5, Panel E). In explants treated with FGF10, plus TGFβ, little if any fluorescence could be detected, suggesting that the *SpC* promoter activity is inhibited by TGFβ (Fig. 5, Panel F). The reduced expression of *Nkx2.1* and *SpC* were further verified by semi-quantitative PCR analysis (Fig. 7).

Another downstream target of NKX2.1 (Zhu et al., 2004) and a key mediator of endodermal morphogenesis in the lung is bone morphogenetic protein 4 (Weaver et al., 2003). We used a *Bmp4*$^{LacZ/+}$ reporter knock-in mouse line to localize *Bmp4*-dependent LacZ expression in the lung endodermal explants. As expected, in FGF10-treated explants, *Bmp4* showed a restricted pattern of expression in the terminal tips of the budding endoderm (Fig. 5, Panel H). In explants treated with TGFβ inhibition of morphogenesis was accompanied by absence or reduced levels of *Bmp4* (LacZ) gene expression (Fig. 5, Panel I). Thus, TGFβ also represses *Bmp4* gene expression in embryonic endodermal explants.

TGFβ1 inhibits FGF10-induced cell proliferation

TGFβ is known to stimulate apoptosis (Bright et al., 1997) and inhibit cellular proliferation (Goey et al., 1989). To examine whether TGFβ inhibition of FGF10-induced endodermal morphogenesis

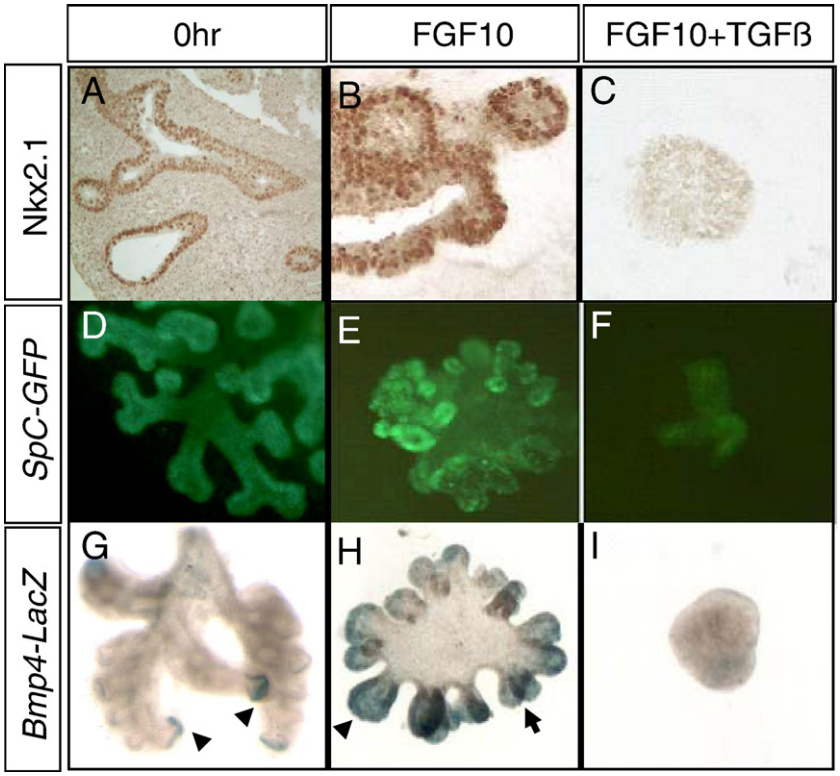

Fig. 5. TGFβ represses morphogenetic gene expression in embryonic endodermal explants. Immunohistochemistry showed significant reduction in NKX2.1 transcription factor in TGFβ-treated endodermal explants (compare Panel C to A or B). Similarly, TGFβ reduced expression of two NKX2.1 target genes, SpC and Bmp4 as documented by reduced fluorescence in Spc-Gfp, (compare Panel F to D or E) and reduced LacZ staining in $Bmp4^{LacZ/+}$ endodermal explants (compare Panel I to G or H). Arrows show positive LacZ staining at the tip of buds.

observed in Fig. 2 is accompanied by changes in cellular proliferation, we used the BrdU labeling approach. These analyses showed that when compared to FGF10 alone, explants treated with FGF10 plus TGFβ had approximately 25% to 35% decrease in cellular proliferation (Fig. 6, Panels A–D and H). The endodermal explants were also analyzed for apoptosis by TUNEL staining. We found no evidence of apoptosis as assessed by TUNEL in either FGF10 treated explants (Fig. 6, Panel C) or those treated with FGF10+TGFβ (Fig. 6, Panel F). E11.5 wild-type whole lung section was used as positive control (Fig. 6, Inset Panel G). Therefore, TGFβ does not appear to impact apoptosis.

Pten mediates the effect of TGFβ-induced inhibition of cell proliferation

Semiquantitative PCR analysis of mRNA was conducted for a number of genes including *Nkx2.1*, *SpC* and *Pten*, a lipid phosphatase closely associated with regulation of cell proliferation (Andrés-Pons et al., 2007). The results for *Nkx2.1* and *SpC* confirmed the observations in Fig. 7; *SpC* mRNA became undetectable, whereas a nearly 74% reduction was observed in *Nkx2.1* mRNA in response to TGFβ treatment. Importantly, the PCR analysis showed increased *Pten* mRNA in the endodremal explants treated with TGFβ (Fig. 7). Thus, we hypothesized that the inhibitory activity of TGFβ on endodermal cell proliferation may be mediated through increased *Pten*. To test this hypothesis, we first validated the PCR results by precise measurement of mRNA for *Pten* and its transcriptional regulator EGR1 (Okamura et al., 2005; Virolle et al., 2001). Real-time PCR analysis showed a 4-fold increase in *Pten* mRNA in endodermal explants treated with TGFβ (Fig. 8, Panel A). Treatment with TGFβ also increased *Egr1* mRNA level by nearly 14 fold (Fig. 8, Panel B). To test the functional involvement of *Pten*, the response to TGFβ of lung endodermal explants from conditionally deleted *Pten Nkx2.1-cre*-driven recombination was examined (Fig. 9). Deletion of one copy of *Pten* gene [$Pten^{\Delta/+}$] in the endoderm was sufficient to cause major changes in response to FGF10 alone. In contrast to the control [$Pten^{flox/flox}$] which showed normal morphogenesis when treated with FGF10, only marginal budding was observed in $Pten^{\Delta/+}$ endoderm (Fig. 10, Panel G). This observation suggested that even reduced *Pten* interferes with normal processes involved in morphogenesis (haploinsufficiency) at least in the mesenchyme-free endodermal explant culture model. Consistent with this conclusion, homozygous deletion of *Pten* [$Pten^{\Delta/\Delta}$], entirely abrogated budding altogether (Fig. 10, Panel K). Importantly however, both heterozygous and homozygous deletion of *Pten* overcame the TGFβ-induced inhibition of cell proliferation as assessed by size of the explant and BrdU labeling index measurements (Fig. 11). This finding demonstrates that the PTEN/AKT pathway plays a key role in the TGFβ-induced inhibition of lung endodermal cell proliferation.

Discussion

The current study examined the role of TGFβ in lung morphogenesis in explant culture. Treatment of mouse embryonic lungs had little discernible effect on the process of branching morphogenesis. However, morphogenesis of mesenchyme-free lung endodermal explants in response to FGF10 was profoundly inhibited by concentrations as low as 1 ng/ml of all three isoforms of recombinant TGFβ ligands. TGFβ inhibited endodermal morphogenesis concomitant with repression of *Nkx2.1* and its downstream targets *SpC* and *BMP4.* Deletion of either *Smad3* or *Smad4* only partially relieved the inhibitory effect of TGFβ, whereas conditional deletion of *TβRII* completely blocked it. TGFβ inhibited endodermal cell proliferation in association with increased expression of *Pten*. Endodermal-specific deletion of *Pten* inhibited morphogenesis in the absence of TGFβ. Importantly however, $Pten^{\Delta/\Delta}$ endodermal explants were refractory to TGFβ-induced inhibition of cell proliferation. Therefore, TGFβ-

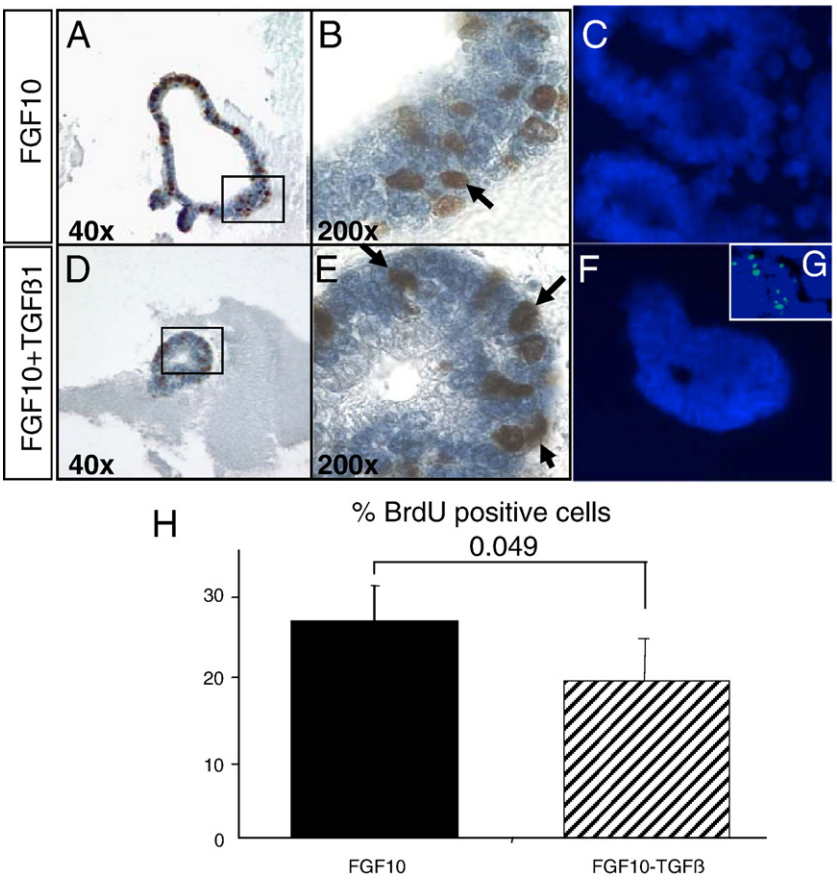

Fig. 6. TGFβ represses endodermal cell proliferation. Endodermal explants cultured in presence of FGF10 (400 ng/ml) (Panels A–C) or FGF10 (400 ng/ml))+TGFβ (10 ng/ml) (Panels D–F) were assayed for cell proliferation by BrdU labeling (Panels A, B, D and E). Panels B and E are high magnification of Panels A and D (arrows show BrdU positive cells). Panel H shows quantification of the BrdU positive cells (*p* value of 0.049). TUNEL analysis showed no detectable apoptosis (Panels C and F). Panel G (inset) shows positive control for apoptosis in whole lung explant culture.

induced inhibition of morphogenesis is mediated through TβRII signaling and likely repression of *Nkx2.1*, but is not entirely Smad-dependent. Inhibition of cell proliferation on the other hand, as a component of TGFβ's inhibitory role on morphogenesis is in large part due to its impact on the PTEN/AKT pathway.

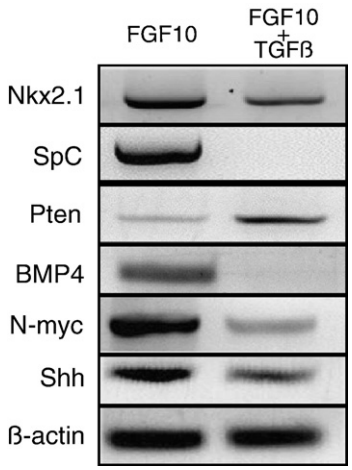

Fig. 7. Analysis of gene expression in endodermal explants treated with TGFβ. RNA isolated from E12.5 wild-type endodermal explants treated with FGF10 or FGF10+TGFβ for 2 days was analyzed by semi-quantitative RT-PCR. mRNA for SpC and Bmp4 became undetectable, whereas *Nkx2.1*, N-myc and Shh mRNA were reduced. *Pten* mRNA was increased.

Contrary to previous reports (Zhao et al., 1999a; Serra et al., 1994), we found no inhibition of branching by 10 or 50 ng/ml of TGFβ in whole lung explants. Serra et al. (1994) reported TGFβ inhibition at relatively high doses (30 ng/ml to100 ng/ml). Thus, the observed phenotype may simply reflect toxic/pathologic consequences of high TGFβ concentrations. The TGFβ concentration used in our studies was the same as that used by Zhao et al (1999a). Plasminogen activator inhibitor, PAI-1 is induced by TGFβ in a number of cell types (Liu et al., 1997). The finding by Real-time PCR that TGFβ increased *PAI*-1 mRNA in whole lung explants, confirmed that the TGFβ in our studies was indeed functional. Exposure of embryonic lungs in culture to TGFβ expressing adenoviruses also had no impact on branching morphogenesis, whereas injection of viruses into the lumen of the embryonic lungs did (Zhao et al., 1999b). It is therefore possible that recombinant TGFβ, much like the TGFβ-adenoviruses does not diffuse adequately through the mesenchyme to reach the endodermal layer. This is the most likely explanation for the discrepancy between our findings and the other two previous reports. The latter is also consistent with the report by Zhou et al who found inhibition of lung morphogenesis in transgenic lungs over-expressing TGFβ specifically in the lung epithelial layer (Zhou et al., 1996).

In the current study, we utilized a mesenchyme-free endodermal explant model to examine the direct impact of TGFβ on isolated murine lung endoderm. TGFβ inhibited FGF10-induced endodermal morphogenesis in association with alterations in specific gene expression. A notable change occurred in the level of NKX2.1, a homeodomain transcription factor whose activity is critical for lung endodermal morphogenesis in vivo (Minoo et al., 1995, 1999; Kimura et al., 1999). In *Nkx2.1*$^{-/-}$ embryos, FGF10 is produced by the

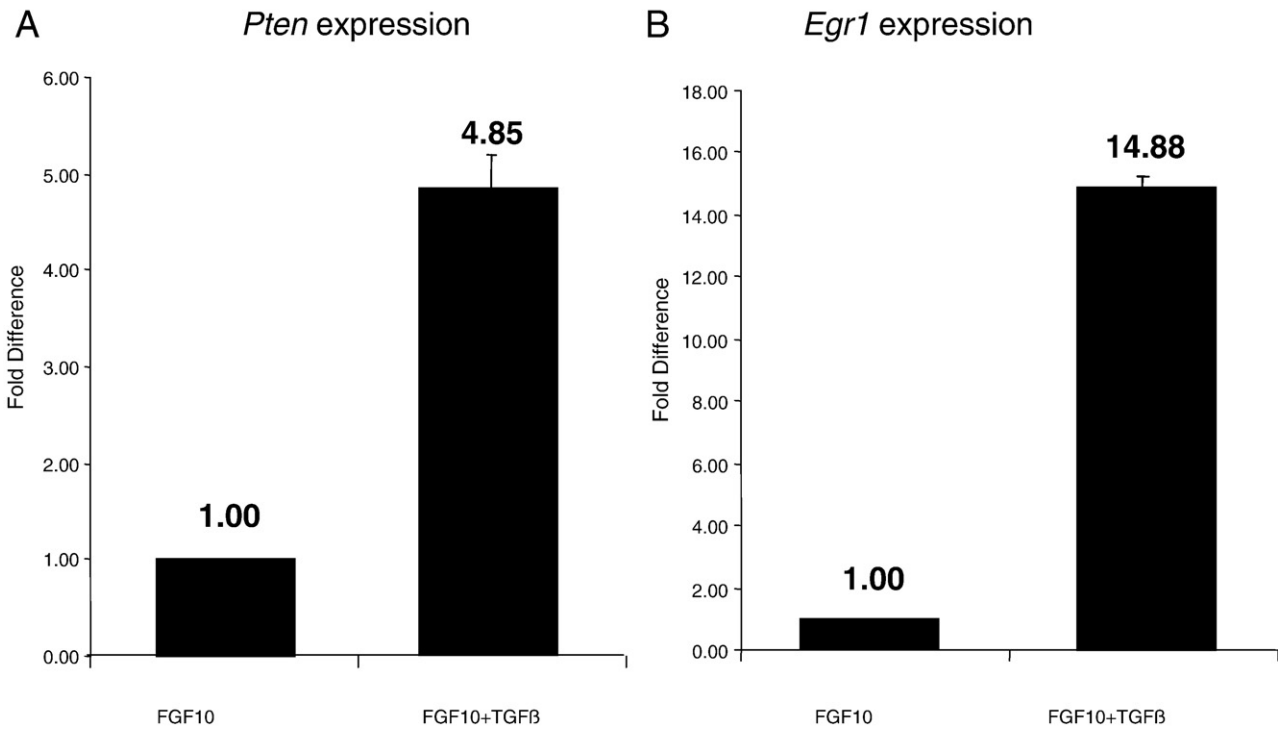

Fig. 8. Real-time PCR quantification of *Pten* and Egr1 mRNA in endodermal explants. RNA from endodermal explants treated with FGF10 alone or FGF10+TGFβ, after 2 days in culture was analyzed for *Pten* (Panel A) and *Egr1* (Panel B) gene expression by Real-time PCR. Both *Pten* and Egr1 mRNA were increased.

mesenchyme, but endodermal branching fails to occur. A role for NKX2.1 may be to interpret and implement mesenchymal FGF10 instructional signaling. In our studies, we found significant reduction in NKX2.1 protein by immunohistochemistry. This finding was confirmed by PCR analysis of mRNA (Fig. 7). Furthermore, expression of a lung-specific target of NKX2.1, *SpC* was also profoundly inhibited by TGFβ (Figs. 5 and 7). The latter occurs either as a consequence of changes in the level of NKX2.1 and/or indirectly through interactions between NKX2.1 and Smad3 as we have previously shown in cell culture for another NKX2.1 target, *SpB* (Li et al., 2002). Another downstream target of NKX2.1 in the lung endoderm is *Bmp4* (Zhu et al., 2004). *Bmp4* is thought to be induced by FGF10 and dynamic interactions between the two signaling pathways may be necessary for normal endodermal branching (Weaver et al., 2000). Using endoderm from $Bmp4^{LacZ/+}$ mouse embryo, we found that treatment with TGFβ strongly inhibited *Bmp4*. Inhibition of *Bmp4* through over-expression of Noggin, its cellular inhibitor or a dominant negative *Bmp4* receptor results in abnormal branching morphogenesis (Eblaghie et al., 2006). Other regulators of branching morphogenesis whose expression decreased in the lung endoderm by TGFβ were *Shh* and *N-Myc* (Fig. 8). SHH, the vertebrate homologue of Drosophila HH, which is highly expressed by embryonic lung endoderm is a major signaling pathway in lung morphogenesis. SHH is thought to control *Fgf10* gene expression in the lung mesenchyme. Targeted disruption of *Shh* leads to arrested endodermal branching in mouse embryos (Pepicelli et al., 1998). The mechanism by which TGFβ represses *Shh* remains to be elucidated.

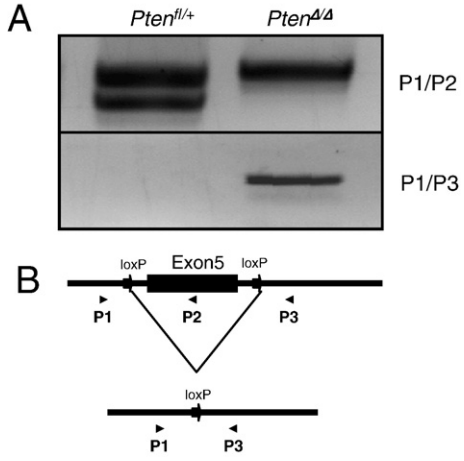

Fig. 9. Inactivation of mouse *Pten* gene and generation of $Pten^{\Delta/\Delta}$ allele. Tissue-specific inactivation of the $Pten^{\Delta/\Delta}$ conditional allele by *Nkx2.1-cre* transgene was detected by PCR analysis (Panel A). Primers for recombination analysis (Panel B) were designed as described by Freeman et al., 2006. The PCR products and the identity of the genotypes are as follows: P1/P2, upper band in $Pten^{\Delta/\Delta}$ lungs is the result of mesenchymal DNA amplification. P1/P3 primers amplify only the deleted *Pten* allele.

Many biological effects of TGFβ are ascribed to SMAD-dependent pathways. In particular, TGFβ inhibition of cell differentiation in osteoblasts and muscle progenitor cells is mediated by SMAD3 (Aliston et al., 1994; Liu et al., 2001). In the lung, although normal embryonic development is not perturbed in $Smad3^{-/-}$ mice, adult animals develop an emphysema like condition (Datto et al., 1999; Chen et al., 2005; Bonniaud et al., 2004). In contrast, absence of *Smad3* in mutant mice attenuates *bleomycin*-induced pulmonary fibrosis indicating at least a partial role for SMAD3 (Zhao et al., 2002). In pulmonary adenocarcinoma cells, SMAD3 and not SMAD2 mediates the TGFβ inhibition of *SpB* transcription via interactions with NKX2.1 which reduces the binding affinity of NKX2.1 for its target DNA elements (Li et al., 2002). The results of the current study indicate that the role of SMAD3 in TGFβ-induced inhibition of lung endodermal morphogenesis, at least in explant culture is limited. Absence of SMAD3 resulted in some protection from TGFβ-induced inhibition, but did not restore normal branching. Since another potential pathway is that which is activated by SMAD2, we opted to abrogate the entire SMAD-dependent pathway by targeted, conditional deletion of *Smad4*. Endodermal explants from $Smad4^{\Delta/\Delta}$ embryos also exhibited only partial restoration of

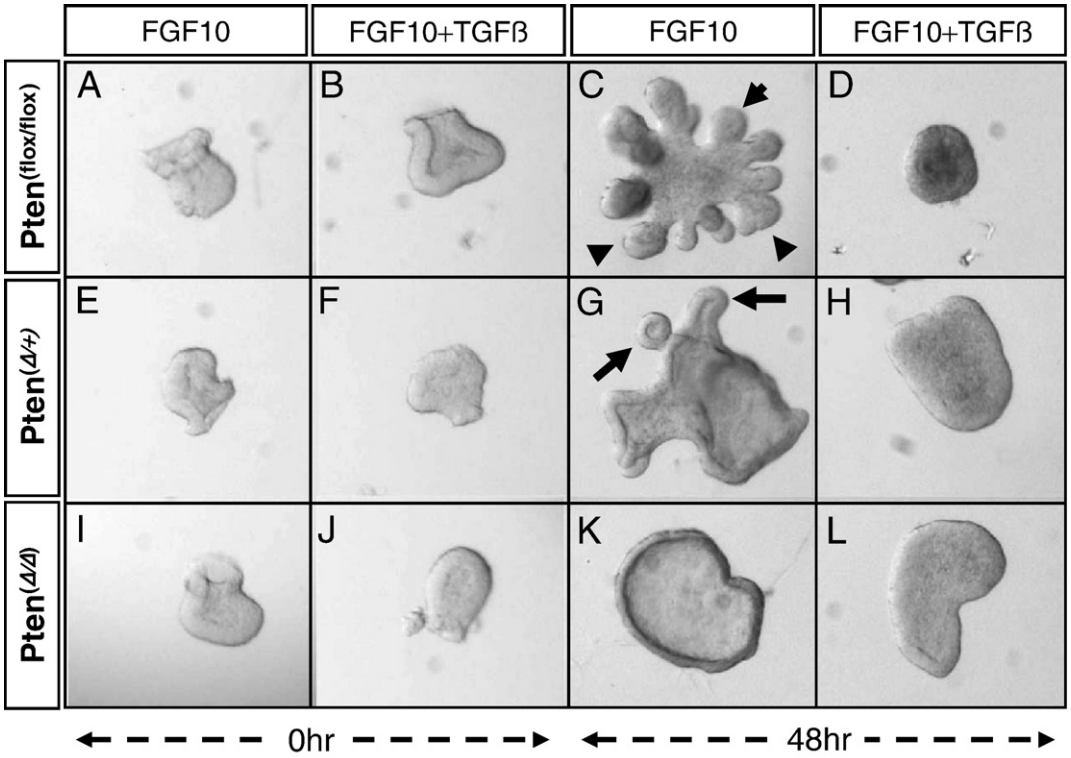

Fig. 10. *Pten* mediates the effect of TGFβ-induced inhibition of cell proliferation. Endodermal explants from E12.5 $Pten^{\Delta/\Delta}$ lungs were treated with FGF10 (400 ng/ml) alone or FGF10 (*400 ng/ml*) + TGFβ (*10 ng/ml*). Explants at time 0 h are shown in Panels A, B, E, F, I and J. Culturing for 48 h showed robust morphogenesis of wild-type explants in presence of FGF10 alone (Panel C) and profound inhibition in response to TGFβ (Panel D). Compared to wild-type explants, both $Pten^{\Delta/flox}$ (Panel G) and $Pten^{\Delta/\Delta}$ (Panel K) endodermal explants showed significant decrease in FGF10-induced morphogenesis. TGFβ did not inhibit the growth of either $Pten^{\Delta/flox}$ or $Pten^{\Delta/\Delta}$ explants (Panels H and L, respectively). (Arrows & and arrowheads show sites of budding).

morphogenesis. These results argue strongly that SMAD-dependent pathways do not play a major role in TGFβ-induced inhibition of endodermal morphogenesis.

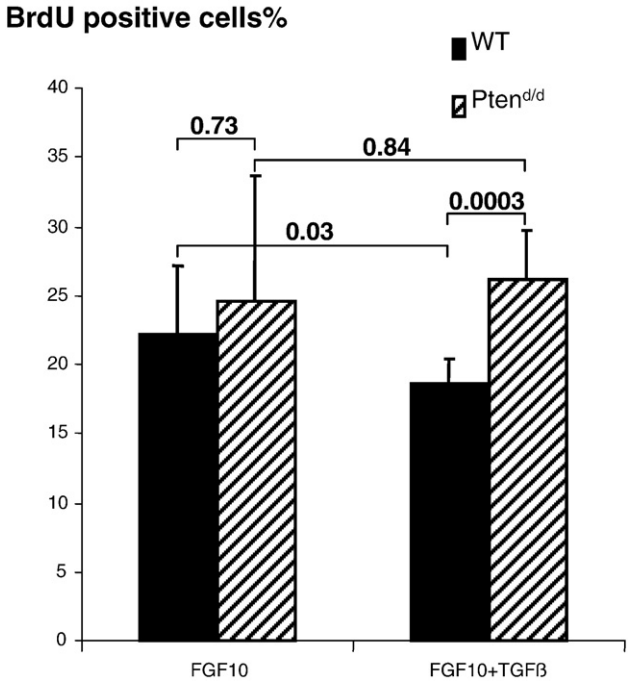

Fig. 11. TGFβ does not inhibit $Pten^{\Delta/\Delta}$ endodermal cell proliferation. Endodermal explants were treated with BrdU for 3 h. Immunohistochemistry was used to identify the BrdU positive cells, which were counted on multiple random fields. Percentages of BrdU positive cells in total cells are shown. *P* values are given above bars that indicate comparisons between samples.

Recent findings have indicated that certain effects of TGFβ, including its classical impact on induction of fibronectin as well as the ability to inhibit cell proliferation may involve SMAD-independent pathways (Niculescu-Duvaz et al., 2007; Giehl et al., 2000). Thus, TGFβ activates other signaling cascades, including ERK, JNK and P38 MAP kinases. Some of these pathways regulate SMAD activation but others might induce responses unrelated to transcription (Engel et al., 1999). In addition, mutated TGFβ type I receptor, defective in SMAD activation, nevertheless activate p38 MAPK signaling in response to TGFβ (Yu et al., 2002). Extreme sensitivity of the isolated embryonic lung endoderm to toxicity from MAPK inhibitors, as an experimental tool, did not permit investigation of the potential role of this pathway. Undoubtedly, elucidating the links between TGFβ-MAPK and FGF-MAPK may reveal additional mechanisms for TGFβ1 inhibition of FGF-induced budding morphogenesis in lung development.

Growth inhibitory effects of TGFβ have been recognized. This can be accomplished either by increased apoptosis or decreased cellular proliferation (Yamamura et al., 2000). For example, TGFβ inhibits proliferation by blocking cyclin and cyclin-dependent kinases (Datto et al., 1995) or by directly suppressing *c-Myc* expression (Pietenpol et al., 1990). Treatment of lung endodermal explants with TGFβ did not change the incidence of endodermal apoptosis (Fig. 6), but reduced cellular proliferation by 25 to 35% when compared to untreated tissue (Fig. 6). Concomitant with this decrease, TGFβ also significantly repressed the expression of *N-myc* that encodes a basic helix-loop-helix-leucine zipper (bHLHZ) transcription factor (Serra et al., 1994). Normal expression of *N-myc* is essential for lung development through its impact on cell proliferation and branching morphogenesis (Moens et al., 1992, 1993; Sawai et al., 1993; Stanton et al., 1992).

A notable finding of the current study is the involvement of PTEN, a phosphatase cancer suppressor, in TGFβ-induced inhibition of lung endodermal cell proliferation. Direct evidence for the latter was derived by examining the response of $Pten^{\Delta/\Delta}$ endodermal explants to

TGFβ. Deletion of *Pten* restored cell proliferation in explants treated with combination of FGF10 and TGFβ (Figs. 10 and 11). Initial identification of *Pten* was based on its decreased expression in response to TGFβ in a human keratinocyte cell line, HaCaT (Li and Sun, 1997). In contrast to the latter, we found increased *Pten* and its upstream transcription factor *Egr1* in embryonic lung endoderm, treated with TGFβ. The reason for this discrepancy remains unknown, but it may simply reflect differences between normal, embryonic tissue used in the current study, compared to a transformed cell line in the previous work (Li and Sun, 1997). However, this finding correlates well with the known function of *Pten* as a regulator of cell proliferation through the PTEN/AKT pathway. PTEN antagonizes PI3K, which is activated downstream of tyrosine kinase receptors, a major growth factor signaling pathway (For Reviews, Goberdhan and Wilson, 2003; Stiles et al., 2004). Thus, increased expression of *Pten* is expected to block cell proliferation as we have found in endodermal explants treated with TGFβ. To our knowledge this is the first demonstration of the role of PTEN in TGFβ-induced inhibition of lung endodermal cell proliferation.

In summary, the TGFβ-induced inhibition of lung endodermal morphogenesis appears to entail at least two related mechanisms. First, TGFβ via stimulation of the transcription factor EGR1 and its target gene, *Pten* has a profound inhibitory effect on endodermal cell proliferation. Inhibition of cell proliferation undoubtedly slows, if not block morphogenesis. In addition, however, TGFβ has a direct impact on morphoregulatory genes, such as *Nkx2.1* and *Bmp4*, two molecules that are absolutely required for endodermal morphogenesis. Both of the latter mechanisms are dependent on intact TβRII-mediated signaling. The partial role of SMADs in this pathway is a novel finding that indicates a key role for alternative, SMAD-independent TGFβ signaling pathways.

Acknowledgments

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