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Misexpression of ELF5 Disrupts Lung Branching and Inhibits Epithelial Differentiation

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Abstract

ELF5, an Ets family transcription factor found exclusively in epithelial cells, is expressed in the distal lung epithelium during embryogenesis, then becomes restricted to proximal airways at the end of gestation and postnatally. To test the hypothesis that ELF5 represses distal epithelial differentiation, we generated a transgenic mouse model in which a doxycycline inducible HA-tagged mouse *Elf5* transgene was placed under the control of the lung epithelium-specific human *SFTPC* promoter. We found that expressing high levels of ELF5 during early lung development disrupted branching morphogenesis and produced a dilated epithelium. The effects of ELF5 on morphogenesis were stage dependent, since inducing the transgene on E16.5 had no effect on branching. ELF5 reduced expression of the distal lung epithelial differentiation markers *Erm*, *Napsa* and *Sftpc*, and type II cell ultrastructural differentiation was immature. ELF5 overexpression did not induce the proximal airway epithelial markers *Ccsp* and *Foxj1*, but did induce expression of p63, a marker of basal cells in the trachea and esophagus. High ELF5 levels also induced the expression of genes found in other endodermal epithelia but not normally associated with the lung. These results suggest that precise levels of ELF5 regulate the specification and differentiation of epithelial cells in the lung.

Keywords

lung development; Ets; ELF5; gene transcription; lung differentiation

INTRODUCTION

The mouse lung first appears at E9.5 as two outgrowths from the foregut endoderm that invade the adjacent splanchnic mesoderm. Following this initial event, the foregut endoderm undergoes stereotypic branching within the lung mesenchyme to form the basic structure of the pulmonary tree. Later in gestation and in early postnatal life, the lung goes through stages of distal airway expansion, distal and proximal airway cellular differentiation, septation and alveolarization. These events are all dependent on the correct spatiotemporal expression and

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regulation of genes and proteins such as transcription factors and secreted factors (Cardoso and Lu, 2006; Maeda et al., 2007; Warburton et al., 2005).

Secreted factors are known to be responsible for intercellular interactions that occur between the lung epithelium and mesenchyme (Shannon and Hyatt, 2004). For example, FGF10 secreted by the lung mesenchyme signals through FGFR2b in the epithelium and acts as a chemoattractant on the epithelium to drive branching morphogenesis (Bellusci et al., 1997; Hyatt et al., 2004; Min et al., 1998; Ohuchi et al., 2000; Park et al., 1998; Sekine et al., 1999) and to maintain a population of progenitors that later differentiate into the peripheral cell types of the lung (Hokuto et al., 2003; Perl et al., 2002b; Ramasamy et al., 2007). Other factors also influence the branching epithelium, notably BMPs and Wnts, which are important for distal/proximal specification of the epithelium as well as maintenance of lung epithelial progenitors (Bellusci et al., 1996; Dean et al., 2005; Eblaghie et al., 2006; Hyatt et al., 2002; Hyatt et al., 2004; Mucenski et al., 2003; Okubo and Hogan, 2004; Shu et al., 2005; Weaver et al., 2000; Weaver et al., 1999).

Secreted ligands such as FGF10 bind their receptors and initiate signaling cascades that activate downstream target genes. In a previous study we identified *Elf5* (*Ese2*) as a gene activated in the embryonic lung epithelium by FGF signaling (Metzger et al., 2007). ELF5 is a member of the Ese (Epithelial Specific Ets) family that was originally reported as being expressed in differentiating keratinocytes and branching epithelia such as salivary gland, prostate, mammary gland and kidney (Oettgen et al., 1999; Zhou et al., 1998). We found that *Elf5* expression was dynamic throughout lung development. During early branching morphogenesis *Elf5* expression was restricted to the distal lung epithelium, then became enriched in the proximal airway epithelium and diminished distally at the end of gestation and into postnatal lung development. From these results we hypothesized that ELF5 may initially act to regulate epithelial branching, then later in development increased ELF5 in the proximal epithelium may negatively regulate distal epithelial gene expression and differentiation. To test these hypotheses, we have now performed gain-of-function studies by conditionally expressing HA-tagged ELF5 under the control of the lung epithelium-specific human *SFTPC* promoter. We found that ELF5 misexpression disrupts branching morphogenesis and negatively regulates distal lung epithelial differentiation. Microarray analysis of lungs overexpressing ELF5 reveals the repression of some type II cell-specific genes, but the induction of others. High levels of ELF5 expression also induced the expression of genes not typically found in the distal lung epithelium, suggesting that ELF5 can influence eventual cell fate.

MATERIALS AND METHODS

Generation of HA-Elf5 mice

The full-length mouse *Elf5* cDNA was amplified from E12.5 lung by RT-PCR using primers 5’-CGGGATCCCGATGGCGTACCCATACGACGTGCCTGACTACGCCTCCCTCATGTTGGAACTCCGTA-3’ and 5’-CCATCGATGGCCATCAAATGAGCCTGGTGT-3’ to add an N-terminal HA-tag to the ELF5 protein and 5’ BamHI and 3’ ClaI cut sites to facilitate cloning. The PCR product was then ligated into the rtTA inducible pTRE-Tight vector (Clontech, Mountain View, CA) digested with BamHI and ClaI. The *pTRE-Tight-HA-Elf5* vector was cut with XhoI to give a fragment that was used for injection into FVB/N oocytes by the Cincinnati Children’s Hospital Research Transgenic Core Facility. Genotyping was performed by PCR on genomic DNA using 2 different primer sets. The first primer set, 5’-CGTATGTCGAGGTAGGCGTGTA-3’ and 5’-TCAGGTTCAGGGGGAGGTGTG-3’, was specific for the pTRE-Tight promoter while the second primer set, 5’-TACCCATACGACGTGCCTGAC-3’ and 5’-CCATCGATGGCCATCAAATGAGCCTGGTGT-3’ was specific for the *HA-Elf5* transgene.

Conditional expression of the *HA-Elf5* transgene to lung epithelium was accomplished by breeding *SFTPC-rtTA* mice (Perl et al., 2002a) to *HA-Elf5* mice (hereafter *SFTPC/Elf5* mice) and then treating the mice with doxycycline (Dox). Except as noted below, pregnant dams were treated with Dox from the day of conception. Transgene expression was traced using anti-HA immunohistochemistry (IHC). Littermates bearing a single transgene (*SFTPC-rtTA* or *HA-Elf5*) were used as controls.

Immunohistochemistry

Tissues were fixed in 4% paraformaldehyde or formalin, embedded in paraffin, and 5 μm sections were cut for IHC. Primary antibodies used were: HA (rabbit, 1:200, Santa Cruz Biotech; Santa Cruz, CA), mouse IgG1 (1:200, Cell Signaling; Danver, MA), ELF5 (goat, 1:50, Santa Cruz Biotech), phosphohistone H3 (pHH3; mouse, 1:500, Santa Cruz Biotech), Cleaved Caspase3 (rabbit, 1:5000; R & D; Minneapolis, MN), p63 (mouse 4A4, 1:50, Santa Cruz Biotech), Pro-SP-C (rabbit, 1:1000 and guinea pig, 1:1000), TTF1 (rabbit, 1:500), FoxA1 (guinea pig, 1:1000), FoxA2 (rabbit, 1:1000), CCSP (rabbit, 1:1000), FoxJ1 (rabbit, 1:5000). The antibodies against Pro-SP-C, TTF1, FoxA1, FoxA2, CCSP and FoxJ1 were generous gifts from Dr. Jeffrey Whitsett. Double IHC staining was performed using the ELF5 antibody with the rabbit Pro-SP-C antibody, or using the HA antibody with the guinea pig Pro-SP-C antibody. Biotinylated secondary antibodies were used with the ABC Vectastain kit (Vector Laboratories; Burlingame, CA). DAB with nickel enhancement and the AEC peroxidase substrate kit (Vector Laboratories) were used for visualization.

Proliferation analysis

The percentages of pHH3 positive epithelial and mesenchymal cells in E16.5 *SFTPC-rtTA* and *SFTPC/Elf5* lungs were counted after pHH3 staining. Specifically, three 20X micrographs of representative fields were taken from three different control or double transgenic lungs, and pHH3 positive and negative cells were counted. A minimum of 1000 epithelial cells and mesenchymal cells were counted per slide. Proliferation data were statistically analyzed using a Student's t-test on GraphPad Prizm version 4.0 (GraphPad Software, San Diego, CA).

Microarray analysis

Cysts from the lungs of E16.5 *SFTPC/Elf5* fetuses and spatially similar areas from *SFTPC-rtTA* control lungs were dissected, and RNA was isolated using an RNeasy Micro Kit (Qiagen, Valencia, CA). RNA from three *SFTPC/Elf5* lungs and three *SFTPC-rtTA* littermate lungs were used in the analysis. The microarray analysis was performed as previously described (Metzger et al., 2007). Differentially expressed genes between experimental and control samples were determined by performing a Student's t-test ($p \leq 0.05$) and filtering for genes that increased or decreased at least 2-fold. Differentially expressed genes were further compared to genes from a previously published microarray (Matsuzaki et al., 2006) using genes with two present calls in wild-type adult type II cells.

RT-PCR and Quantitative PCR

Adult alveolar type II cells were isolated by dispase digestion and differential adherence (Rice et al., 2002) and MLE15 cells, which are immortalized distal epithelial cells, were grown in HITES medium (Wikenheiser et al., 1993). RNA for RT-PCR was isolated and reverse transcribed into cDNA by standard methods. The primers used for RT-PCR were as follows:

Elf5, forward: 5'-GGACTCCGTAACCCATAGCA-3'

reverse: 5'-TACTGGTCGCAGCAGAATTG-3';

Sftpc, forward: 5'-CATACTGAGATGGTCCTTGAG-3'

reverse: 5'-TCTGGAGCCATCTTCATGATG-3'

β-actin, forward: 5'-TGGAATCCTGTGGCATCCATGAAAC-3'

reverse: 5'-TAAAACGCAGCTCAGTAACAGTCCG-3'

For quantitative PCR (qPCR), we dissected cysts from E16.5 *SFTPC/Elf5* lungs treated with doxycycline from E0 – E16.5, isolated the RNA, and generated cDNA. Spatially similar areas from nontransgenic or single transgenic *SFTPC-rtTA* or *HA-Elf5* littermate lungs served as controls. Analysis was done on 3–5 independent tissue isolations. We performed qPCR using Taqman probe and primer sets (Applied BioSystems, Foster City, CA) specific for *Elf5* (Assay ID: Mm00479832_m1), *Ese3/Ehf* (Mm00468193_m1), *Spdef* (Mm00600221_m1), *Ese1/Elf3* (Mm00468224_m1), *Sftpc* (Mm00488144_m1), *Erm/Etv5* (Mm00465816_m1), *Pea3/Etv4* (Mm01245872_m1), *Spink5* (Mm00511522_m1), *Spink4* (Mm00803437_m1), *Scgb3a1* (Mm00446493_m1), *Scgb3a2* (Mm00504412_m1), *TFF3* (Mm00495590_m1) and *p63/Trp63* (Mm00495788_m1), and *Napsa* (Mm00492829_m1). A probe and primer set for 18s rRNA was used as the normalization standard. The PCR reactions and relative quantifications were performed using 25ng of cDNA per reaction in a 7300 Real-Time PCR Sytem (Applied BioSystems). Relative quantification data from qPCR analysis were statistically analyzed using Student's t-tests on GraphPad Prizm version 4.0.

RESULTS

Expression of ELF5 during lung development

We previously reported that *Elf5* mRNA is initially expressed in the distal tip lung epithelium at E11.5, becomes highly expressed in the proximal airway epithelium as development progresses, then decreases distally (Metzger et al., 2007). We have now extended these observations to determine expression of ELF5 protein in lungs by IHC and found an identical pattern throughout gestation. ELF5 was localized to the nuclei of epithelial cells in the branching distal tips, but absent from the proximal epithelium at E14.5 (Fig. 1A). At E15.5 ELF5 was expressed in the proximal epithelium as well as the distal epithelium (Fig. 1B), and there was a low level of the distal epithelial marker Pro-SP-C expressed in the distal tips (Fig. 1C). E16.5 lungs showed increased ELF5 expression in the proximal airway epithelium and continued ELF5 expression in the distal epithelium (Fig. 1D), while Pro-SP-C, a marker specific to distal lung epithelium in the fetus and to alveolar type II cells postnatally, was still expressed at a relatively low level (Fig. 1E). At E17.5, cells of the airway epithelium showed strong staining for ELF5 compared to cells in the lung parenchyma (Fig. 1G,I), and Pro-SP-C was strongly expressed in presumptive alveolar type II cells (Fig. 1H). To determine the identity of the ELF5 positive cells in the lung parenchyma at E17.5, we performed double IHC for ELF5 and Pro-SP-C and found that ELF5 positive cells in the lung parenchyma were presumptive type II cells (Fig. 1K). Because alveolarization continues after birth, we further evaluated the co-expression of ELF5 and Pro-SP-C protein in the lungs of postnatal day 6 (PND6) and 4-week old mice (Fig 1L,M). ELF5 was undetectable in Pro-SP-C positive type II cells at these time points, which is consistent with our previous ISH results (Metzger et al., 2007), and a previous study that used a different ELF5 antibody (Lapinskas et al., 2004). We observed some apical staining with the ELF5 antibody, but determined that this was non-specific (Fig. 1J). Because the IHC data showed that distal expression of *Elf5* decreased as *Sftpc* increased, we performed RT-PCR for *Elf5* and *Sftpc* on isolated adult type II cells and on the immortalized distal epithelial cell line MLE15. The results (Fig. 1F) revealed an inverse relationship between *Elf5* and *Sftpc* expression. Although the RT-PCR data indicate that *Elf5* is present at low levels in adult type II cell preparations, this may be due to the fact that isolated type II cell preparations typically contain some contaminating proximal epithelial cells. Overall the data indicate that *Elf5* expression is very low in fully differentiated distal epithelial cells.

Misexpression of ELF5 in the distal epithelium disrupts lung branching morphogenesis

Our previous study (Metzger et al., 2007) indicated that the distal epithelial expression of *Elf5* during the pseudoglandular stage of lung development was regulated by FGF signaling and was specifically downstream of FGFR2b. We hypothesized that ELF5 might play a downstream role in FGF signaling that is important for lung morphogenesis and differentiation. In order to investigate the function of ELF5 during branching morphogenesis and its role in distal epithelial differentiation, we used the 3.7 kb human *SFTPC* promoter to direct expression of a Dox-inducible *HA-Elf5* transgene to the distal lung epithelium (*SFTPC/Elf5* mice). Recently, a longer isoform of ELF5 has been described that contains an additional 31 amino acids at the N-terminus (Choi and Sinha, 2006); these isoforms were designated as ESE2 and ESE2L (with the additional 31 amino acids). Our misexpressed ELF5 protein corresponds to the ESE2 isoform.

We identified a transgenic line in which *Elf5* was expressed at a high level; at E16.5 the lungs of these mice showed a 16-fold increase over endogenous *Elf5* expression (Fig. 8). When we examined the effects of increased ELF5 expression on E14.5 we found that, compared to the typical fine branches in lungs of single transgenic littermate control animals (Fig. 2A), the lungs from double transgenic embryos that were treated with Dox from conception had dilated distal branches (Fig. 2B). This phenotype was not uniform throughout the lungs of double transgenic embryos, because branching appeared normal in some areas. When we traced expression of the HA-ELF5 fusion protein using HA IHC, we found that the *HA-Elf5* transgene was expressed in the distal lung epithelium (Fig. 2I), and that its expression appeared to be mosaic, which likely contributed to the variability in phenotype. Lungs from E16.5 embryos treated with Dox from conception gave similar phenotypes (Fig. 2C–F), with the distal epithelium in *SFTPC/Elf5* lungs displaying dilated, cystic branches (Fig. 2D,F,H) compared to the fine branches of single transgenic controls (Fig. 2C,E,G). We confirmed expression of the HA-ELF5 fusion protein in the E16.5 *SFTPC/Elf5* lung epithelium by IHC (Fig. 2J) and again observed mosaic expression.

High ELF5 expression inhibits epithelial proliferation

Proper branching morphogenesis requires a high rate of cellular proliferation to generate the number of cells required for tissue expansion, and a cystic phenotype may result in the absence of sufficient proliferation. One possible reason for the inhibition of branching we observed in *SFTPC/Elf5* lungs is that proliferation was decreased. We therefore assessed the rate of proliferation in lungs from E16.5 *SFTPC/Elf5* and control mice by IHC for pHH3, a marker for dividing cells (Fig. 3A,B). Epithelial cells in the periphery of single transgenic control lungs stained strongly for pHH3 (Fig. 3A), indicating a high rate of proliferation. We observed much lower epithelial pHH3 staining in the lungs of *SFTPC/Elf5* fetuses (Fig. 3B). Quantification of the percentage of proliferating epithelial cells revealed a significant decrease of epithelial proliferation in double transgenic lungs (Fig. 3C). There was no significant change in mesenchymal proliferation between control and experimental lungs (Fig. 3D), demonstrating that the effect of high ELF5 expression on proliferation was specific to the epithelium. To determine if the HA-ELF5 fusion protein was inducing cell death in double transgenic lungs, we stained for the apoptosis marker cleaved caspase 3. We saw very few apoptotic cells in either control (Fig. 3E) or double transgenic lungs, even in areas of high transgene expression (Fig. 3F').

High ELF5 expression inhibits distal epithelial differentiation

The results in Fig. 1 demonstrate that although distal epithelial expression of ELF5 is high during branching morphogenesis, it is extinguished postnatally. In contrast, proximal epithelial ELF5 expression is low early in gestation, but progressively increases in late gestation and after birth. One possible explanation for this dynamic expression pattern is that ELF5 plays a

role in suppressing distal differentiation. To determine the effects of ELF5 overexpression on distal epithelium differentiation, we evaluated the lungs of E16.5 embryos that had been exposed to Dox since conception for markers of lung epithelial differentiation (Fig. 4). As expected, we found that Pro-SP-C protein was uniformly expressed in distal epithelial cells in control lungs (Fig. 4A). We did not detect Pro-SP-C expression in the dilated cysts of *SFTPC/Elf5* lungs, although histologically normal lung tissue in the same section expressed the protein (Fig. 4B). We also assessed the expression of other proteins known to be important in distal lung epithelial differentiation. We found that virtually all of the epithelial cells in *SFTPC/Elf5* lungs were positive for FOXA1 (Fig. 4E,F) and FOXA2 (Fig. 4G,H), which are markers of endoderm. Similarly, all of the epithelial cells stained positive for TTF1 (Fig. 4C,D), indicating that the cells had entered the respiratory lineage.

We next examined lungs at E18.5, when saccule development has begun in the normal lung (Fig. 5A). We found the same morphology of dilated epithelial cysts in *SFTPC/Elf5* lungs (Fig. 5B). The dilated cysts persisted in the lungs of adults (Fig. 5D), whereas controls were extensively alveolarized (Fig. 5C). Similar to lungs from E14.5 and E16.5 embryos, the extent of phenotype was variable, with some lobes in adult lungs apparently unaffected (data not shown). Because Pro-SP-C was undetectable in the cysts of E18.5 *SFTPC/Elf5* lungs, we examined these regions by electron microscopy for evidence of changes in type II cell ultrastructural differentiation. We found that *SFTPC-rtTA* control lungs contained many glycogen-laden type II cells with microvilli and cytoplasmic lamellar bodies, which are the storage organelle for pulmonary surfactant. Secreted surfactant was present in the alveolar lumina (Fig. 5E). In contrast, the epithelial cells forming the dilated cysts of *SFTPC/Elf5* E18.5 lungs were undifferentiated. Low cuboidal epithelial cells contained copious glycogen deposits, but did not contain lamellar bodies. Secreted surfactant was never seen in luminal spaces of cystic regions. The interstitial space adjacent to the epithelial lining was very loosely organized, and the resident fibroblasts contained many lipid droplets (Fig. 5F).

Pro-SP-C immunostaining on lungs from fetuses continuously exposed to Dox through day E18.5 (DOX E0–E18.5), gave results similar to those observed on E16.5: Pro-SP-C expression was undetectable in the dilated cysts of *SFTPC/Elf5* lungs, whereas control lungs and the histologically normal parenchyma of double transgenic lungs strongly expressed the protein (Fig. 6A,B). Accordingly, HA IHC of these lungs revealed that the *HA-Elf5* transgene expression was only seen in the epithelium of dilated cysts and was absent from unaffected areas (Fig. 6K).

In order to determine if the effects of Elf5 overexpression were dependent on the developmental stage at which the transgene was induced, we waited until E16.5 to treat dams with Dox (DOX E16.5–E18.5). This delayed induction of the *HA-Elf5* transgene until the end of the pseudoglandular stage, when patterning of the pulmonary tree has been completed, but formation of acini and saccules is still ongoing. We found that the morphology of DOX E16.5–E18.5 embryos was very similar lung morphology to control littermates. We did not observe cystic dilation of the distal epithelium, even when transgene expression was widespread (Fig. 6L). However, we still observed decreased Pro-SP-C expression in *SFTPC/Elf5* lungs (Fig. 6H) compared to those of single transgene littermate controls (Fig. 6G). Double IHC staining revealed that HA positive cells did not co-express Pro-SP-C (Fig. 6I,J), confirming that high ELF5 levels in late gestation still repressed Pro-SP-C expression.

Since ELF5 is highly expressed in the airway epithelium in late gestation we speculated that ELF5 might induce genes specific to the proximal airway epithelial cell differentiation. We therefore evaluated CCSP, a marker of Clara cells, in DOX E0–E18.5 lungs by IHC. We found that endogenous CCSP was present in the proximal airway epithelium of both DOX E0–E18.5 and control lungs (Fig. 6C,D). Notably, the distal cystic epithelium of *SFTPC/Elf5* lungs was

negative for CCSP (Fig. 6D). In parallel with these results, expression of the ciliated cell marker, FOXJ1, was restricted to the proximal airway epithelium in both control and experimental lungs, and was not seen in the cystic epithelium (Fig. 6E,F).

Microarray analysis

The lack of Pro-SP-C expression and the appearance of immature cuboidal epithelial cells in double transgenic lungs suggested that distal epithelial cell differentiation was impaired; however, the fate of the cells in the cysts was unknown. To gain some insight into the identity of the cells, we isolated cystic regions of E16.5 *SFTPC/Elf5* lungs and spatially similar regions from the lungs of *SFTPC-rtTA* littermates for microarray analysis. Since transcriptional targets of ELF5 and other ESE transcription factors have not been well characterized (Choi and Sinha, 2006), we hypothesized that microarray analysis would provide candidate ELF5 target genes. We found that overexpression of ELF5 activated 202 genes at least 2-fold and decreased the expression of 123 genes. These included genes involved in transcriptional regulation, transport, receptor activity, intracellular signaling, adhesion, and a number of secreted proteins (Supplement).

Since differentiation of type II cells in E18.5 *SFTPC/Elf5* lungs was defective, we compared the list of differentially expressed genes to those present in isolated wild-type adult mouse type II cells; 40 of the 123 genes that decreased with ELF5 overexpression were present in differentiated type II cells (Table 1). Some of these genes are associated with type II cell differentiation. Both *Erm* (*Etv5*) and *Napsa*, which are expressed in differentiated type II cells, were decreased in the microarray, a result we confirmed by qPCR (Fig 8). Conversely, 92 of 202 genes that increased with ELF5 overexpression were present in isolated type II cells (Table 2). Some of these genes, such as *Sftpd, Spink5*, and *Scgb3a1*, are not only present in type II cells but are also expressed in the proximal airway epithelium. The increases in *Spink5* and *Scgb3a1* were confirmed by qPCR (Fig. 7A).

ELF5 also induced the expression of genes not typically associated with the distal lung, such as *Spink4, Guca2a, Tff3* and *Dcpp* (Supplement); we verified the induction of *Spink4* and *Tff3* by qPCR (Fig. 7A). We identified other genes increased in *SFTPC/Elf5* lungs that are not present in type II cells but are expressed in the proximal airway epithelium (Supplement), such as *Scgb3a2* (Reynolds et al., 2002), whose upregulation in *SFTPC/Elf5* lungs was verified by qPCR (Fig. 7A). ELF5 overexpression also induced the expression of the basal cell marker, p63, which is normally found in the pseudostatified epithelium of the trachea and bronchus (Fig. 7H) as well as the stratified esophageal epithelium. Because we observed *Elf5* expression in these tissues at E17.5 (Fig. 7F,G), we analyzed p63 expression in DOX E0–E16.5 *SFTPC/Elf5* lungs by IHC. We found that p63 was detectable in the cysts of double transgenic lungs (Fig. 7C), while control lungs lacked p63 expression in the distal epithelium (Fig. 7B). Similarly, p63 was also induced in DOX E16.5–E18.5 double transgenic E18.5 lungs (Fig. 7E), but not in controls (Fig. 7D). Esophageal epithelium, previously reported to have a high expression of p63 (Daniely et al., 2004; Que et al., 2007), was used as an internal positive control for experimental samples (Fig. 7B,D, insets).

ELF5 regulates the expression of other Ets factors in the lung

Along with the predicted increase of *Elf5*, another Epithelial-Specific Ets factor, *Ese3*, was increased in the microarray and was also present in type II cells (Table 2); these data were confirmed with qPCR (Fig.8). We then evaluated by qPCR the expression of other Epithelial Specific Ets factors, *Spdef* and *Ese1*, which were not changed as determined by microarray. We found that *Spdef*, previously described as a marker for goblet cells in the airway epithelium (Park et al., 2007), was significantly increased in *SFTPC/Elf5* lungs, but *Ese1* expression did not change (Fig. 8). *Erm*, which has previously been shown to be highly expressed in type II

cells, to directly activate the *Sftpc* promoter, and to be critical for distal epithelium differentiation (Lin et al., 2006;Liu et al., 2003) was decreased in both microarray and qPCR analyses. We also found that *Sftpc* expression was reduced by microarray and qPCR (Fig. 8), supporting our IHC results (Fig. 6). *Pea3*, which is thought to play a redundant role to *Erm* (Liu et al., 2003), did not change (Fig. 8).

DISCUSSION

ELF5 expression during lung development

We previously reported that *Elf5* is expressed in the distal tip lung epithelium at E11.5, becomes more highly expressed in the proximal airway epithelium as development progresses, then decreases distally at the end of gestation and postnatally (Metzger et al., 2007). We have now extended these observations to show that ELF5 protein expression follows a parallel pattern throughout gestation. As late as E17.5, the distal epithelial cells that expressed ELF5 co-expressed Pro-SP-C, identifying them as presumptive type II cells. ELF5 expression was no longer detectable in Pro-SP-C positive type II cells after birth, which is consistent with our ISH results (Metzger et al., 2007), and a previous study using a different ELF5 antibody (Lapinskas et al., 2004). These data indicate that *Elf5* expression is very low in differentiated type II cells, and suggested the possibility that ELF5 may play a role in negatively regulating *Sftpc*. Although our microarray data (Table 2) indicate that *Elf5* is present in type II cell preparations, our RT-PCR results indicate that the expression is at low levels. We believe that this low level of expression is due to the fact that isolated type II cell preparations typically contain some contaminating proximal epithelial cells, which are *Elf5* positive in the postnatal lung.

ELF5 overexpression causes lung branching defects

Our previous study indicated that the distal epithelial expression of *Elf5* during the pseudoglandular stage of lung development was regulated by FGF signaling, and was specifically downstream of FGFR2b. Because of the importance of FGF10, which uses FGFR2b as its primary receptor, in lung development (Min et al., 1998; Sekine et al., 1999), we hypothesized that ELF5 might act downstream in regulating morphogenesis and differentiation. We tested this hypothesis by overexpressing ELF5 using the human *SFTPC* promoter, which is active in the epithelium from the beginning of mouse lung development (Perl et al., 2002b). We found that misexpressed ELF5 disrupted branching morphogenesis, as evidenced by the formation of dilated cysts in the distal epithelium. This phenotype, which we detected as early as E14.5, persisted throughout gestation and into postnatal life. HA IHC verified that the transgene was being expressed in the distal epithelium, but its expression was mosaic, causing a partially cystic phenotype. We also observed that only a subset of cells in the cystic regions were transgene positive as gauged by HA IHC. The basis for this mosaic expression pattern is unknown. One possible explanation stems from our observation that ELF5 decreases *Sftpc* expression (Fig. 4): since we drove ELF5 expression using the *SFTPC* promoter, it could have the secondary effect of reducing its own expression, but only in a subset of cells. Alternatively, transgene expression may have only been high enough to detect in a subset of cells, and these affected their neighbors through crosstalk via secreted factors. The effects of ELF5 overexpression on lung branching were restricted to a window during development, however, because morphogenesis was unaffected when the transgene was induced beginning on E16.5, when basic patterning of the lung has been mostly completed.

Cell proliferation not only sustains overall lung growth in the embryo, but also influences lung patterning, since the rate of epithelial proliferation is higher in branching regions (Weaver et al., 2000). One possible result of insufficient epithelial proliferation is decreased branching and cyst formation. The phenotype we observed in *SFTPC/Elf5* embryos, where ELF5

specifically inhibited epithelial proliferation in the cysts, is consistent with this concept. Analysis of a lung-specific *Elf5* knockout mouse model will be required to determine if ELF5 directly inhibits lung epithelial cell proliferation. The possibility that ELF5 acts as a negative regulator of epithelial proliferation may be tissue type-dependent, however, since mammary epithelial cell proliferation decreases in mice heterozygous for a null *Elf5* allele (Zhou et al., 2005).

Increased ELF5 represses distal epithelial cell differentiation

Although distal epithelial expression of ELF5 is high during branching morphogenesis, it is silenced postnatally. One possible explanation for this dynamic expression pattern is that ELF5 negatively regulates distal differentiation. Our results showing that overexpression of ELF5 in the distal epithelium causes decreased expression of *Sftpc* and the loss of ultrastructural hallmarks of type II cell differentiation are consistent with this possibility. In addition to inhibiting *Sftpc* expression and type II cell differentiation, microarray analysis revealed that 40 genes normally expressed in differentiated adult type II cells were decreased in *SFTPC/Elf5* lungs (Table 1). Two of these genes, *Erm (Etv5)* and *Napsa*, have previously been shown to be associated with differentiated type II cells (Brasch et al., 2003;Chuman et al., 1999;Lin et al., 2006;Liu et al., 2003;Ueno et al., 2004). ERM directly binds the *Sftpc* promoter to drive its transcription (Lin et al., 2006). High ELF5 expression may therefore indirectly inhibit *Sftpc* expression by reducing *Erm* expression. This may be occurring in the normal adult airway epithelium, where ELF5 is highly expressed and *Erm* is absent (Lin et al., 2006). It is also possible that ELF5 may compete with ERM for Ets binding sites on the *Sftpc* promoter, which would directly inhibit *Sftpc* transcription. The ETS domain of ELF5 can function as a repressor (Choi and Sinha, 2006), suggesting the possibility that ELF5 may directly inhibit Sftpc transcription. Reduced levels of *Napsa* have also been associated with defective lung epithelial differentiation in double heterozygous $Gata6^{+/-}/Nkx2.1^{+/-}$ mutant embryos (Zhang et al., 2007). Notably, we found that the effects of ELF5 misexpression on distal lung epithelial differentiation occurred both when the transgene was expressed throughout development and when it was induced only from E16.5 to E18.5. This observation was distinct from the effect of ELF5 misexpression on lung morphogenesis, which was stage-dependent, suggesting that ELF5 disrupts lung epithelial branching and differentiation by affecting different target genes.

In addition to *Elf5* (*Ese2*), the Epithelial-Specific Ets family also includes *Ese1* (*Elf3*), *Ese3* (*Ehf*) and *Spdef* (*Pdef*) (Feldman et al., 2003), all of which are expressed in the lung (Kas et al., 2000; Lei et al., 2007; Oettgen et al., 1999; Park et al., 2007; Silverman et al., 2002; Zhou et al., 1998). We found that *SFTPC/Elf5* lungs contained increased *Ese3* and *Spdef* levels, suggesting the possibility that ELF5 may control their expression. SPDEF, which is expressed in airway epithelial cells, appears to regulate cell differentiation and gene expression, since misexpression of SPDEF in the airway epithelium induced goblet cell hyperplasia (Park et al., 2007). We saw no evidence of goblet cell hyperplasia in the distal epithelial cysts of *SFTPC/Elf5* lungs, indicating that the influence of SPDEF on target gene expression and cell phenotype may be regionally specific. ESE-3 is also expressed in the airway epithelium, especially in serous and mucous glands, (Tugores et al., 2001). Little is known about the function of ESE-3 in the lung, other than that it is required for induction of the squamous differentiation marker *SPRR1B* in human bronchial epithelial cells (Reddy et al., 2003). In other tissues, however, ESE-3 functions as a context-dependent transcriptional repressor that lies downsteam of MAPK signaling (Tugores et al., 2001), in cellular senescence (Fujikawa et al., 2007), and as a tumor suppresor in prostate cancer (Cangemi et al., 2007). Since ELF5 expression increases in proximal airway epithelium late in gestation, and because ELF5 induced the proximal airway epithelial genes *Spdef* and *Ese3*, we asked whether the high levels of ELF5 seen in *SFTPC/Elf5* lungs induced the distal epithelial cells to adopt a proximal epithelial cell phenotype. We found, however, that *CCSP* and *FoxJ1*, markers of Clara and ciliated cells, respectively, were

not induced in the cystic distal epithelium, indicating that ELF5 by itself cannot induce fully differentiated airway epithelial cells.

ELF5 in the maintenance of lung epithelial cell fate

Although a primary role of FGF10 in lung morphogenesis is to act as an epithelial cell chemoattractant (Park et al., 1998), it has also been proposed to maintain epithelial cells as undifferentiated progenitor cells (Norgaard et al., 2003; Nyeng et al., 2007; Ramasamy et al., 2007). Our data showing that ELF5 misexpression represses type II cell maturation and the expression of *Sftpc*, *Erm*, and *Napsa* suggest that *Elf5*, a target gene of FGF10 (Metzger et al., 2007), may act in this process. If this is the case, the silencing of *Elf5* in the distal epithelium that normally occurs in late gestation would be required for normal differentiation to proceed. In support of the concept that ELF5 maintains undifferentiated progenitors, we found that ELF5 overexpression induced *p63* and *Aqp3*, genes that normally mark undifferentiated basal cells of the tracheobronchial epithelium (Avril-Delplanque et al., 2005; Daniely et al., 2004; Liu et al., 2007; Rawlins and Hogan, 2006; Sato et al., 2004). Basal cells of pseudostratified epithelia (e.g. tracheal epithelium) and stratified epithelium (e.g. esophageal epithelia and epidermis) are a source of progenitor cells in these tissues (Daniely et al., 2004; Yang et al., 1998). Basal cells in the adult trachea and bronchi can repopulate the injured epithelium and give rise to fully differentiated cell types, suggesting they behave as stem cells (Hong et al., 2004a; Hong et al., 2004b). The fact that we observed many p63 positive cells in *SFTPC/Elf5* lungs suggests the possibility that the dilated cysts may represent a population of progenitor cells. Some reports have suggested that p63 is important for the maintenance of progenitor/stem cells, but this is not fully resolved and is complicated by the existence of various p63 isoforms that can perform distinct functions (Blanpain and Fuchs, 2007; Koster et al., 2007; Koster et al., 2004; Koster et al., 2005; Senoo et al., 2007; Yang et al., 1999).

The identity of the cells constituting the epithelial cysts is unclear, but the fact that they express TTF1 indicates that they are in the respiratory lineage (Zhou et al., 1996). Although our microarray data showed that high expression of ELF5 repressed some genes that were present in adult type II cells, it simultaneously induced others, such as *Sftpd*, *Spink5*, and *Scgb3a1*. It should be noted, however, that these genes are not restricted to type II cells, since they are also expressed in proximal airway epithelium (Crouch et al., 1992; Reynolds et al., 2002; Walden et al., 2002; Wong et al., 1996). These cells, however, are not fully differentiated airway epithelial cells, since they lack markers for type II, Clara, or ciliated cells.

Disruption of the normal program of epithelial differentiation by high levels of ELF5 may activate new downstream targets that allow the cells to adopt a different fate, as evidenced by our observation that *Spink4, Guca2a, Tff3* and *Dcpp* were induced in the cysts of *SFTPC/Elf5* lungs. These are genes that are normally expressed in the intestine and not the lung parenchyma. A previous study has shown that hyperactivation of β-catenin transcriptional activity directed to lung epithelial progenitors produced dilated epithelial cysts and suppressed type II cell differentiation (Okubo and Hogan, 2004). Some of the cells in the cysts expressed *Spink4, Guca2a, Tff3* and *Dcpp*, and the authors concluded that these cells had been induced to change fate by a process termed transdetermination. Although we observed that these genes were induced in *SFTPC/Elf5* lungs, our microarrays showed no changes in *Wnt*-related gene expression. Furthermore, Okubo et al. did not report a change in *Elf5*, indicating that *Elf5* is not downstream of WNT signaling. A more recent study by Nyeng et al. showed that FGF10 misexpressed under control of the *Pdx1* promoter caused cystic dilation of the branching lung with induced expression of *Tff3* (Nyeng et al., 2008). The epithelial cells in these lungs co-expressed markers associated with the early distal lung epithelium (e.g. SP-C and TTF1), but did not show evidence of transdetermination, leading the authors to conclude that excessive FGF10 signaling arrested epithelial cell differentiation and maintained the cells as progenitors.

This phenotype is similar, but not identical, to that which we observed in *SFTPC/Elf5* mice, which were SP-C negative. Because we have previously shown that *Elf5* is a downstream target of FGF10 (Metzger et al., 2007), we speculate that some of the effects of FGF10 misexpression on maintaining epithelial cells in a progenitor state may be due to increased expression of ELF5.

In summary, we have used an inducible transgenic mouse model to show that high levels of ELF5 in the distal lung epithelium disrupt normal lung morphogenesis and epithelial cell differentiation. Our results suggest that ELF5 must be present at precise levels for the proper specification and subsequent differentiation of lung epithelial cells. Our data also suggest that the role of ELF5 in lung epithelial cell differentiation is complex, and may depend on other components in the transcriptional networks regulating the differentiation of individual lung epithelial cell types.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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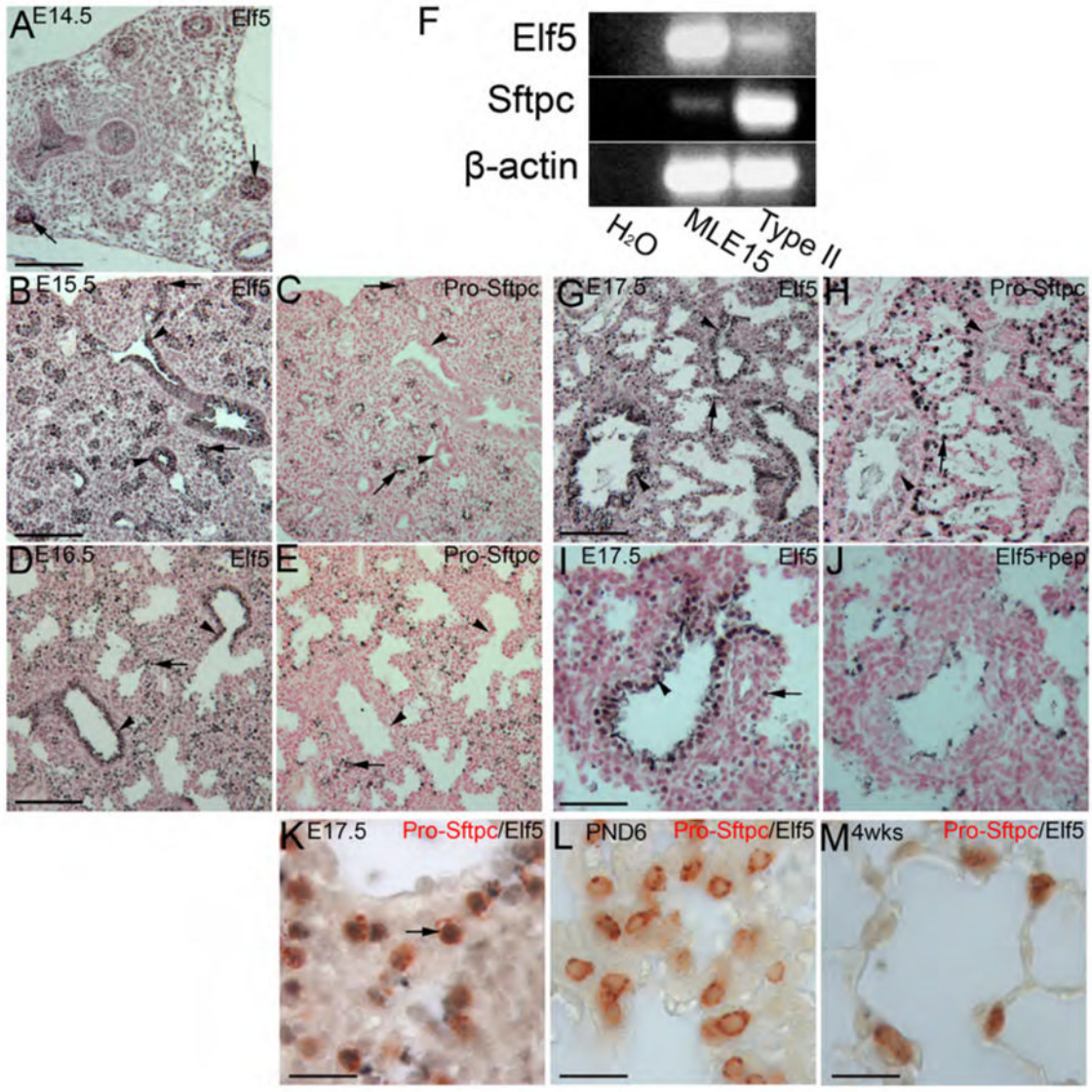

Fig. 1.

Endogenous expression of ELF5 and Pro-SP-C protein expression during lung development. ELF5 is expressed at the tips of branching lung epithelium (arrows) at E14.5 (A). ELF5 expression continues in branching epithelium (arrows) at E15.5 but is also expressed in the developing airway epithelium (arrowhead) (B). Pro-SP-C is expressed in tips of branching epithelium (arrow) but is not expressed in the proximal airway (arrowheads) (C). ELF5 is present in the airways (arrowheads) and in lung parenchymal cells (arrows) at E16.5 and E17.5 (D,G), while Pro-SP-C is found exclusively in cells in the distal lung parenchyma (arrows) and not the airway (arrowhead) (E,H). ELF5 is seen in many cells in the E17.5 airway (arrowhead) and parenchyma (arrow) (I). Staining on the apical surfaces of some luminal cells is nonspecific as determined by the ELF5 peptide block control (J). Double IHC staining of E17.5 lungs for ELF5 and Pro-SP-C indicates that distal cells expressing ELF5 also stain for Pro-SP-C (K), whereas ELF5 is undetectable in Pro-SP-C positive type II cells in postnatal day 6 (PND6) (L) and 4 week lungs (M). RT-PCR shows an inverse relationship between *Elf5* expression and *Sftpc* expression in MLE15 cells and in isolated type II cells (F). Scale bars: 100 μm in A–E, G, H; 50 μm in I,J and 20 μm in K–M.

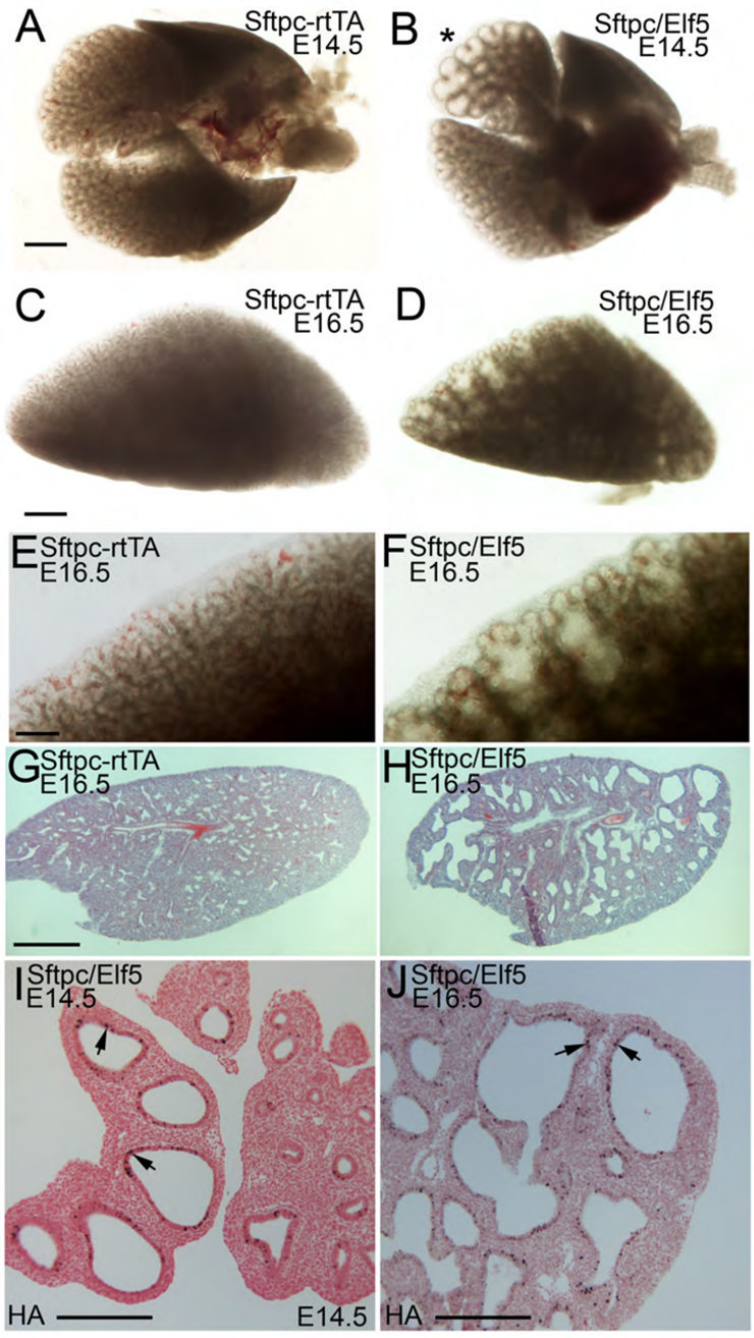

Fig. 2.

Misexpression of ELF5 in distal lung epithelial cells disrupts branching morphogenesis at E14.5. Control lungs from an E14.5 *SFTPC-rtTA* embryo show typical fine branches (A), whereas the lungs from an E14.5 *SFTPC/Elf5* embryo display areas of epithelial dilation (B). Note that in this preparation only one lobe appears to be affected (*). A left lung from a control E16.5 *SFTPC-rtTA* embryo, showing finely branched distal tips (C). The left lung from an E16.5 *SFTPC/Elf5* embryo (D) showing widespread dilated epithelium, which are readily apparent at higher magnification (E,F). Histology of E16.5 *SFTPC-rtTA* (G) and *SFTPC/Elf5* (H) lungs demonstrates that the dilated epithelium is widespread, although some areas appear unaffected. Immunostaining for HA in *SFTPC/Elf5* lungs on E14.5 (I) and E16.5 (J)

shows those cells expressing the *HA-Elf5* transgene (arrows). Note that not all of the epithelial cells stain positive. Scale bars: 500 μm in A–D, G, H; 200 μm in E, F, I, J.

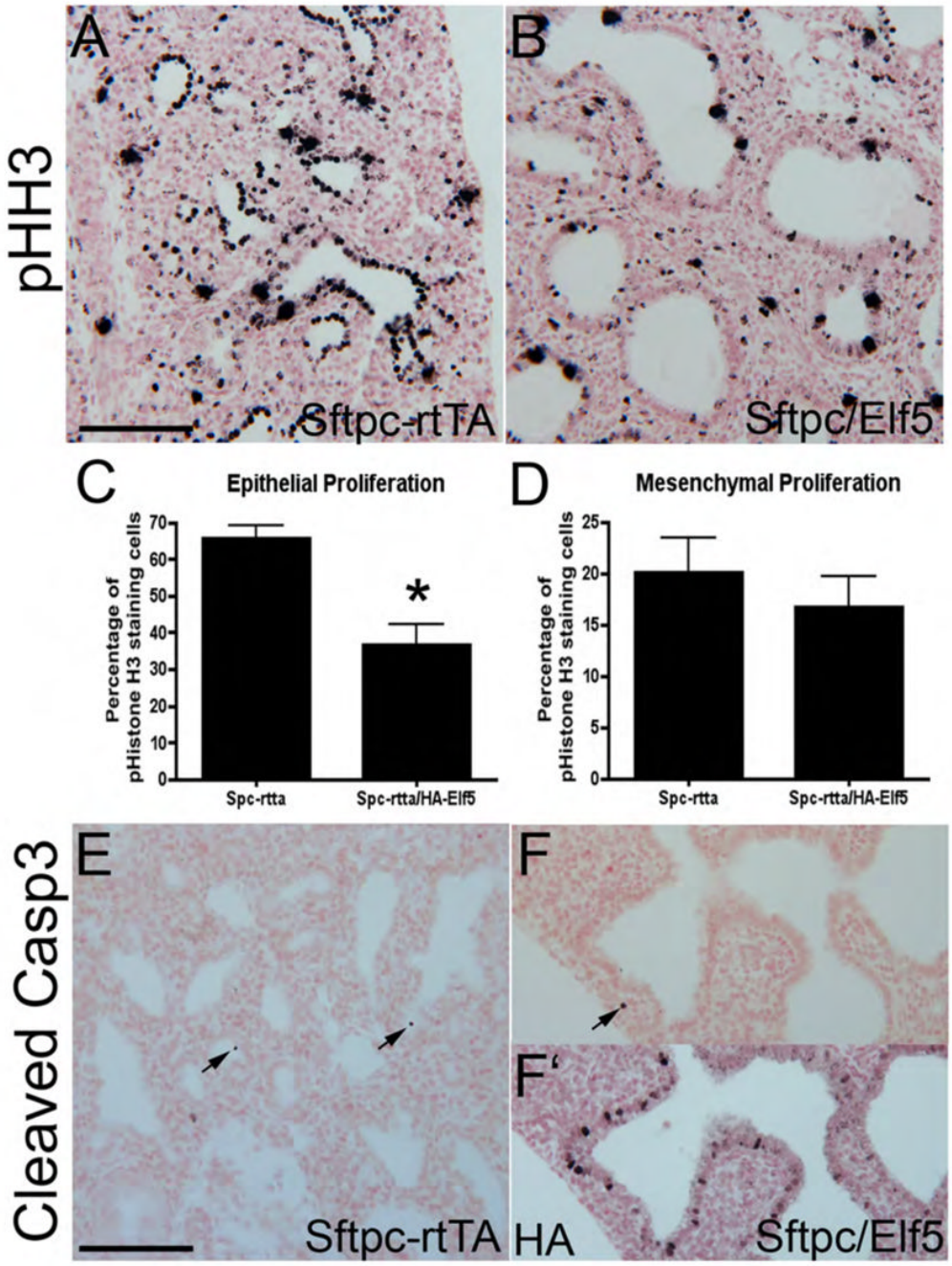

Fig. 3.

ELF5 overexpression inhibits epithelial proliferation. IHC for the proliferation marker phospho-Histone H3 (pHH3) in E16.5 *SFTPC-rtTA* (A) and *SFTPC/Elf5* (B) lungs shows decreased epithelial staining in *SFTPC/Elf5* lungs. Quantification of the percentage of epithelial cells and mesenchymal cells staining for pHH3 in *SFTPC/Elf5* lungs compared to *SFTPC-rtTA* lungs shows that epithelial proliferation (C) was significantly decreased in *SFTPC/Elf5* lungs ($p < 0.05$), whereas mesenchymal proliferation (D) did not change. IHC for the apoptosis marker cleaved caspase 3 shows that few positive cells are found in either *SFTPC-rtTA* control lungs (E) or *SFTPC/Elf5* lungs (F). A consecutive section to that shown in F

stained for HA shows HA-ELF5 is expressed in the area of the epithelium evaluated for cleaved caspase 3 (F'). Scale bar: 100 μm.

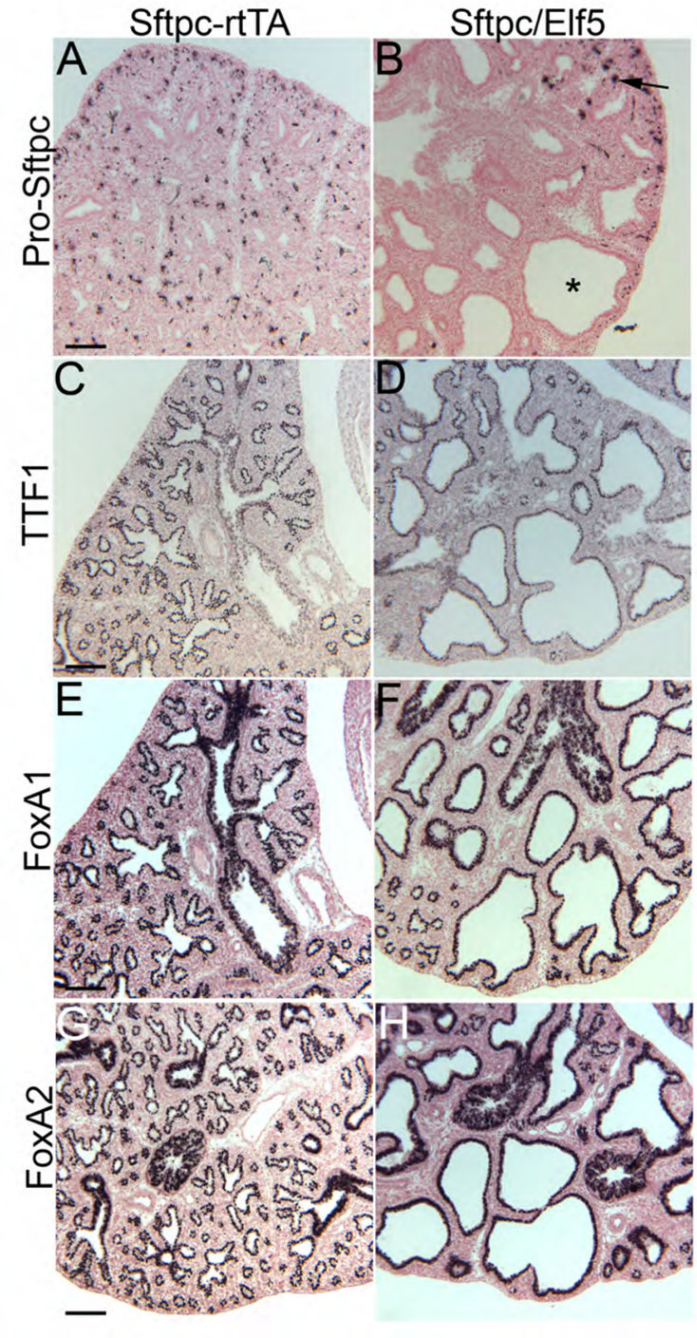

Fig. 4.

Effects of ELF5 misexpression on gene expression in E16.5 lungs. Sections of *SFTPC-rtTA* control lungs (A) and *SFTPC/Elf5* lungs (B) stained for Pro-SP-C protein show that it is not detectable in the cystic epithelium of *SFTPC/Elf5* lungs (asterisks), although histologically normal regions still express the protein (B; arrow). Control lungs stain uniformly for Pro-SP-C (A). IHC for TTF1 shows strong expression in the epithelium of both control (C) and *SFTPC/Elf5* (D) lungs. IHC for FOXA1 (E,F) and FOXA2 (G,H) shows no differences in staining for either protein in *SFTPC/Elf5* lungs compared to *SFTPC-rtTA* controls. Scale bar: 100 μm.

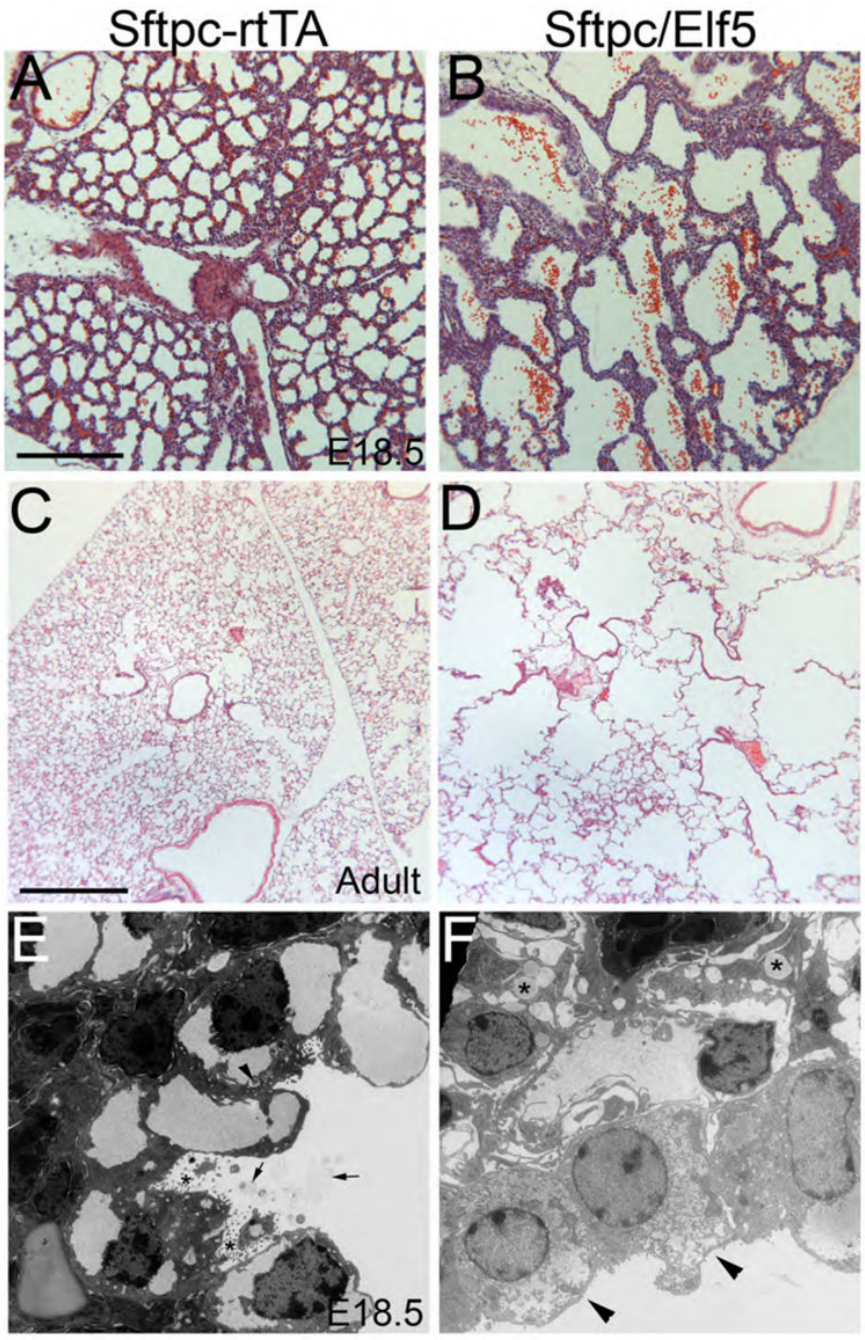

Fig. 5.

Histology of E18.5 *SFTPC-rtTA* (A) and *SFTPC/Elf5* (B) lungs shows that expression of ELF5 transgene disrupts acinar formation, and this persists in adult control (C) and double transgenic (D). Electron micrographs of E18.5 *SFTPC-rtTA* control (E) and *SFTPC/Elf5* (F) lungs reveal ultrastructural differences. *SFTPC-rtTA* lungs (E) contain normal type II cells that exhibit apical microvilli (*) and cytoplasmic lamellar bodies (arrowhead); surfactant is seen in the alveolar lumen (arrows). In contrast, the cystic epithelium of *SFTPC/Elf5* lungs (F) is predominantly composed of immature cuboidal cells lacking apical microvilli (arrowheads) and cytoplasmic lamellar bodies; secreted surfactant could not be found in the cyst lumina.

Fibroblasts in the loosely-organized interstitium adjacent to the cystic epithelium contain many lipid droplets (*). Scale bars: 200 μm in A,B; 500 μm in C,D.

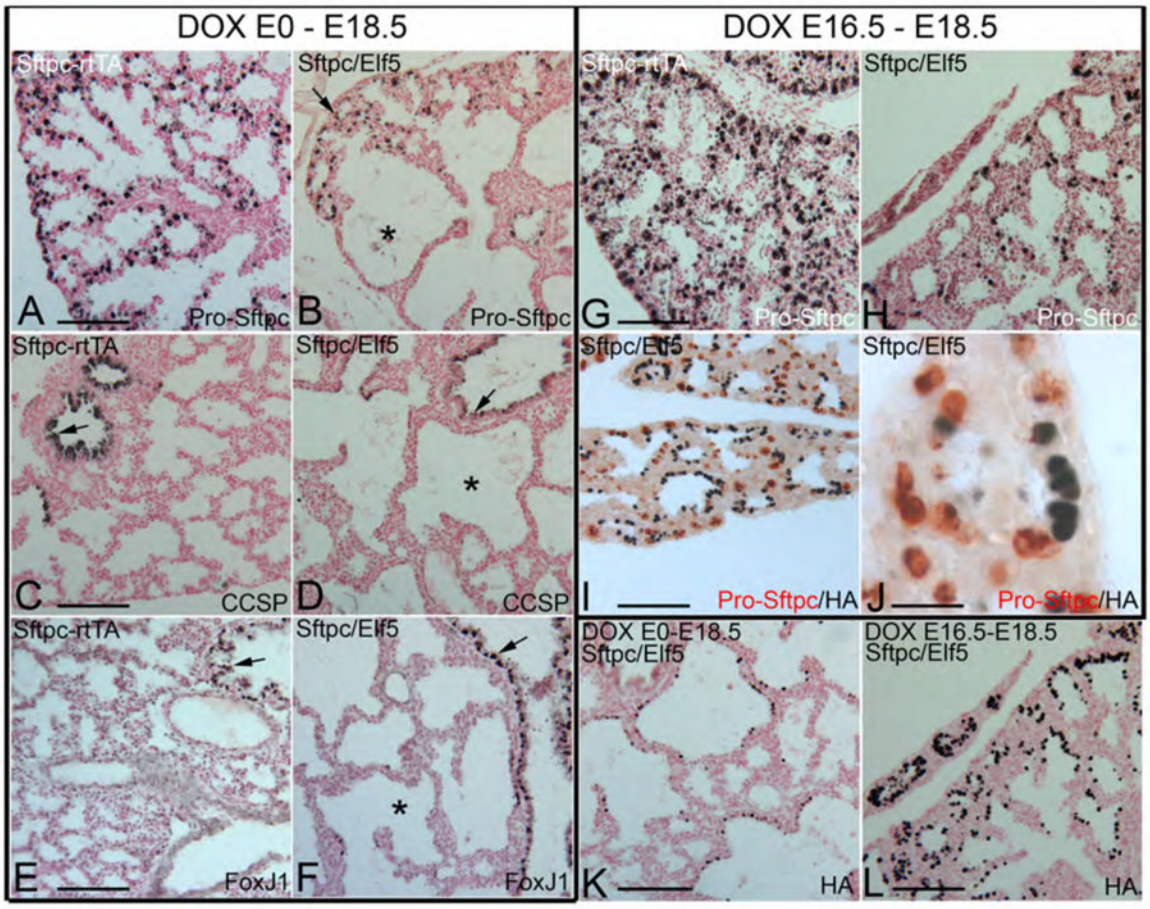

Fig. 6.

ELF5 represses SP-C expression in E18.5 lungs. IHC for Pro-SP-C in the lungs of *SFTPC/Elf5* embryos that had been exposed to Dox since conception (DOX E0–E18.5) reveals a lack of Pro-SP-C in the epithelial cysts (*) (B), whereas *SFTPC-rtTA* control lungs show widespread expression Pro-SP-C (A). IHC for CCSP shows endogenous protein expression in the airway epithelium (arrows) of both control (C) and *SFTPC/Elf5* lungs (D). Note the lack of CCSP induction in the epithelium of the cysts (*) of double transgenic lungs (D). Similarly, IHC for FOXJ1 in control (E) and *SFTPC/Elf5* lungs (F) shows endogenous protein expression in the airway epithelium, but no induction in the cystic epithelium (*) of *SFTPC/Elf5* lungs (F). IHC for Pro-SP-C in the lungs of embryos that had been exposed to Dox from E16.5 to E18.5 (DOX E16.5 – E18.5) shows a decided decrease in Pro-SP-C positive cells in *SFTPC/Elf5* lungs (H) compared to *SFTPC-rtTA* controls (G). Double IHC of HA and Pro-SP-C in DOX E16.5 – E18.5 *SFTPC/Elf5* lungs (I,J) shows that Pro-SP-C is repressed in cells that stain positive for the *HA-Elf5* transgene. IHC for HA in DOX E0–E18.5 *SFTPC/Elf5* lungs shows that the transgene is expressed in the cystic epithelium, but is not found in histologically normal areas (K). In contrast, transgene expression is widespread in DOX E16.5 – E18.5 *SFTPC/Elf5* lungs (L), but overall histology is normal (compare H and L to A) indicating that the effects of ELF5 misexpression on morphogenesis occur prior to E16.5. Scale bars: 100 μm in A–I,K,L; 20 μm in J.

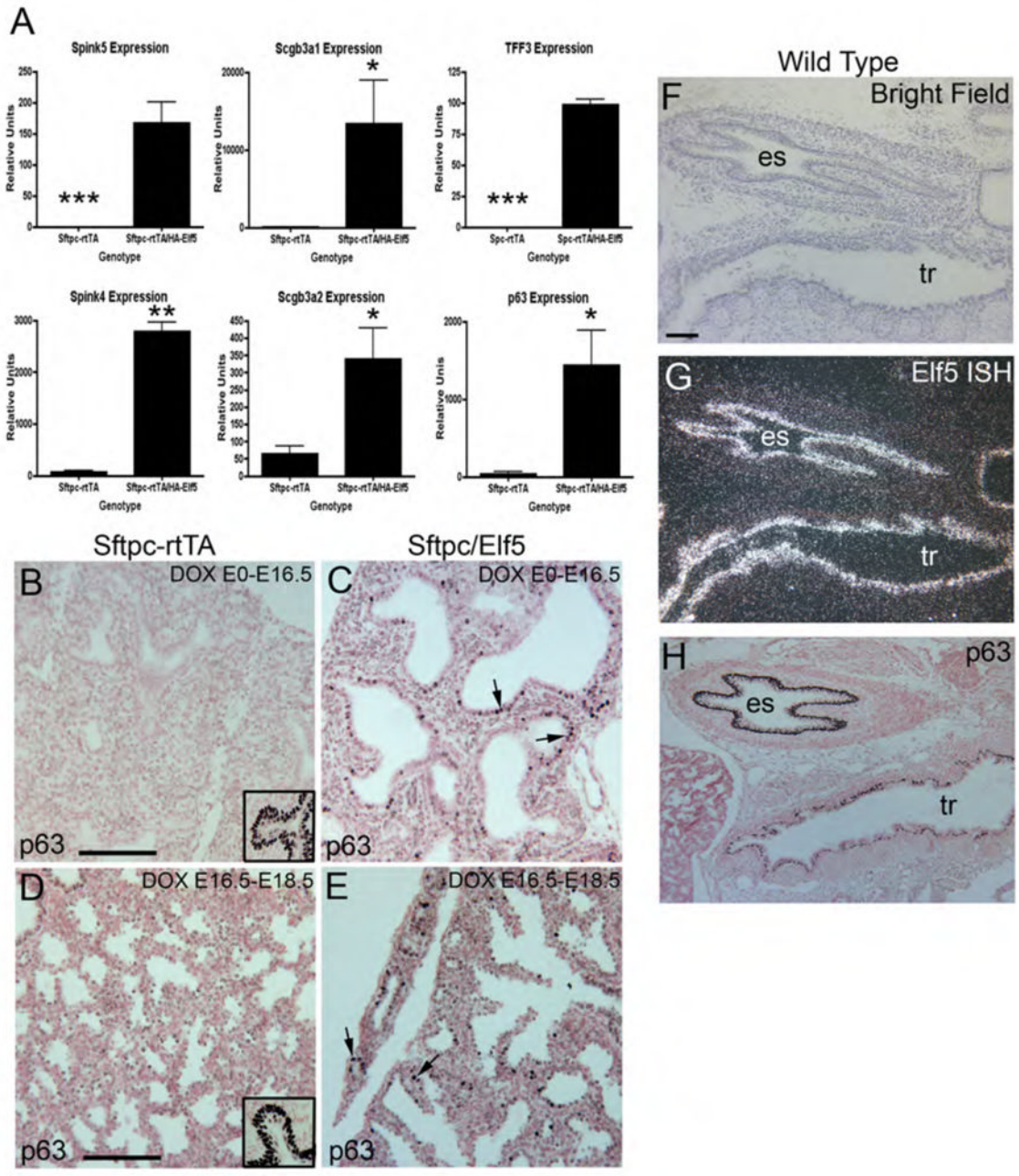

Fig. 7.

Cystic regions of E16.5 *SFTPC/Elf5* lungs were compared by microarray to spatially similar areas of *SFTPC-rtTA* control lungs to identify upregulated genes. qPCR for expression of 6 of the genes identified-- *Spink5, Spink4, Scg3a1, Scgb3a2, Tff3* and *p63* -- confirms that all were induced or increased in *SFTPC/Elf5* lungs (A; n = 3; * = $p < 0.05$; ** = $p < 0.0001$; *** = expression not detected in controls). IHC for p63 in *SFTPC-rtTA* and *SFTPC/Elf5* lungs in E16.5 (DOX E0–E16.5) and E18.5 (DOX E16.5–E18.5) lungs shows that p63 was absent in control lungs (B,D) but induced in double transgenic lungs (C,E). Insets in B and D show control staining for p63 in esophageal epithelium in the same section. Examination of an E17.5 wild-type fetus (F) shows that *Elf5* (G) and p63 (H) are endogenously co-expressed in the esophageal (es) and tracheal (tr) epithelium. Scale bars: 100 μm.

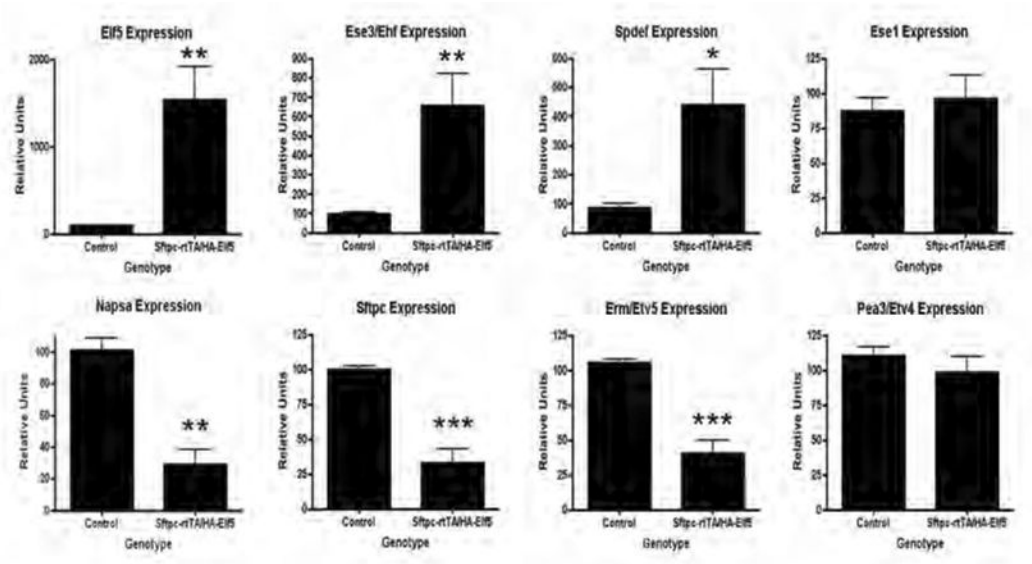

Fig. 8.

E16.5 *SFTPC/Elf5* lungs were compared to controls by qPCR for expression of other Ets transcription factors (n = 5) *Sftpc* (n = 5) and *Napsa* (n = 3). *Elf5* was significantly ($p < 0.005$) increased in *SFTPC/Elf5* lungs, as were the Epithelial Specific Ets genes, *Ese3* ($p < 0.005$) and *Spdef* ($p < 0.05$). The expression of *Sftpc*, *Napsa*, and *Erm*, however, were significantly ($p < 0.0001$) reduced in *SFTPC/Elf5* lungs. The expression of *Pea3* and the Epithelial Specific Ets gene *Ese1* did not change significantly.

Table 1

Genes present in adult type II cells but decreased with Elf5 overexpression.

Fold Decrease	Accession	Abreviation	Name
14.45	AW215442	C130039A10	
5.62	NM_019806	Vapb	Vesicle-associated membrane protein, associated protein B and C
5.56	XM_132070	Cyt1	Cytokine like 1
5.05	NM_008437	Napsa	Napsin A aspartic peptidase
4.35	NM_026085	3110049J23Rik	
3.85	NM_026055	Rpl39	Ribosomal protein L39
3.31	XM_620753	Zfp291	Zinc finger protein 291
3.15	NM_027041	1700003M02Rik	
3.04	NM_001024617	Inpp4b	inositol polyphosphate-4-phosphatase, type II
2.92	NM_007663	Cdh16	Cadherin 16
2.82	NM_010819	Clec4d	C-type lectin domain family 4, member d
2.79	XM_137239	Ppp1r12a	Protein phosphatase 1, regulatory (inhibitor) subunit 12A
2.68	NM_021475	Adamdec1	ADAM-like, decysin 1
2.67	NM_139144	Ogt	O-linked N-acetylglucosamine (GlcNAc) transferase
2.65	NM_011018	Sqstm1	Sequestosome 1
2.62	NM_011909	Usp18	Ubiquitin specific protease 18
2.58	NM_010266	Gda	Guanine deaminase
2.58	NM_013835	Ssa2	Sjogren syndrome antigen A2
2.38	NM_031374	Tex15	Testis expressed gene 15
2.36	NM_021344	Tesc	Tescalcin
2.35	NM_019394	Mia1	Melanoma inhibitory activity 1
2.34	NM_007780	Csf2rb1	Colony stimulating factor 2 receptor, beta 1, low-affinity
2.34	AK028312	3526402J09Rik	
2.31	NM_145374	BC020002	
2.31	NM_007499	Atm	Ataxia telangiectasia mutated gene
2.30	NM_013514	Epb4.9	Erythrocyte protein band 4.9
2.28	NM_009105	Rsu1	Ras suppressor protein 1
2.28	NM_010448	Hnrpab	Heterogeneous nuclear ribonucleoprotein A/B
2.25	XM_135030	Ibtk	Inhibitor of Bruton agammaglobulinemia tyrosine kinase, transcript variant 1
2.24	NM_011378	Sin3a	
2.23	NM_030143	Ddit4l	DNA-damage-inducible transcript 4-like
2.17	BQ175321	2900022I03Rik	
2.16	NM_010762	Mal	Myelin and lymphocyte protein
2.16	NM_144820	1700009P13Rik	
2.16	NM_011638	Tfrc	Transferrin receptor
2.10	NM_013785	Ihpk1	Inositol hexaphosphate kinase 1
2.09	NM_023794	Etv5	Ets variant gene 5
2.07	NM_021559	Zfp191	Zinc finger protein 191
2.07	NM_019472	Myo10	Myosin X
2.03	NM_013825	Ly75	Lymphocyte antigen 75

Table 2

Genes present in adult type II cells and upregulated by Elf5 everpression.

Fold Increase	Accession	Abbreviation	Name
255.9	XM_283487	Spink5	Serine protease inhibitor, Kazal type 5
120.3	NM_009789	S100g	S100 calcium binding protein G
99.16	NM_010125	Elf5	E74-like factor 5
71.93	XM_619652	LOC545340	RIKEN cDNA 5031415C07 gene
69.17	NM_054037	Scgb3a1	Secretoglobin, family 3A, member 1
40.54	XM_129166	Mlana	Melan-A
25.65	NM_010165	Eya2	Eyes absent 2 homolog (Drosophila)
17.84	NM_025786	Rnf186	Ring finger protein 186
12.79	NM_029639	1600029D21Rik	
11.27	NM_175397	5830484A20Rik	
10.8	NM_012031	Spag1	Sperm associated antigen 1
9.504	NM_011783	Agr2	Anterior gradient 2 (Xenopus laevis)
8.611	AK008789	2210401J11Rik	
8.319	NM_009897	Ckmt1	Creatine kinase, mitochondrial 1, ubiquitous
7.317	NM_172613	Atp13a4	ATPase type 13A4
6.361	NM_009132	Scin	Scinderin
5.921	NM_183187	BC055107	
5.862	NM_181579	Pof1b	Premature ovarian failure 1B
5.534	NM_009897	Ckmt1	Creatine kinase, mitochondrial 1, ubiquitous
5.291	BM933741		
5.042	NM_011700	Vill	Villin-like
4.803	NM_007914	Ehf	Ets homologous factor
4.526	NM_028618	1110014F24Rik	
4.119	NM_007995	Fcna	Ficolin A
3.854	NM_146050	Oit1	Oncoprotein induced transcript 1
3.85	NM_008339	Cd79b	CD79B antigen
3.797	NM_029639	1600029D21Rik	
3.679	NM_007914	Ehf	Ets homologous factor
3.609	NM_010330	Emb	Embigin
3.526	NM_023270	Rnf128	Ring finger protein 128
3.492	NM_009402	Pglyrp1	Peptidoglycan recognition protein 1
3.411	NM_009700	Aqp4	Aquaporin 4
3.339	NM_133775	9230117N10Rik	
3.326	NM_010330	Emb	Embigin
3.259	NM_015761	Dos	Downstream of Stk11
3.235	NM_008125	Gjb2	Gap junction membrane channel protein beta 2
3.215	NM_009199	Slc1a1	Solute carrier family 1, member 1
3.188	NM_008528	Blnk	B-cell linker
3.158	NM_146117	BC004853	
3.124	NM_027286	Ace2	Angiotensin I converting enzyme 2
3.117	NM_177905	9230101H05Rik	
3.046	XM_128291	Lypdc2	Ly6/Plaur domain containing 2
3.032	NM_001001179	BC048546	
3.016	NM_018754	Sfn	Stratifin
3.01	AK014573	4632413I24Rik	
2.998	NM_023270	Rnf128	Ring finger protein 128
2.964	NM_009434	Phlda2	Pleckstrin homology-like domain, family A, member 2
2.961	NM_152823	Unc5cl	Unc-5 homolog C-like
2.902	NM_008330	Ifi47	Interferon gamma inducible protein 47
2.874	NM_011315	Saa3	Serum amyloid A 3
2.802	BG175786		Transcribed locus
2.79	NM_011382	Six4	Sine oculis-related homeobox 4 homolog
2.675	NM_009199	Slc1a1	Solute carrier family 1, member 1
2.652	NM_145838	st8sia6	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 6
2.635	NM_153404	Liph	Lipase, member H
2.606	NM_134072	9030611N15Rik	
2.603	NM_007494	Ass1	Argininosuccinate synthetase 1
2.601	NM_021297	Tlr4	Toll-like receptor 4
2.591	NM_019980	Litaf	LPS-induced TN factor
2.563	NM_021451	Pmaip1	Phorbol-12-myristate-13-acetate-induced protein 1
2.553	NM_133681	Tspan1	Tetraspan 1
2.496	NM_027096	2310032D16Rik	
2.476	NM_011382	Six4	Sine oculis-related homeobox 4 homolog
2.407	NM_008118	Gif	Gastric intrinsic factor
2.376	BI499978		
2.364	NM_009763	Bst1	Bone marrow stromal cell antigen 1
2.332	NM_197945	Prosapip1	Proline-rich synapse-associated protein-interacting protein 1

Fold Increase	Accession	Abbreviation	Name
2.304	NM_008719	Npas2	Neuronal PAS domain protein 2
2.29	NM_033612	Ela1	Elastase 1, pancreatic
2.288	NM_029007	AW125753	
2.252	NM_178715	Tmem30b	Transmembrane protein 30B
2.238	NM_019738	Nupr1	Nuclear protein 1
2.235	NM_009663	Alox5ap	Arachidonate 5-lipoxygenase activating protein
2.228	XM_283054	Colec11	Collectin sub-family member 11
2.227	NM_020047	Tacstd2	Tumor-associated calcium signal transducer 2
2.224	NM_018811	Abhd2	Abhydrolase domain containing 2
2.203	NM_021297	Tlr4	Toll-like receptor 4
2.202	NM_145403	Tmprss4	Transmembrane protease, serine 4
2.198	NM_011382	Six4	Sine oculis-related homeobox 4 homolog
2.186	NM_019738	Nupr1	Nuclear protein 1
2.166	NM_054095	Efcbp2	EF hand calcium binding protein 2
2.159	NM_009259	Spn	Sialophorin
2.154	NM_134134	A630042L21Rik	
2.153	NM_023755	Tcfcp2l1	Transcription factor CP2-like 1
2.127	NM_144533	Nmnat3	Nicotinamide nucleotide adenylyltransferase 3
2.112	NM_181650	Prdm4	PR domain containing 4
2.11	NM_009657	Aldoc	Aldolase 3, C isoform
2.067	NM_010008	Cyp2j6	Cytochrome P450, family 2, subfamily j, polypeptide 6
2.066	NM_197999	2210023G05Rik	
2.048	BF138688	BC064078	
2.017	XM_129913	Dock10	Dedicator of cytokinesis 10
2.013	XM_286373	1810010H24Rik	RIKEN cDNA 1810010H24 gene