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Kruppel-like factor 5 is required for perinatal lung morphogenesis and function

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The transition to air breathing after birth requires both anatomic and biochemical maturation of the lung. Lung morphogenesis is mediated by complex paracrine interactions between respiratory epithelial cells and mesenchymal cells that direct transcriptional programs guiding patterning and cytodifferentiation of the lung. In the present study, transgenic mice were generated in which the Kruppel-like factor 5 gene (*Klf5*) was conditionally deleted in respiratory epithelial cells in the fetal lung. Lack of KLF5 inhibited maturation of the lung during the saccular stage of development. $Klf5^{\Delta/\Delta}$ mice died of respiratory distress immediately after birth. Abnormalities in lung maturation and morphogenesis were observed in the respiratory epithelium, the bronchiolar smooth muscle, and the pulmonary vasculature. Respiratory epithelial cells of both the conducting and peripheral airways were immature. Surfactant phospholipids were decreased and lamellar bodies, the storage form of surfactant, were rarely found. mRNA microarray analysis demonstrated that KLF5 influenced the expression of genes regulating surfactant lipid and protein homeostasis, vasculogenesis, including *Vegfa*, and smooth muscle cell differentiation. KLF5 regulates genes controlling paracrine interactions during lung morphogenesis, as well as those regulating the maturation of the respiratory epithelium that is required for lung function after birth.

KEY WORDS: Pulmonary, Transcription factor, Vasculogenesis, Paracrine signaling, VEGF, Mouse

INTRODUCTION

Lung morphogenesis and homeostasis are highly coordinated and ordered processes that require the precise regulation of epithelial-mesenchymal interactions among various cell types in the lung. During lung development, progenitor cells lining embryonic lung tubules differentiate into multiple cell types that vary along the cephalo-caudal axis of the lung. Distinct epithelial cell types contribute to mucociliary clearance, fluid and electrolyte transport, the production of innate host defense molecules, and the production of pulmonary surfactant, which is required for gas exchange after birth. Abnormalities in epithelial cell proliferation and differentiation are associated with both acute and chronic lung diseases, including infantile respiratory distress syndrome, bronchopulmonary dysplasia, asthma, chronic obstructive lung disease, cystic fibrosis and pulmonary tumorigenesis (Shi et al., 2007). Understanding the genes and processes regulating maturation of the respiratory epithelium has provided a basis for the diagnosis and treatment of disorders affecting perinatal lung function.

KLF5, a member of the Kruppel-like family of transcription factors, was first identified as an intestinal-enriched member of the zinc-finger transcription factors of the Sp1 subfamily (Conkright et al., 1999). In the mouse, *Klf5* mRNA is expressed in the posterior endoderm associated with the primitive streak in the embryonic day (E) 7.5 embryo (Moore-Scott et al., 2007). Later in embryonic development, KLF5 is detected in epithelial cells of the gastrointestinal tract, the outer layer of the tongue, the epidermis, the trachea, and bronchial epithelial cells (Ohnishi et al., 2000). *Klf5* expression is influenced by important developmental pathways, including the WNT, retinoic acid (RA), RAS and FGF signaling pathways, which, in turn, influence proliferation and differentiation in many organ systems, including the lung (Chanchevalap et al., 2004; Kawai-Kowase et al., 1999; Nandan et al., 2004; Ziemer et al., 2001). Although these pathways are known to be involved in lung morphogenesis, there is increasing evidence that they are also involved in the pathogenesis of lung disease, being induced during inflammation, repair and tumorigenesis (Shaw et al., 2007).

In the mouse embryo, KLF5 is required for formation of the endoderm. $Klf5^{-/-}$ mice die at approximately E8.5, well before lung formation (Shindo et al., 2002). Although *Klf5* is expressed at relatively high levels in epithelial cells lining the fetal and postnatal lung, the role of KLF5 in lung development and function is unknown. In the present study, we generated mice in which the *Klf5* gene was conditionally deleted from respiratory epithelial cells in the developing lung to assess its potential role in lung development and function.

MATERIALS AND METHODS

Mouse models and analysis

Animal protocols were approved by the Institutional Animal Care and Use Committee in accordance with NIH guidelines. A targeting vector containing approximately 9.2 kb of the murine *Klf5* gene was constructed from mouse S6 ES cell genomic DNA. The targeting vector contained loxP sites flanking exons 2 and 3 of the mouse *Klf5* gene, and a selection cassette containing a *frt-pgkneopA-frt* insert. Correctly recombined G418-resistant clones were identified by PCR and Southern blot analyses. $Klf5^{loxP}$ ES cell clones were injected into C57BL/6J mouse blastocysts. Germline chimeras were crossed to FVB/N mice. Heterozygous offspring were mated to generate homozygous $Klf5^{loxP/loxP}$mice. Homologous recombination between loxP sites was accomplished by using the $(TetO)_7$ CMV-Cre transgenic mouse line (Sauer, 1998), kindly provided by Dr Corrinne Lobe, University of Toronto. For lung-specific, doxycycline-induced recombination, the FVB.Cg-^{Tg}(*SFTPC*-rtTA) 5Jaw/J transgenic line was

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used (The Jackson Laboratory, Bar Harbor, Maine). Triple transgenic mice, termed *Klf5*$^{\Delta/\Delta}$ after exposure to doxycycline, were generated by crossing (TetO)$_7$-Cre$^{-/tg}$;*Klf5*$^{loxP/loxP}$ and *SFTPC*-rtTA$^{-/tg}$;*Klf5*$^{loxP/loxP}$ mice. *Klf5*$^{loxP/loxP}$ littermates lacking rtTA and/or Cre alleles served as controls. Gestation was determined by identification of a vaginal plug (E0.5). Dams bearing double- and triple-transgenic pups were maintained on doxycycline-containing food (25 mg/g; Harlan Teklad, Madison, Wisconsin) until E14.5. The mice were killed by injection of anesthetic and then exsanguinated for evaluation. Transgenic mice were identified by PCR using genomic DNA isolated from the tails as previously described (Perl et al., 2002). *Klf5*$^{loxP/loxP}$ PCR was carried out using primers that can distinguish between the wild-type allele and the floxed allele: primer 1, CCT GCG TGC AAT CCA TCT TGT TCA ATG GC; primer 2, TCA CCC TCT GCA GAT CTT AGG C; and primer 3, GCT TGG CTC AAA ATT CCG TTC C.

Histology and immunohistochemistry

Fetal lung tissue was immersion-fixed, embedded, sectioned and immunostained as previously described (Davé et al., 2006). Guinea pig anti-KLF5 antibody was raised against a His-KLF5 peptide containing amino acids 72-245 of the mouse KLF5 protein, a region lacking sequence identity with other KLF family members. To generate the recombinant KLF5 peptide, a fragment of *Klf5* cDNA was amplified and cloned into pTrcHis-TOPO for expression in *E. coli* (Invitrogen, Carlsbad, CA). His-KLF5 peptides were purified using a His-tag protein purification kit (Novagen, Madison, WI). The antibody was tested by ELISA, western blot and immunohistochemistry for specificity and expression in mouse tissues.

For immunohistochemistry, CCSP, FOXJ1, phosphohistone H3, CEBPα, αSMA, and PECAM staining were performed as previously described (Bell et al., 2008; Davé et al., 2006; Martis et al., 2006). Additional antibodies used were as follows: KLF5 (1:2000), VEGFR2 (1:250, rabbit monoclonal, 55B11 Cell Signaling Technology, Danver, MA), and pan-cytokeratin (1:500, mouse monoclonal, C1801, Sigma-Aldrich). For dual immunolabeling, antibodies from two different species were used: guinea pig KLF5 (1:100); rabbit anti-CCSP (1:500); rabbit anti-proSP-C (1:200); rabbit anti-FOXJ1 (1:1000). All experiments shown are representative of findings from at least two independent dams, generating at least four triple transgenic offspring that were compared with littermate controls.

Ultrastructural analysis

Electron microscopy was performed on lung tissue obtained from *Klf5*$^{\Delta/\Delta}$ and littermate controls at approximately E18 (n=3 for each genotype). Tissue was fixed and embedded as previously described (Zhou et al., 1997).

RNA isolation and analysis

RNA was isolated from whole E18.5 lung and reverse transcribed, according to the manufacturer's protocol (Verso^{TM} cDNA kit, Thermo Fisher Scientific, Waltham, MA), prior to RT-PCR analysis. Densitometric quantitation of the PCR products was carried out using Quality One software (Bio-Rad Laboratories, Philadelphia, PA). The relative concentrations of each mRNA were normalized to the concentration of β-actin mRNA in each sample. Primer sequences are available on request. *Sftpb*, *Sftpc* and *Scgbla1* mRNAs were quantified by S1 nuclease protection assays using ribosomal protein L32 as an internal control (Dranoff, 1994). Differences were assessed by Student's t-test.

RNA microarray analysis

RNAs from three different control and *Klf5*$^{\Delta/\Delta}$ mouse lungs were isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA), and amplified using an Ovation Biotin RNA application and labeling system (NuGen Technologies, San Carlos, CA). Lung cRNA was hybridized to the murine genome MOE430_2 chips, consisting of approximately 39,000 transcripts (Affymetrix, Santa Clara, CA), using the manufacturer's protocol. The RNA quality and quantity assessment, probe preparation, labeling, hybridization and image scan were carried out in the CCHMC Affymetrix Core using standard procedures. Affymetrix MicroArray Suite version 5.0 was used to scan and quantify signals using default scan settings. Six chips from three pair-wise experiments were used. Normalization was performed using the Robust Multichip Average Model (Irizarry et al., 2003a; Irizarry et al., 2003b). Data were further analyzed using affylmGUI from the R/Bioconductor package (Smyth, 2004). Differentially expressed genes were selected with a threshold of Student t-test P-value ≤0.05, False Discovery Rate (FDR) ≤5%, fold change ≥1.5 and a minimum of two present calls by Affymetrix algorithm in three samples. Gene ontology analysis was performed using the publicly available web-based tool DAVID (database for annotation, visualization, and integrated discovery) (Dennis et al., 2003). Pathways that were overly represented were identified by comparing the overlap of differentially expressed genes and all genes in the MOE430 mouse genome. Gene sets were associated with known pathways and disease states from KEGG (http://www.genome.ad.jp/kegg/), GenMAPP (http://www.genmapp.org/) and GEArrays (http://www.superarray.com/). A pathway was considered to be overly represented when it showed a probability P-value ≤0.01 and >10 gene hits.

Surfactant analysis

Saturated phosphatidylcholine (SatPC) was isolated from lipid extracts of lung homogenates from six mice of each genotype and analyzed as previously described (Wan et al., 2004). Mature SP-B western blot was performed with antibody AB3426 (Chemicon, Temecula, CA) against mature SP-B peptide.

Transient transfection assays

H441 (a human pulmonary adenocarcinoma cell line) and JEG (a human choriocarcinoma cell line) cells were grown to 70% confluence in six-well plates and transfected with either plasmid (0.5 μg) or siRNA (100 pmole) using Lipofectamine 2000 (catalog number 11668-027, Invitrogen, Carlsbad, CA). Promoter activity was determined by the measurement of luciferase activity normalized to β-galactosidase activity 48 hours after transfection. All experiments were done in duplicate in three independent experiments. The mean of the control was set to 1 and relative promoter activities were shown as mean±s.e.m. and compared by the two-tailed Student t-test (*P<0.05). pGL2-*Vegfa*-Luc was kindly provided by Dr Mukhopadhyay (Mukhopadhyay et al., 1997). pGL3-3TP-Luc, a TGFβ responsive promoter construct, was kindly provided by Dr Molkentin (Wrana et al., 1992). A human HIF2α expression vector in pcDNA3 was a gift from Dr Richard K. Bruick (Tian et al., 1997).

RESULTS

Klf5 is expressed in pulmonary epithelial cells throughout lung development

To determine the pattern of *Klf5* expression during lung morphogenesis, immunohistochemistry was performed using an anti-mouse KLF5 polyclonal antibody. At E12.5, KLF5 staining was observed primarily in the nuclei of subsets of epithelial cells lining the proximal bronchial tubules, and exhibited a difference in mediolateral expression with increased staining in the medial aspect of the tubules (Fig. 1A). From E14.5 to E18.5, KLF5 was more widely expressed in both proximal and peripheral epithelium, the expression levels varying among different subsets of epithelial cells (Fig. 1B,C). After birth, KLF5 was found in subsets of epithelial cells in both conducting airways and alveoli (Fig. 1D). Dual immunolabeling for KLF5 and various epithelial cell specific markers was performed (Fig. 1E-G). At E18.5, KLF5 staining was detected in a subset of cells expressing proSP-C (surfactant protein C), a type II alveolar epithelial cell marker. In conducting airways, KLF5 was expressed most robustly in cells staining for the non-ciliated bronchiolar cell marker CCSP (Clara cell secretory protein). Under these conditions, KLF5 was not co-expressed with FOXJ1, a ciliated cell marker. However, by more sensitive immunohistochemistry, KLF5 was detected at low levels in ciliated bronchiolar cells (as compared with the level observed in nonciliated bronchiolar cells, Fig. 1D).

KLF5 is required for normal lung morphogenesis: conditional deletion of *Klf5* in the lung

LoxP sites flanking exons 2 and 3 of the *Klf5* gene were introduced into the gene-targeting vector, which was designed to delete most of the protein-coding region, including part of the KLF5 DNA-binding

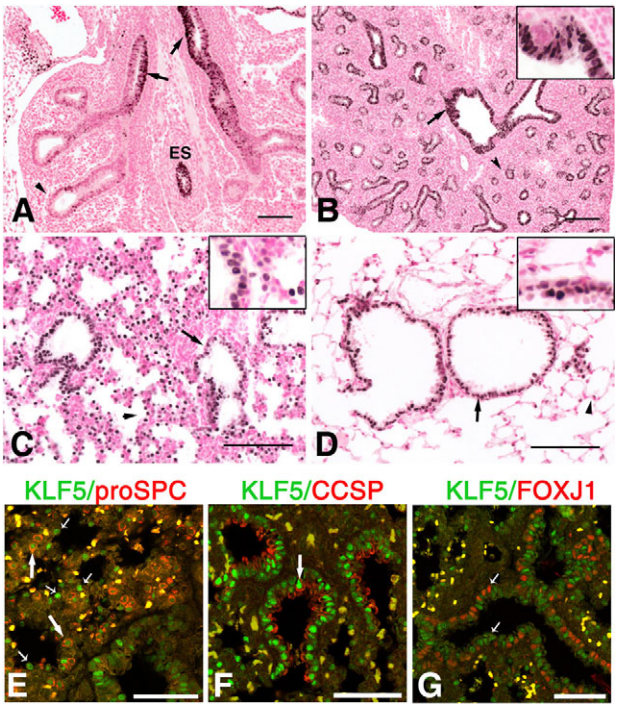

Fig. 1. Immunohistochemical analysis of KLF5 in the developing mouse lung. (**A**) At E12.5, during the early pseudoglandular stage of development, KLF5 was detected in nuclei of epithelial cells in the proximal bronchial tubules (arrow). Staining was generally more intense along medial aspects of the tubules. ES, esophagus. Arrowhead indicates peripheral epithelial cells. (**B**) At E15.5, KLF5 was detected at highest levels in bronchiolar tubules (arrow); however, staining of subsets of cells lining the peripheral lung buds (arrowhead) was also noted. (**C**) At E18.5, KLF5 was present in both peripheral (arrowhead) and proximal (arrow) airways. (**D**) In mature lung, KLF5 was detected in nuclei of subsets of epithelial cells in both conducting airways (arrow) and alveolar regions (arrowhead). Insets are higher magnifications of the corresponding figures. (**E-G**) Dual immunolabeling for KLF5 (green nuclei) and (E) proSPC (red cytoplasm), (F) CCSP (red cytoplasm), and (G) FOXJ1 (red nuclei) was performed on E18.5 lung sections. KLF5 staining was observed in proSPC-positive (large arrow) and -negative (small arrow) cells (E), in CCSP-positive cells (large arrow, F), and in FOXJ1-negative cells (small arrow, G). Yellow signal is due to the autofluoresence of red blood cells, detectable in both channels. Scale bars: 100 μm.

domain (Fig. 2). Germline chimeras and mice heterozygous for the $Klf5^{loxP}$ allele were produced from targeted ES cells. Heterozygous offspring were mated to generate homozygous $Klf5^{loxP/loxP}$mice. Adult $Klf5^{loxP/loxP}$ mice were healthy, fertile and expressed KLF5 normally in the lung, indicating no apparent interference with endogenous KLF5 expression. These animals were bred into the $(TetO)_7$-$Cre^{-/tg}$ and *SFTPC*-$rtTA^{-/tg}$ transgenic lines to enable us to create triple transgenic animals in which *Klf5* could be deleted in respiratory epithelial cells upon doxycycline treatment. $Klf5^{\Delta/\Delta}$ mice were generated by maintaining pregnant dams on doxycycline from E0 to E14.5. Lungs were isolated from fetuses at E15.5 and E18.5 for further analysis.

The extent and sites of *Klf5* deletion were determined by immunohistochemistry, demonstrating variable but extensive deletion of *Klf5* in epithelial cells of the lung. In control and $Klf5^{\Delta/+}$ mice, KLF5 was detected in the nuclei of both alveolar and bronchiolar epithelial cells (Fig. 3A,C,E), whereas nuclear staining of KLF5 was absent in most respiratory epithelial cells in the $Klf5^{\Delta/\Delta}$ mice (Fig. 3B,D,F).

Klf5 is required for perinatal lung function at birth

When mice were maintained on doxycycline from E0 to E14.5, offspring were born in the expected Mendelian distribution for all genotypes. At E15.5, morphological abnormalities were not observed in the lungs of $Klf5^{\Delta/\Delta}$ mice at the light microscopic level (Fig. 3B). At E18.5, no morphological abnormalities were observed in the lungs of $Klf5^{\Delta/+}$ fetuses (Fig. 3E) and these fetuses survived normally after birth. By contrast, all $Klf5^{\Delta/\Delta}$ mice died of respiratory distress shortly after birth. At E18.5, the lung morphology of $Klf5^{\Delta/\Delta}$ mice was perturbed (Fig. 3D,F). The alveolar saccules appeared hypercellular, lacking the dilated peripheral saccules that are characteristic of the normal lung at this stage of development. The severity of the morphological abnormalities correlated with the efficiency of *Klf5* deletion. A dramatic reduction in the number of squamous epithelial cells were found in the lungs of $Klf5^{\Delta/\Delta}$ mice, as visualized by staining with pan-cytokeratin antibody (Fig. 3G,H), indicating a lack of alveolar type I cell differentiation after the deletion of *Klf5*. Morphometric analysis demonstrated that the luminal area of the alveolar saccules in the lungs of $Klf5^{\Delta/\Delta}$ mice was 26.5±5.3% and that of the controls was 37.3±3.8%. Thus, luminal area was significantly decreased in the lungs of $Klf5^{\Delta/\Delta}$ mice ($P<0.05$, $n=3$).

KLF5 did not affect proliferation of pulmonary epithelial cells

The wet lung/body weight ratio for controls was 0.029±0.005 ($n=10$) and for $Klf5^{\Delta/\Delta}$ mice was 0.022±0.003 ($n=11$). The lung/body weight ratio was slightly but significantly decreased in $Klf5^{\Delta/\Delta}$ mice ($P<0.05$). Because of the failure of sacculation in lungs of the $Klf5^{\Delta/\Delta}$ mice, significant differences in the wet lung/body weight ratio could be caused by the lack of fluid in the alveolar saccules in the $Klf5^{\Delta/\Delta}$ mice. Previous studies indicated that KLF5 influenced the RAS/MAPK signaling pathway and promoted cell proliferation in various cell types, including fibroblasts, smooth muscle cells, bladder cancer and intestinal epithelial cells (Bateman et al., 2004; Chanchevalap et al., 2004; Chen, C. et al., 2006; Nandan et al., 2005; Nandan et al., 2004; Sun et al., 2001). In order to determine whether KLF5 affected the proliferation of epithelial or mesenchymal cells, phosphohistone H3 (p-histone-3) immunostaining was performed and the average number of immunolabeled cells per mm^2 in the mesenchyme and in the epithelium was determined at E15.5, a time that active proliferation is

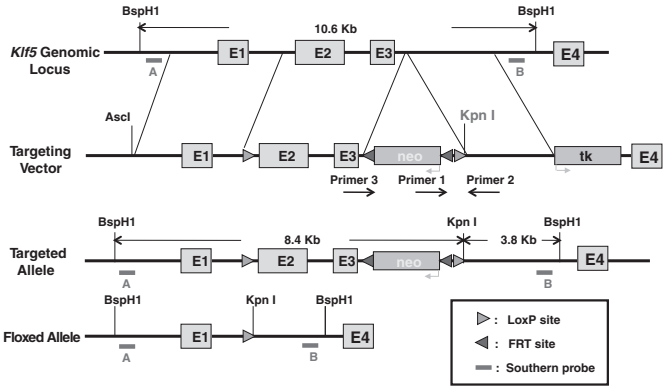

Fig. 2. Targeting of the *Klf5* gene. Indicated are the structures of the endogenous *Klf5* locus, the targeting vector, targeted allele, and recombined allele. Exons 1 to 4 are indicated by the boxes. The 5′ and 3′ probes used for Southern blot analysis are indicated.

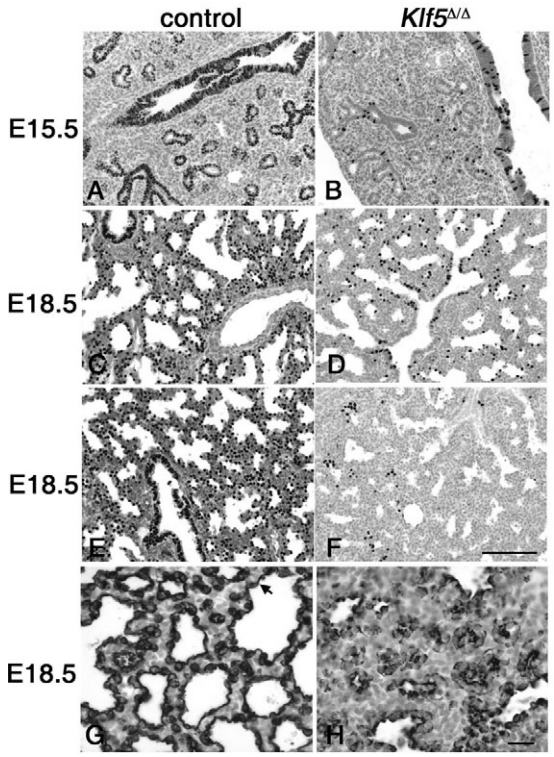

Fig. 3. Deletion of *Klf5* disrupts lung morphogenesis. (**A-H**) Lung sections were prepared from the fetus at E15.5 (A,B) and E18.5 (C-H), and were stained with a guinea pig anti-mouse KLF5 polyclonal antibody (A-F) or pancytokeratin (G,H), which outlined the epithelial cells. Nuclear staining of KLF5 was observed in epithelial cells lining the conducting and peripheral airways in control (A,C) and $Klf5^{\Delta/+}$ (E) mice. Staining was absent or decreased in $Klf5^{\Delta/\Delta}$ mice (B,D,F). Morphological changes in the lungs of $Klf5^{\Delta/\Delta}$ mice were not observed at E15.5 (A versus B). At E18.5, lung morphology was markedly perturbed in $Klf5^{\Delta/\Delta}$ mice (D,F,H), note the thickened walls of the mutant saccules, compared with $Klf5^{+/+}$ (C,G) and $Klf5^{\Delta/+}$ mice (E). Squamous epithelial cells (arrow) were rarely observed in the lungs of $Klf5^{\Delta/\Delta}$ mice (H), compared with controls (G). Scale bars: 50 μm.

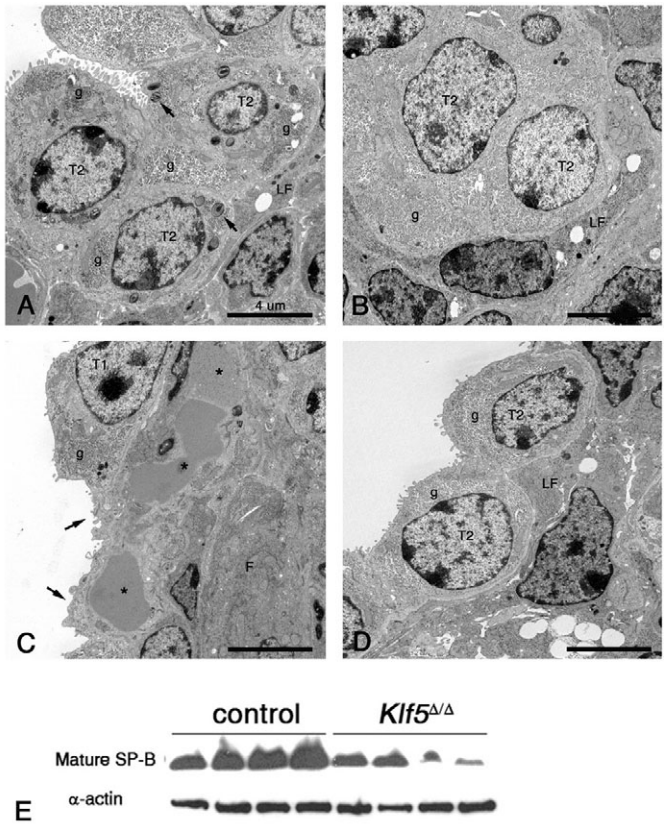

Fig. 4. Ultrastructural and biochemical analysis of lungs from $Klf5^{\Delta/\Delta}$ mice. (**A-D**) Lungs from control (A,C) and $Klf5^{\Delta/\Delta}$ (B,D) mice (approximately E18) were fixed and processed for electron microscopy as described in the Materials and methods. (B) Immature Type 2 cells (T2) with no lamellar bodies and abundant glycogen particles (g), distributed diffusely throughout the cytoplasm, were observed in the lungs of the $Klf5^{\Delta/\Delta}$ mice. Lipofibroblasts (LF) were found ensheathing the undilated acinar tubules and buds in the periphery of the lung. (A) By comparison, T2 cells (T2) from control mice were more mature, exhibiting multiple lamellar bodies (arrows), smaller patches of glycogen (g), and increased amounts of rough endoplasmic reticulum. (D) In the $Klf5^{\Delta/\Delta}$ mice, immature T2 cells (T2) with no lamellar bodies were observed lining the more dilated acinar tubules found in the central regions of the $Klf5^{\Delta/\Delta}$ lungs. Note the prominent lipofibroblasts (LF) directly adjacent to these epithelial cells. (C) By contrast, differentiating T1 cells (T1) with thin cytoplasmic extensions (arrows) and centrally located glycogen patches (g) were found in the dilated alveolar saccules of control mice. Pulmonary capillaries filled with red blood cells (*) were found in close apposition to squamous type I cells (arrows). Fibroblasts (F) without lipid droplets were observed in the interior of the alveolar septa. (**E**) Whole lung homogenates were prepared at E18.5 and 100 μg protein used for western blot using antiserum against the mature SP-B peptide. Mature SP-B was decreased in the lungs of $Klf5^{\Delta/\Delta}$ mice at E18.5, as assessed in four individual animals. Scale bars: 4 μm.

normally present in both of these cell types. No differences in p-histone-3 staining were observed in pulmonary epithelial or mesenchymal cells after deletion of *Klf5* (data not shown). Similarly, BrdU labeling at E15.5 revealed no differences between the lungs of $Klf5^{\Delta/\Delta}$ and control mice (data not shown).

Abnormalities in lung ultrastructure after deletion of *Klf5*

At the ultrastructural level, lungs from $Klf5^{\Delta/\Delta}$ mice were immature. Alveolar type II cells contained abundant glycogen but no lamellar bodies (Fig. 4B). In KLF5 sufficient (non-deleted) mice, cuboidal alveolar type II cells contained numerous lamellar bodies, rough endoplasmic reticulum, apical microvilli, and smaller patches of glycogen (Fig. 4A). Secreted surfactant was observed in KLF5 sufficient mice but not in $Klf5^{\Delta/\Delta}$ mice (data not shown), and squamous type I cells were found in close apposition to adjacent capillaries (Fig. 4C). In $Klf5^{\Delta/\Delta}$ mice, squamous type I epithelial cells were not observed (Fig. 4D). The interstitial tissue was hypercellular and contained numerous capillaries that were more centrally located (data not shown). In both control and $Klf5^{\Delta/\Delta}$ mice, bronchioles were lined by differentiating ciliated cells containing little residual glycogen and by immature, glycogen-containing, columnar, Clara cells (data not shown). There were no clear differences in the ultrastructure of epithelial cells lining the bronchioles caused by deletion of *Klf5*.

KLF5 is required for normal surfactant protein and lipid production

Surfactant proteins and lipids are crucial for lung function at birth, being required for the reduction of surface tension and the maintenance of lung expansion. Lack of surfactant proteins and

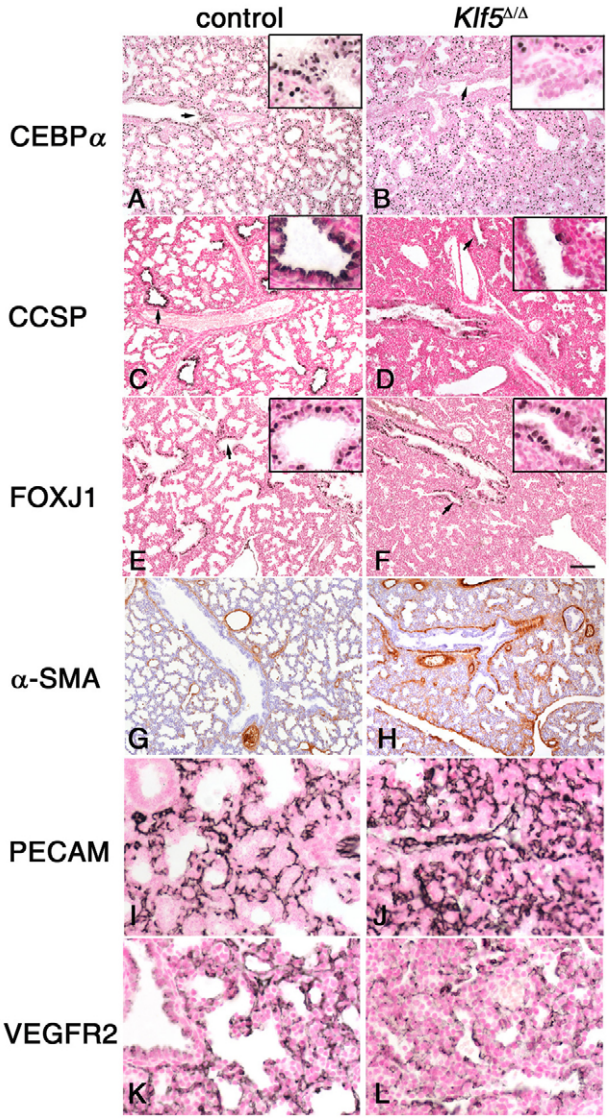

Fig. 5. KLF5 influences the differentiation of lung epithelium and mesenchyme. (**A-L**) Lung sections from *Klf5*$^{\Delta/\Delta}$ mice and littermate controls were prepared at E18.5 and immunostained for CEBPα (A,B), CCSP (C,D), FOXJ1 (E,F), αSMA (G,H), PECAM (I,J) and VEGFR2 (K,L). Significantly decreased staining of CEBPα (B) and CCSP (D) was detected in the airway epithelial cells, whereas no change in FOXJ1 staining was observed (F). The intensity and distribution of αSMA staining were increased in the peripheral lung of the *Klf5*$^{\Delta/\Delta}$ mice (H). Although staining for PECAM (J) and VEGFR2 (L) was readily detected, the pulmonary mesenchyme was thickened and the blood vessels were not found in close proximity to the adjacent epithelial cells. Insets are higher magnification views of the regions indicated by the arrows. Scale bar: 50 μm.

lipids causes infantile respiratory distress syndrome in preterm infants at birth. Saturated phosphatidylcholine (SatPC) is the major surfactant phospholipid that is crucial for lung function. Lung SatPC increases dramatically in late gestation in most mammals and is a useful indicator of lung maturation in preterm infants. Consistent with the lack of lamellar bodies observed by electron microscopy, the SatPC content in the lungs of E18.5 *Klf5*$^{\Delta/\Delta}$ mice [3.302±0.12 nmol/lung weight (mg)] was significantly decreased (n=6, P<0.05), compared with controls [4.86±0.37 nmol/lung weight (mg)]. Western blot analysis was performed to detect mature SP-B in lung homogenates, as previously described (Wan et al., 2004). The active SP-B peptide was decreased in lung from the *Klf5*$^{\Delta/\Delta}$ pups (Fig. 4E).

KLF5 influences epithelial cell gene expression

Cell-specific markers were used to evaluate the effects of *Klf5* gene deletion on epithelial cell differentiation at E18.5. CEBPα, an epithelial-specific transcription factor normally expressed in type II cells and non-ciliated bronchiolar epithelial cells (Fig. 5A), was dramatically decreased in conducting airway epithelial cells, but not in peripheral pre-type II cells (Fig. 5B). CCSP staining, a marker of differentiated non-ciliated bronchiolar epithelial cells, was also markedly decreased in the lungs of *Klf5*$^{\Delta/\Delta}$ mice (Fig. 5D). The lack of CCSP and the association of KLF5 expression with Clara cells suggest a potential role of KLF5 in bronchiolar cell differentiation. By contrast, FOXJ1 staining was not changed by deletion of *Klf5* (Fig. 5F). CCSP and CEBPα staining was decreased in the bronchiolar epithelium in the *Klf5*$^{\Delta/\Delta}$ mice. Taken together with the robust KLF5 staining in the Clara cells of control mice, these data suggest that KLF5 regulates differentiation or gene expression in non-ciliated cells. Promoter constructs of pGL3-CCSP-Luc (promoter construct of *Scgbla1* gene) and pGL3-*Cebpa*-Luc were co-transfected with a *Klf5* expression plasmid or *Klf5*-targeting siRNA into H441 cells, a human

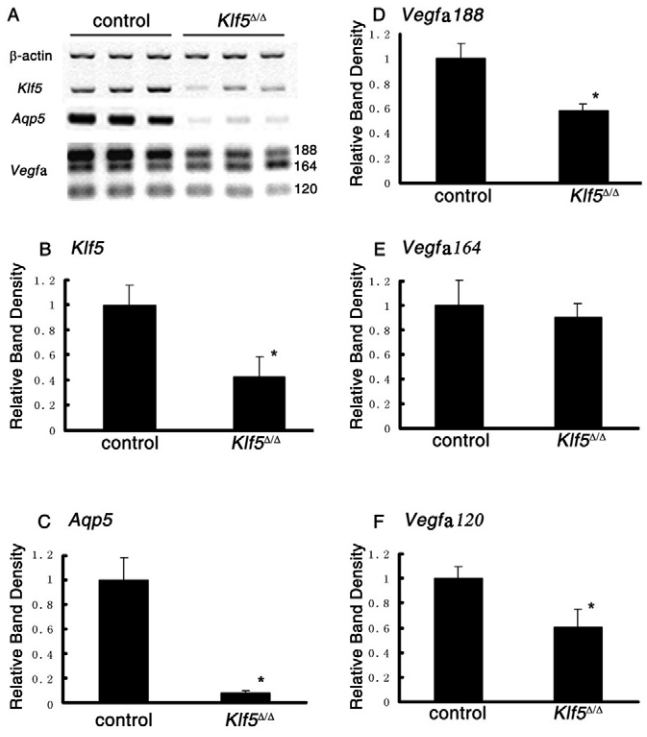

Fig. 6. Decreased *Vegfa*188, *Vegfa*120 and *Aqp5* mRNA in lungs from *Klf5*$^{\Delta/\Delta}$ mice. mRNAs from *Klf5*$^{\Delta/\Delta}$ mice and littermate controls were isolated at E18.5. (**A**) RT-PCR was performed for β-actin, *Klf5*, *Aqp5* and *Vegfa*. *Vegfa* isoforms were determined by using primers flanking exon 6 and exon 7 of *Vegfa*. (**B-F**) Densitometric quantitation of the PCR products, which were normalized to β-actin in each sample, revealed significantly decreased levels of *Klf5* (B), *Aqp5* (C), *Vegfa*188 (D) and *Vegfa*120 (F) mRNA, but not *Vegfa*164 mRNA (E) in the lungs of *Klf5*$^{\Delta/\Delta}$ mice. The mean of the control was set to 1; relative mRNA levels are shown as mean±s.e.m. and were compared by a two-tailed Student's *t*-test (*P<0.05).

Table 1. Cellular pathways affected by deletion of *Klf5*

Genes overlapping with pathways	Gene number	*P*-value
Breast cancer and estrogen receptor signaling	29	2.88×10^{-10}
Androgen signaling and prostate cancer	23	6.58×10^{-7}
Stress response to cellular damage	24	3.15×10^{-6}
Cell cycle	15	7.47×10^{-6}
MAP kinase signaling pathway	21	0.000176
ECM-receptor interaction	11	0.000587
cAMP Ca^{2+} signaling pathway finder	16	0.000838
p53 signaling pathway	19	0.001910
TGFβ BMP signaling pathway	16	0.003400
Signal transduction in cancer	14	0.008640

pulmonary adenocarcinoma cell line that expresses high levels of *Klf5*. Changes in KLF5 expression did not significantly alter the expression of the *Scgbla1* and *Cebpa* gene promoters in vitro (data not shown). As CEBPα is known to regulate the *Scgbla1* promoter (Martis et al., 2006), the lack of CEBPα in the bronchial/bronchiolar epithelial cells may provide a potential mechanism by which KLF5 influences the expression of *Scgbla1* in those cells. Aquaporin 5 (*Aqp5*) mRNA, an alveolar type I cell marker, was significantly decreased in the lungs of $Klf5^{\Delta/\Delta}$ mice as analyzed by RT-PCR and densitometric quantitation of the PCR products (Fig. 6C), consistent with the absence of alveolar type I cells seen morphologically.

KLF5 is required for sacculation and influences epithelial-mesenchymal interactions

Deletion of *Klf5* in lung epithelial cells perturbed the organization of the adjacent mesenchyme. Expression of αSMA, a marker of bronchiolar and vascular smooth muscle cell differentiation, was markedly increased in the bronchioles of the lungs from $Klf5^{\Delta/\Delta}$ mice (Fig. 5H). Although the pulmonary vasculature was present in the lungs of $Klf5^{\Delta/\Delta}$ mice, as indicated by PECAM (Fig. 5I,J) and VEGFR2 (Fig. 5K,L) immunostaining, the normal alignment of epithelial cells with alveolar capillaries in the lung periphery was perturbed. These findings support the ultrastructural changes seen in the lungs of $Klf5^{\Delta/\Delta}$ mice, in which the normal association of epithelial and endothelial cells was disrupted.

As *Vegfa* is expressed in respiratory epithelial cells of the developing lung and is essential for pulmonary vascular development, the effects of KLF5 on the expression of *Vegfa* were examined by RT-PCR using primers flanking exons 6 and 7 (Healy et al., 2000). The splicing-variant *Vegfa188*, *Vegfa164* and *Vegfa120* mRNAs were detected as uniquely sized products. Controls included β-actin and *Klf5* itself. Densitometric quantitation of the PCR products revealed a significant decrease in *Vegfa188* and *Vegfa120* mRNA in the lungs of $Klf5^{\Delta/\Delta}$ mice (Fig. 6D,F). Although selectively expressed in epithelial cells, KLF5 is required for normal morphogenesis of the capillary bed, indicating a role for KLF5 in the regulation of paracrine interactions in the developing lung.

Genomic responses to the deletion of *Klf5*

To identify other genes influenced by the conditional deletion of *Klf5*, mRNA expression profiles were compared in lungs from E18.5 $Klf5^{\Delta/\Delta}$ mice and their littermate controls using Affymetrix murine genome MOE 430_2 GeneChips. Genes with a fold change of at least 1.5 were selected. Gene set enrichment analysis using the differentially expressed genes overlapping with pathways from KEGG, GenMAPP and Superarray indicated that KLF5 influenced the expression of genes associated with cancer, cell cycle, MAP kinase signaling, angiogenesis, and the TGFβ and BMP signaling pathways (Table 1). As deletion of *Klf5* affects perinatal lung maturation and function at birth, we chose to determine the effects of KLF5 on subsets of genes and pathways regulating perinatal lung maturation, lipid metabolism, angiogenesis and TGFβ signaling.

The microarray study of the $Klf5^{\Delta/\Delta}$ mice at E18.5 demonstrated changes in the expression of genes known to regulate surfactant and surfactant lipid homeostasis, including *Sftpa*, *Sftpb*, *Sftpd*, *Abca3*, *Aytl2* (*Lpcat1* – Mouse Genome Informatics), *Srebf1* and *Srebf2* (Besnard et al., 2007; Chen, X. et al., 2006; Shulenin et al., 2004) (see Table 2). Decreased expression of *Sftpb* was confirmed by S1 nuclease protection assay (data not shown), and by western blot analysis (Fig. 4). To assess whether KLF5 directly regulated the transcription of *Sftpa*, *Sftpb*, *Sftpd*, *Aytl2* and *Abca3*, promoter constructs were co-transfected with a *Klf5* expression plasmid or *Klf5* siRNA into H441 cells. In contrast to previous studies demonstrating that some of these promoters were directly regulated by NKX2.1, FOXA2 and CEBPα (Bohinski et al., 1994; Martis et al., 2006), KLF5 did not significantly alter the activity of these gene promoter constructs in vitro (data not shown).

Microarray analysis and extensive literature mining identified changes in several genes known to be involved in pathways that mediate epithelial-mesenchymal interaction in the lungs of $Klf5^{\Delta/\Delta}$ mice, including VEGF, PDGF, FGF and TGFβ (Fig. 7), supporting

Table 2. Genes involved in surfactant protein and lipid production

Gene symbol	Common gene name	Ratio
Abca3	ATP-binding cassette, sub-family A (ABC1), member 3	–1.78
Abca4	ATP-binding cassette, sub-family A (ABC1), member 4	–1.97
Acox2	Acyl-coenzyme A oxidase 2, branched chain	–2.99
Aytl2	Acyltransferase like 2	–3.09
Cdipt	CDP-diacylglycerol–inositol 3-phosphatidyltransferase	–1.69
Fasn	Fatty acid synthase	–1.60
Ldlr	Low-density lipoprotein receptor	–2.35
Pon1	Paraoxonase 1	–11.81
Scd1	Stearoyl-coenzyme A desaturase 1	–7.95
Scd2	Stearoyl-coenzyme A desaturase 2	–2.07
Srebf1	Sterol regulatory element binding factor 1	–1.63
Srebf2	Sterol regulatory element binding factor 2	–1.56
Sftpa1	Surfactant associated protein A1	–4.24
Sftpb	Surfactant associated protein B	–2.08
Sftpd	Surfactant associated protein D	–12.80
Adrb2	Adrenergic receptor, beta 2	–1.75
Ndst1	N-deacetylase/N-sulfotransferase 1	–1.56

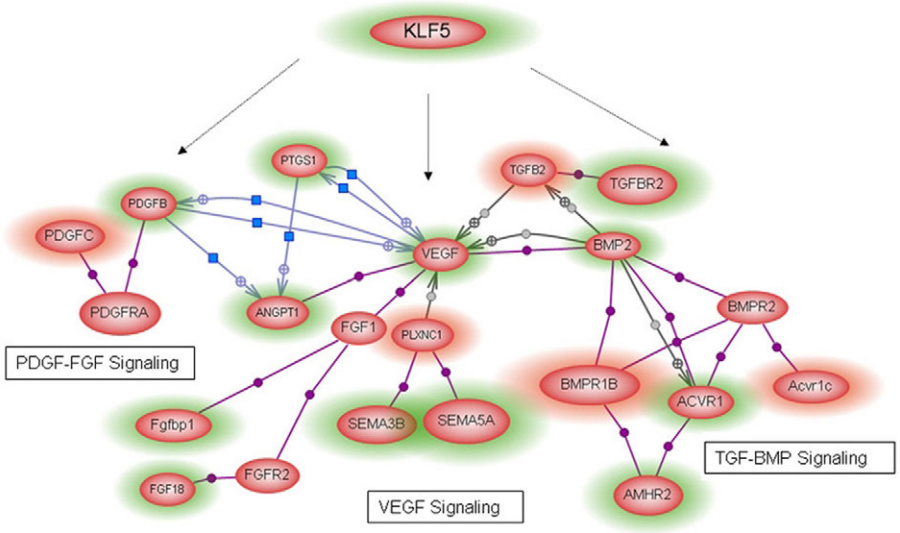

Fig. 7. Model of the paracrine signaling network influenced by deletion of *Klf5*. mRNAs that were significantly altered in lungs from $Klf5^{\Delta/\Delta}$ mice were subjected to literature mining to identify potential functional associations and regulatory relationships using Pathway Studio 5.0 (Ariadne Genomics). KLF5 influenced the expression of genes previously associated with various signaling pathways, including those regulated by PDGF, FGF and TGFβ. mRNAs increased in $Klf5^{\Delta/\Delta}$ mice are framed in red, those decreased are framed in green. Each line indicates a regulatory relationship between gene nodes based upon literature references. Regulatory relationships are denoted by line colors and patterns: purple lines represent binding; blue lines represent regulation of expression; the gray lines represent 'regulation'; and arrows with a plus indicate positive regulation.

the morphological findings that KLF5 regulates paracrine signaling from the epithelium, influencing mesenchymal cell differentiation. Table 3 lists genes altered in the E18.5 $Klf5^{\Delta/\Delta}$ lung that mediate paracrine signaling. The increase in *Tgfb2* (1.9-fold) and *Acvr1c* (2.87-fold), and the decrease in *Smad6* (–1.25-fold) expression were confirmed by real-time PCR (data not shown).

KLF5 regulates *Vegfa* gene expression in vivo and in vitro

As *Vegfa* mRNA was significantly decreased in the microarray, and *Vegfa188* and *Vegfa120* isoforms were found by RT-PCR to be decreased, we tested whether KLF5 influenced the transcriptional activity of *Vegfa*. A luciferase reporter construct containing 2.6 kb

Table 3. Genes involved in paracrine signaling

Gene symbol	Gene name	Fold change	Signaling pathway
Acvr1	Activin A receptor, type 1	–1.595	TGFβ
*Acvr1c**	Activin A receptor, type IC	5.001	TGFβ
Amhr2	Anti-Mullerian hormone type 2 receptor	–8.928	TGFβ
Bmp2	Bone morphogenetic protein 2	–1.898	TGFβ
Bmp5	Bone morphogenetic protein 5	1.565	TGFβ
Bmp8b	Bone morphogenetic protein 8b	–1.522	TGFβ
Bmpr1b	Bone morphogenetic protein receptor, type 1B	1.659	TGFβ
Cdkn2b	Cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	–4.232	TGFβ
Gdf5	Growth differentiation factor 5	–1.506	TGFβ
Inhbb	Inhibin beta-B	–3.029	TGFβ
Ltbp1	Latent transforming growth factor beta binding protein 1	1.571	TGFβ
*Smad6**	MAD homolog 6 (Drosophila)	–1.638	TGFβ
Nodal	Nodal	–1.719	TGFβ
Rbl1	Retinoblastoma-like 1 (p107)	1.536	TGFβ
Thbs2	Thrombospondin 2	1.526	TGFβ
Thbs3	Thrombospondin 3	2.238	TGFβ
Tgfb2	Transforming growth factor, beta 2	1.626	TGFβ
*Tgfbr2**	Transforming growth factor, beta receptor 2	–1.761	TGFβ
*Vegfa**	Vascular endothelial growth factor A	–2.017	VEGF
Vegfr1(Flt1)	FMS-like tyrosine kinase 1	–1.502	VEGF
Vegfr3(Flt4)	FMS-like tyrosine kinase 4	–1.569	VEGF
Edg1	Endothelial differentiation sphingolipid G-protein-coupled receptor 1	–1.703	VEGF
Edg6	Endothelial differentiation, G-protein-coupled receptor 6	–1.953	VEGF
Edg7	Endothelial differentiation, lysophosphatidic acid G-protein-coupled receptor 7	–2.134	VEGF
Efna3	Ephrin A3	–1.667	VEGF
Sema5a	Sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 5A	–1.713	VEGF
Sema3b	Sema domain, immunoglobulin domain (Ig), short basic domain, secreted, (semaphorin) 3B	–1.878	VEGF
Hbegf	Heparin-binding EGF-like growth factor	–1.553	VEGF
Pdgfb	Platelet derived growth factor, B polypeptide	–1.533	PDGF, FGF
Pdgfc	Platelet-derived growth factor, C polypeptide	1.667	PDGF, FGF
Fgf18	Fibroblast growth factor 18	–2.148	PDGF, FGF
Fgfbp1	Fibroblast growth factor binding protein 1	–1.506	PDGF, FGF
Frag1	FGF receptor activating protein 1	–1.737	PDGF, FGF

*Changes in expression were confirmed by real-time PCR of RNAs from independent samples (n=5, P<0.05).

of the regulatory region of the *Vegfa* gene (Mukhopadhyay et al., 1997) was co-transfected with a *Klf5* expression vector in vitro. KLF5 enhanced *Vegfa* luciferase activity in a dose-dependent manner in JEG-3 cells, a human choriocarcinoma cell line that does not normally express lung-specific transcription factors (Fig. 8A) (Bachurski et al., 2003). Whereas no change in *Vegfa* luciferase activity was detectable in H441 cells, a cell line that expresses *Klf5* (data not shown). Decreasing the level of *Klf5* expression by using *Klf5* siRNA inhibited *Vegfa*-luciferase activity in H441 cells (Fig. 8B). As HIF2α is a strong activator of the *Vegfa* promoter in H441 cells (Maeda et al., 2008), the effect of KLF5 on HIF2α-dependent *Vegfa*-luciferase activity was assessed. *Klf5* siRNA significantly decreased HIF2α-dependent *Vegfa*-luciferase activity in H441 cells (Fig. 8B), indicating that KLF5 might influence *Vegfa* expression, at least in part, via a transcriptional mechanism.

KLF5 affected TGFβ1-dependent pGL2-3TP-Luc activity in vitro

In order to test whether KLF5 influenced the TGFβ signaling pathway in vitro, H441 cells were co-transfected with a *Klf5* expression vector and pGL2-3TP-Luc, a construct consisting of a synthetic promoter derived from the TGFβ-responsive (SMAD selective) region of the plasminogen activator inhibitor 1 gene (Wrana et al., 1992). TGFβ1 induced pGL2-3TP luciferase activity more than 20-fold in H441 cells. Increasing the level of KLF5 expressed in H441 cells mildly inhibited pGL2-3TP luciferase activity at baseline and markedly inhibited the induction of pGL2-3TP luciferase activity by TGFβ1 (Fig. 8C). Inhibition of endogenous *Klf5* mRNA levels using siRNA increased pGL2-3TP luciferase activity and markedly enhanced pGL2-3TP luciferase activity in the presence of TGFβ1 (Fig. 8D), indicating that KLF5 acts as a negative regulator of TGFβ signaling in pulmonary adenocarcinoma cells in vitro.

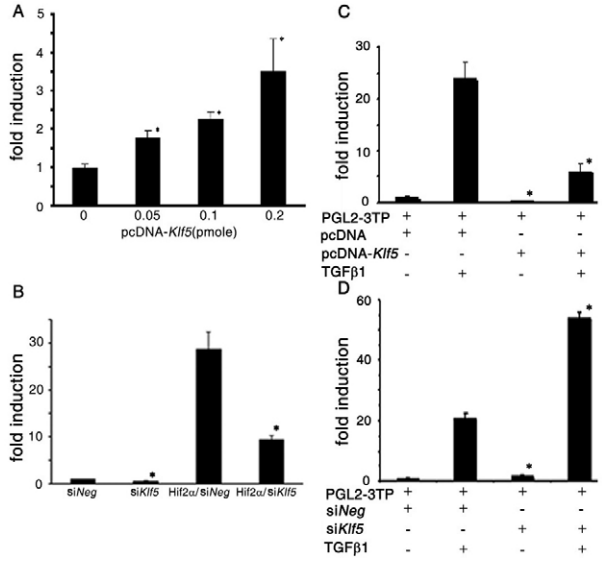

Fig. 8. Transcriptional regulation of *Vegfa* and *Tgfb* promoter activity by KLF5. (**A**) JEG cells were transfected with promoter plasmid pGL2-*Vegfa*-Luc and increasing amounts of the expression plasmid pcDNA-*Klf5*. The plasmid pcDNA was used as an empty control. KLF5 increased the *Vegfa* promoter activity in a dose-responsive manner. (**B**) H441 cells were transfected with promoter plasmid pGL2-*Vegfa*-Luc and *Klf5*-targeting siRNA, with or without an HIF2α expression vector. A control negative siRNA was used as empty control. Inhibition of *KLf5* expression moderately, but significantly inhibited *Vegfa* promoter activity. *Klf5* siRNA significantly inhibited HIF2α-dependent activity of the *Vegfa* promoter. (**C**) H441 cells were transfected with the promoter plasmid pGL3-3TP-Luc and the *Klf5* expression vector, with or without TGFβ1 (2 ng/ml). Expression of *Klf5* inhibited pGL3-3TP luciferase activity and inhibited TGFβ1-dependent activity of pGL3-3TP. The plasmid pcDNA was used as a control. (**D**) H441 cells were transfected with the promoter plasmid pGL3-3TP-Luc and *Klf5*-targeting siRNA, with or without TGFβ1 (2 ng/ml). A control negative siRNA was used as empty control. Inhibition of *Klf5* expression moderately, but significantly enhanced 3TP promoter activity. Inhibition of *Klf5* expression significantly enhanced TGFβ1-dependent activity of 3TP promoter (*P<0.05).

DISCUSSION

KLF5 regulates epithelial cell maturation

During the last third of gestation, peripheral pulmonary epithelial cells undergo dramatic morphological and functional changes associated with the differentiation of alveolar type II and type I cells. The production of pulmonary surfactant lipids and proteins by respiratory epithelial cells increases prior to birth. Squamous type I cells are derived from cuboidal pre-type II or type II cells, and are located in close proximity to the capillary bed, forming the attenuated gas-exchange region characteristic of mature alveoli, which supports efficient gas exchange after birth. Deletion of *Klf5* in the respiratory epithelial cells inhibited morphological and biological maturation of the lung. Defects in surfactant lipid and protein expression, and abnormalities during sacculation and formation of the gas exchange region, which are dependent upon the close association of type I cells and the capillary bed, were observed in the $Klf5^{\Delta/\Delta}$ mice. Taken together, perinatal respiratory failure in the $Klf5^{\Delta/\Delta}$ mice was mediated, in part, by decreased expression of pulmonary surfactant and the failure to form gas-exchange structures typical of normal alveolar saccules.

mRNA microarray studies of the lungs from $Klf5^{\Delta/\Delta}$ mice demonstrated that KLF5 was required for the normal expression of genes regulating surfactant phospholipid homeostasis, including *Abca3*, *Abca4*, *Aytl2*, *Liph*, *Srebf1* and *Srebf2*. *Aytl2* (acyltransferase-like 2) is abundantly expressed in alveolar type II cells and is required for SatPC synthesis (Chen, X. et al., 2006). Consistent with this observation, total SatPC was significantly decreased in the lungs of $Klf5^{\Delta/\Delta}$ mice in association with decreased expression of *Aytl2* mRNA. Expression of *Abca3* in alveolar type II cells is required for the formation of lamellar bodies, and for surfactant storage and function (Shulenin et al., 2004), thus decreased expression of *Abca3* may contribute to the lack of lamellar bodies and the decreased surfactant in $Klf5^{\Delta/\Delta}$ mice. Although no direct regulation of *Abca3* promoter activity by KLF5 was found in vitro, *Srebf1*, an important transcriptional regulator of *Abca3* (Besnard et al., 2007), was decreased significantly in $Klf5^{\Delta/\Delta}$ mice, indicating potential, indirect regulation of *Abca3* by KLF5. Taken together, these data indicate that KLF5 regulates type I and type II epithelial cell maturation and influences the expression of genes that are required for surfactant protein and lipid metabolism, which are crucial for the adaptation to air breathing.

KLF5 regulates proximal airway epithelial cell maturation

In late gestation, epithelial cells lining the conducting airways differentiate into ciliated and non-ciliated cells. In $Klf5^{\Delta/\Delta}$ mice at E18.5, staining of CCSP and CEBPα in non-ciliated bronchiolar cells, i.e. Clara cells, was decreased; however, ultrastructural abnormalities were not observed in the conducting airways. The association of KLF5 expression with Clara cells in the normal fetal and adult lung, and the loss of expression of CCSP and CEBPα (also required for CCSP expression) in the airway of $Klf5^{\Delta/\Delta}$ mice, indicates that KLF5 may influence normal differentiation or gene expression in Clara cells.

Lung maturation is regulated by transcription factors expressed in the developing respiratory epithelial cells. Recent studies demonstrated that conditional deletion, or inhibition, of *Gata6*, *Nfat*, *Foxa2* and *Cebpa*, and mutation of *Titf1*, interfered with pulmonary maturation and caused respiratory distress in newborn mice (Davé et al., 2006; DeFelice et al., 2003; Martis et al., 2006; Wan et al., 2004). As discussed above, these transcription factors directly regulate the transcription of many genes involved in surfactant and lipid metabolism, including *Abca3*, the SFTPs, *Aytl2* and others. We were unable to demonstrate direct activation of the *Sftptb*, *Sftptc*, *Abca3* and *Atyl2* gene promoters by KLF5 in vitro. Thus, the mechanisms by which KLF5 influences the expression of these maturation-dependent genes remains unclear. The lack of a direct effect of KLF5 on these promoter constructs suggests that KLF5 is not a direct transcriptional regulator of these genes, although it is possible that the cis-elements mediating the effects of KLF5 were not present in the promoter constructs used in our study. Alternatively, the cell lines tested in our study may lack crucial transcriptional co-factors needed for KLF5 function. Although transcription of *Cebpa* was not affected by KLF5 in H441 cells, *Cebpa* expression was dependent on KLF5 in bronchiolar cell types in vivo. Expression of *Cebpa* was markedly decreased in the bronchiolar epithelial cells, but not in the peripheral type II cells in the lungs of $Klf5^{\Delta/\Delta}$ mice, indicating cell-specific regulation of *Cebpa* expression by KLF5. As CEBPα regulates CCSP gene expression in vivo and in vitro, an inhibitory effect of KLF5 on CCSP expression may be mediated, at least in part, by CEBPα.

KLF5 influences paracrine signaling between lung epithelium and mesenchyme

Lung morphogenesis requires precise interactions among multiple cell types in both epithelial and mesenchymal cell compartments. Deletion of *Klf5* in epithelial cells perturbed the normal patterning and differentiation of the mesenchyme in the saccular stage of lung development, resulting in thickening of the mesenchyme, increased αSMA staining in bronchiolar smooth muscle, and the failure of pulmonary vessels to migrate into close proximity to alveolar epithelial cells. Whereas previous studies demonstrated that KLF5 enhanced αSMA expression in smooth muscle cells (Liu et al., 2003), the extent and intensity of staining for αSMA was increased in $Klf5^{\Delta/\Delta}$ mice, wherein KLF5 is selectively deleted only in the developing respiratory epithelium. These observations indicate that KLF5 influences paracrine interactions between pulmonary epithelial and mesenchymal cells to regulate pulmonary smooth muscle differentiation.

KLF5 influenced a number of mRNAs regulating morphogenesis of the lung. Microarray analysis and extensive literature mining were used to identify the potential pathways that were influenced by KLF5, including TGFβ, PDGF, FGF and VEGF, all of which are known to mediate paracrine signaling during lung development. Decreased expression of *Vegfa* isoforms, *Vegfr1* and *Vegfr3* are consistent with a role of KLF5 in the regulation of pulmonary vasculogenesis and may have contributed to the abnormalities found in the lungs of the $Klf5^{\Delta/\Delta}$ mice, in which the alignment of capillaries with the peripheral epithelium was disrupted. Consistent with the in vivo findings, KLF5 had a transcriptional effect on the activity of the *Vegfa* promoter. The changes in *Vegfa* expression and isoforms might reflect direct effects of KLF5 on gene expression in respiratory epithelial cells, but might also be influenced by more generalized effects on lung maturational programs in various cell compartments.

The expression of *Pdgfb* was decreased and that of *Pdgfc* was increased in the lungs of $Klf5^{\Delta/\Delta}$ mice. PDGFB is known to play an indispensable role in vasculogenesis (Lindahl et al., 1997). During lung development, PDGFC was detected in epithelial cells of the bronchial tubules until E15.5, after which its expression was localized to smooth muscle cells (Ding et al., 2000). Enhanced expression of *Pdgfc* in the developing respiratory epithelium delayed maturation, causing respiratory failure consistent with our findings in the $Klf5^{\Delta/\Delta}$ mice (Zhuo et al., 2006).

$Klf5^{\Delta/\Delta}$ mice and mice expressing an activated form of TGFβ1 in the respiratory epithelium have morphological similarities, including disrupted alveolar sacculation and enhanced bronchiolar smooth muscle cell differentiation (Zeng et al., 2001; Zhou et al., 1996). A group of genes known to be involved in TGFβ signaling (Ghosh-Choudhury et al., 1994; Nakayama et al., 1998; Takaki et al., 2006) were significantly altered, both negatively and positively, in the lungs of $Klf5^{\Delta/\Delta}$ mice. Increased expression of TGFβ2 was found in the lungs of $Klf5^{\Delta/\Delta}$ animals and a similar increase was observed in $Cebpa^{\Delta/\Delta}$ mice, which also exhibit delayed perinatal lung maturation (Martis et al., 2006). Thus, increased TGFβ2 in the lungs of $Klf5^{\Delta/\Delta}$ mice may act in a paracrine manner to affect both epithelial and mesenchymal cell differentiation and patterning. TGFβ2 regulates the transcription of smooth muscle cell-related genes, including αSMA, and influences (myo)fibroblast differentiation (Wicks et al., 2006). Inhibition of a TGFβ reporter construct by KLF5 in vitro suggests that KLF5 influences transcriptional responses to TGFβ.

Expression of a number of genes involved in FGF signaling was altered in the lungs of $Klf5^{\Delta/\Delta}$ mice, including the expression of *Frag1*, *Fgfbp1* and *Fgf18*, which were decreased. Deletion of *Fgf18* in transgenic mice altered lung morphogenesis and was associated with abnormal patterning of the pulmonary vasculature, resulting in perinatal death (Usui et al., 2004). Heparin sulfate proteoglycans bind to and modulate the activities of various signaling molecules, including FGFs, VEGF, TGFβ1 and TGFβ2 (Bernfield et al., 1999; Forsberg and Kjellen, 2001). Interestingly, the expression of *Ndst1*, a key enzyme regulating heparin sulfate synthesis was reduced 1.56-fold in $Klf5^{\Delta/\Delta}$ animals, and $Ndst1^{-/-}$ animals also exhibit respiratory failure and pulmonary immaturity at birth (Fan et al., 2000).

Taken together, deletion of *Klf5* significantly altered the expression of genes involved in a number of paracrine signaling processes that are crucial for normal lung morphogenesis, including *Vegfa*, *Tgfb* and *Pdgfb*. Thus, KLF5 both is regulated by diverse signaling pathways and participates in the regulation of multiple signaling processes, including those mediated by FGF, WNT, RAS and RA in various tissues (Chanchevalap et al., 2004; Kawai-Kowase et al., 1999; Nandan et al., 2004; Ziemer et al., 2001).

As direct-targeted gene deletion of *Klf5* caused early embryonic lethality, identifying its role in the morphogenesis and differentiation of various organs has not been possible. In the present study, we generated a model for conditional deletion of *Klf5*, demonstrating that KLF5 is required for perinatal lung maturation and the expression of many genes crucial for lung function at birth, and that it regulates important but diverse signaling networks that influence lung morphogenesis and maturation.

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