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Epithelial laminin α5 is necessary for distal epithelial cell maturation, VEGF production, and alveolization in the developing murine lung

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Abstract

Laminin α5 is prominent in the basement membrane of alveolar walls, airways, and pleura in developing and adult lung. Targeted deletion of laminin α5 in mice causes developmental defects in multiple organs, but embryonic lethality has precluded examination of the latter stages of lung development. To identify roles for laminin α5 in lung development, we have generated an inducible lung epithelial cell-specific *Lama5* null (SP-C*Lama5*$^{fl/-}$) mouse through use of the Cre/loxP system, the human surfactant protein C promoter, and the reverse tetracycline transactivator. SP-C*Lama5*$^{fl/-}$ embryos exposed to doxycycline from E6.5 died a few hours after birth. Compared to control littermates, SP-C*Lama5*$^{fl/-}$ lungs had dilated, enlarged distal airspaces, but basement membrane ultrastructure was preserved. Distal epithelial cell differentiation was perturbed, with a marked reduction of alveolar type II cells and a virtual absence of type I cells. Cell proliferation was reduced and apoptosis was increased. Capillary density was diminished, and this was associated with a decrease in total lung VEGF production. Overall, these findings indicate that epithelial laminin α5, independent of its structural function, is necessary for murine lung development, and suggest a role for laminin α5 in signaling pathways that promote alveolar epithelial cell differentiation and VEGF expression.

Keywords: Laminin; Lung development; Basement membrane; Alveolar epithelial cell; Differentiation; VEGF

Introduction

Lung morphogenesis results from highly coordinated interactions between cells of endodermal and mesodermal origin and the extracellular matrix. Basement membranes are distinctive extracellular matrices that have essential roles in tissue organization and development (Miner and Yurchenco, 2004). Components common to all basement membranes include laminin, type IV collagen, entactin/nidogen, and sulfated proteoglycans. Laminins are heterotrimers composed of one α, one β, and one γ chain, which together form at least 15 laminin isoforms from combinations of five α chains, four β chains, and three γ chains. The lung contains multiple laminin chains: the α1–α5, β1–β3, and γ1–γ2 chains are present in embryonic lung, and laminin α2–α5, β1–β3, and γ1–γ3 chains are present in the adult lung (Miner et al., 1997; Pierce et al., 2000; Virtanen et al., 1996, 2000). Studies of lung development have shown that manipulation of laminin or its interactions with cells leads to lung abnormalities. Specifically, addition of either laminin-1 (α1β1γ1) antibodies or proteolytic fragments to lung bud cultures perturbs branching morphogenesis, while ablation of the nidogen-binding site on laminin γ1 in vivo interferes with sacculation (Schuger et al., 1990; Willem et al., 2002). Laminin α2 has been shown to be important for bronchial smooth muscle cell differentiation in vitro (Relan

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et al., 1999). Together, these studies show that laminins affect lung development at multiple stages and in different cellular compartments.

Although the importance of laminin-1 in lung development has been established in vitro, it has not been studied in vivo because *Lama1*$^{-/-}$, *Lamb1*$^{-/-}$, and *Lamc1*$^{-/-}$ embryos die very early in development, well before lung formation (Miner et al., 2004; Smyth et al., 1999). Targeted deletions of other laminin chain genes have been informative because the affected animals survive to stages at which lungs have formed, and even reach postnatal life in some cases. *Lama3*$^{-/-}$ mice die 2–3 days after birth from malnutrition (Ryan et al., 1999), and the lungs of these mice appear to have mildly enlarged airspaces (N.M.N., R.M.S., unpublished). Mice lacking laminin γ2 (*Lamc2*), which assembles with α3 and β3 to form laminin-5, also die 1–2 days after birth (Meng et al., 2003), but their lung phenotype is not as severe as the *Lama3*$^{-/-}$ mice (N.M.N., R.M.S., unpublished). Mutations in *Lama2*, *Lama4*, or *Lamb2* do not cause embryonic or perinatal death and do not result in any described lung phenotype (Noakes et al., 1995a; Thyboll et al., 2002; Xu et al., 1994).

To further define the roles of laminins in lung development, we have examined the laminin α5 chain, the α chain of laminin-10/11, which is present both in embryonic and adult lung. Previously, we observed incomplete to absent lobar septation, lack of visceral pleura basement membrane, and ectopic deposition of laminin α4 in airway basement membranes in lungs of laminin α5 null mice (Nguyen et al., 2002). However, because most *Lama5*$^{-/-}$ mice die between E14 and E17, and a significant amount of lung development, including alveolization, occurs postnatally in rodents, all stages of lung development could not be assessed in *Lama5*$^{-/-}$ mice. While the phenotype during early lung development in the *Lama5*$^{-/-}$ embryos was relatively minor, a more significant contribution from laminin α5 in late lung development could not be ruled out.

To determine the role of laminin α5 in the latter stages of lung development, we have produced mice with a laminin α5 deficiency restricted to lung epithelial cells. This was accomplished using the Cre/loxP system and the human surfactant protein C (SP-C) promoter, which is specific for lung epithelial cells (Wert et al., 1993). With this system, in the presence of doxycycline, only cells activating the SP-C promoter acquire a mutation in *Lama5*. Administration of doxycycline at E6.5, prior to formation of the lung anlage, resulted in ablation of *Lama5* in lung epithelial cells, perinatal death from respiratory failure, and severe abnormalities in lung development. These abnormalities included large, emphysematous-appearing distal airspaces, markedly reduced or delayed differentiation of distal lung epithelial cells, a decrease in capillary density, a decrease in cell proliferation, an increase in apoptosis, and a decrease in VEGF production. Overall, these studies demonstrate a crucial role for laminin α5 in lung development.

Materials and methods

Production, breeding, and genotyping of mice

To generate the floxed *Lama5* allele, a targeting vector was constructed from an 8.3-kb bacteriophage fragment isolated from a 129Sv genomic DNA library (Fig. 1A). A loxP-frt-PGK*neo*-frt selectable marker was inserted into the *Kpn*I site in intron 21 of *Lama5*, and a second loxP site was inserted into the *Afl*II site in intron 14. The 7 exons (15 through 21) flanked by loxP sites (of 79 total) encode 40% of domain V, which consists of laminin EGF-like repeats. Splicing from exon 14 to 22, which should occur once Cre-mediated recombination removes exons 15–21, results in an out-of-frame mRNA. The long arm of homology upstream of the isolated loxP site was 5 kb, and the short arm of homology downstream of *neo* was 1.1 kb. The linearized targeting vector was transfected into R1 embryonic stem cells, and several homologous recombinants were identified by PCR. Two independent clones that maintained the isolated loxP site were injected into C57BL/6J blastocysts to generate germline chimeras. Breeding chimeras from both clones to a Cre deletor mouse excised the 7 exons flanked by loxP sites and generated a null allele. To avoid any effects on *Lama5* gene expression from the PGK*neo* insertion, chimeras were bred to mice expressing FLPe recombinase, which removed the selectable marker and generated the conditional *Lama5*fl allele. *Lama5*fl/fl and *Lama5*fl/− mice were normal and indistinguishable from littermates carrying a wild-type *Lama5* allele.

Production of the original *Lama5*$^{-/-}$ mice has been described (Miner et al., 1998). Mice with the SP-C-rtTA and the tetO-cre transgenes were obtained from Jeffrey Whitsett (University of Cincinnati) (Perl et al., 2002). *Lama5*+/− mice were mated with SP-C-rtTA/tetO-cre mice to produce SP-C-rtTA/tetO-cre/*Lama5*+/− mice that were then mated with the floxed laminin α5 mice to produce SP-C-rtTA/tetO-cre/*Lama5*fl/− (SP-C*Lama5*$^{fl/-}$) mice. Dams received doxycycline (1 mg/mL, Sigma, St. Louis, MO) via drinking water continuously pre- and postpartum beginning at E6.5. Genomic DNA was obtained from tails using the Qiagen DNA kit (Qiagen, Carlsbad CA). SP-C-rtTA, tetO-cre, *Lama5*−, *Lama5*fl, and *Lama5*+ genes were identified by PCR (Table 1). The Washington University Animal Studies Committee approved all experiments.

Antibodies

Rat monoclonal antibody to murine laminin α1 was a gift from Dale Abrahamson (University of Kansas Medical Center). Rabbit polyclonal antibodies to laminin α3A and α3B were gifts from Takako Sasaki (Max Planck Institute of Biochemistry, Martinsreid, Germany). Rabbit polyclonal antibodies to murine laminin α5 and laminin α4 were produced as described (Miner et al., 1997; Nguyen et al., 2002). Antibody against integrin α3 was a gift from C.

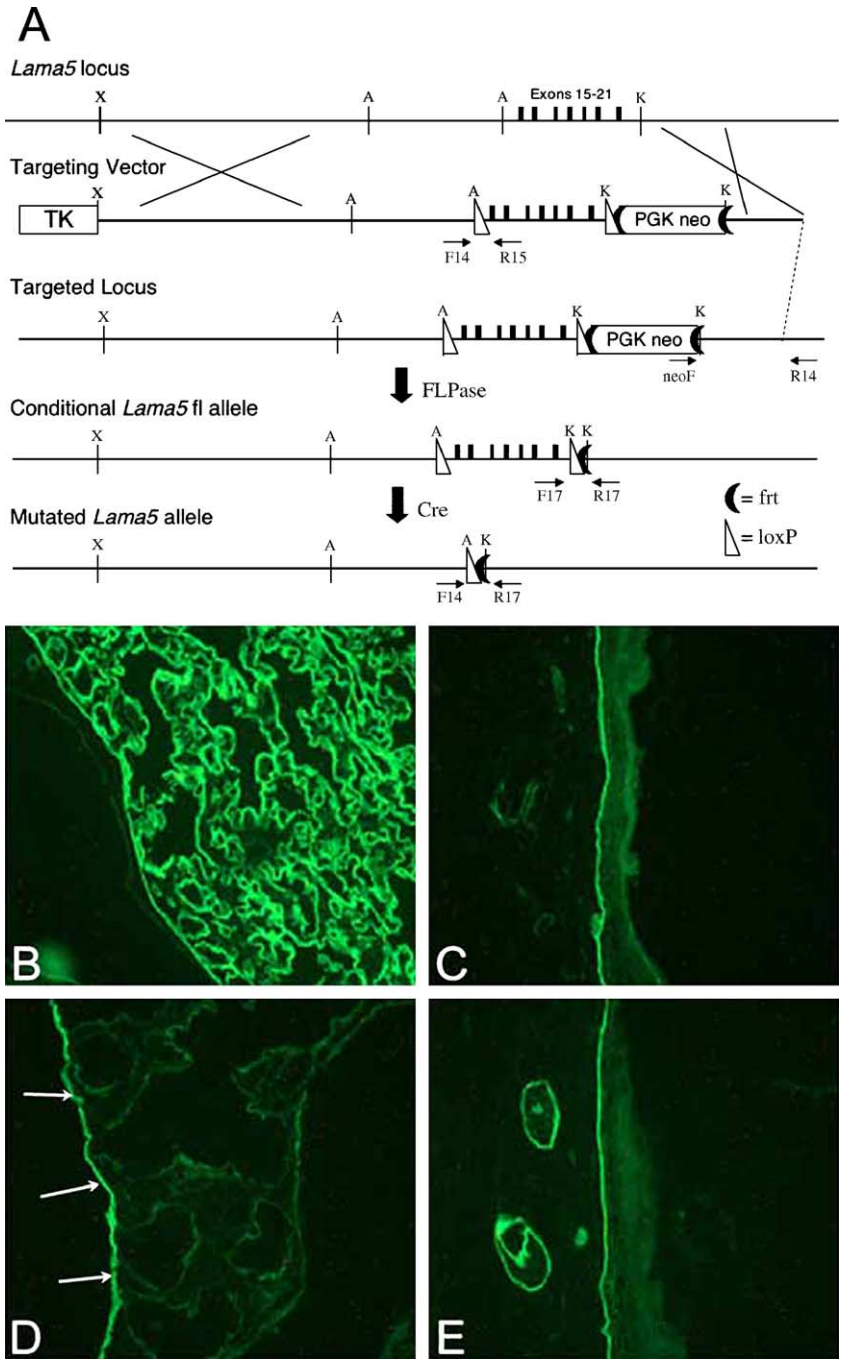


Fig. 1. *Lama5* gene targeting and confirmation of lung-specific mutation. (A) An 8.3-kb fragment of *Lama5* [beginning at an *Xba*I (X) site], which contained exons 7 through 25, was modified by inserting a loxP-frt-PGK*neo*-frt cassette into the *Kpn*I (K) site of intron 21 and a loxP site into the *Afl* II site (A) of intron 14. The HSV thymidine kinase gene (TK) was added for negative selection. After transfection, ES cell colonies surviving G418 and FIAU selection were screened by PCR with primers neoF and R14, the latter being from outside the targeting vector. Homologous recombinants were screened again by PCR with primers F14 and R15 to identify those that maintained the isolated loxP site. Germline chimeras were generated from two such clones, and both were bred to a mouse expressing FLPe to remove the *neo* cassette; screening by PCR with primers F17 and R17 confirmed this. Subsequent breeding to a mouse expressing Cre removed exons 15–21 and created a null allele; screening by PCR with primers F14 and R17 confirmed this. Immunofluorescence staining for laminin α5. Immunofluorescence staining was performed with anti-laminin α5 on lung (B, D) and skin (C, E) sections from newborn control (B, C) and SP-C*Lama5*$^{fl/-}$ (D, E) mice. Strong laminin α5 staining was present only in the visceral pleura basement membrane of SP-C*Lama5*$^{fl/-}$ lungs (arrows, D). Laminin α5 staining in the skin of SP-C*Lama5*$^{fl/-}$ mice was indistinguishable from control (C, E). Original magnification 200×.

Michael DiPersio (Albany Medical College). Antibodies purchased from commercial suppliers were cytokeratin-8 and T1α (8.1.1) (Dev. Studies Hybridoma Bank, Iowa City, IA), laminin α2 (4H8-2, Alexis Biochemicals, San Diego, CA), PECAM and Ki-67 (Pharmingen, San Diego, CA), laminin γ1, GAPDH, integrin α3, aquaporin-5, pro-surfactant protein

Table 1
Genotype PCR primers/conditions

Gene/transgene	Sequence	Anneal (°C)	# Cycles
Laminin α5 sense	5′-TCAATCAGGACCGCTTCATC-3′	60	30
Laminin α5 antisense	5′-CGTGAGCAATCTTCTCACTG-3′		
Laminin α5 null sense	5′-GCTACCAGTCAAGTGCTG-3′	60	30
Laminin α5 null antisense	5′-GGAATTCGCGAGCGTGTG-3′		
Laminin α5 floxed sense	5′-TGTCCGCCCTAACTCCCAAGG-3′	60	30
Laminin α5 floxed antisense	5′-GTTGAAGCCAAAGCGTACAGCG-3′		
Tet-O-Cre sense	5′-CGGTCGATGCAACGAGTGATGAG-3′	62	40
Tet-O-Cre antisense	5′-ACGAACCTGGTCGAAATCAGTGCG-3′		
SP-C-rtTA sense	5′-GACACATATAAGACCCTGGTCA-3′	65	35
SP-C-rtTA antisense	5′-AAAATCTTGCCAGCTTTCCCC-3′		

C (SP-C) (Chemicon, Temecula, CA), cleaved caspase-3 (#9661 Cell Signaling, Beverly, MA), thyroid transcription factor 1 (TTF-1, DAKO Corporation, Carpinteria, CA), and vascular endothelial growth factor [VEGF, sc-507 (immunohistochemical staining), and sc-152 (Western Blot), Santa Cruz Biotech Inc., Santa Cruz, CA]. FITC- and TRITC-conjugated anti-rabbit and anti-rat secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). FITC-anti-mouse IgG2b secondary antibody was from ICN Biomedicals, Inc. (Costa Mesa, CA).

Immunofluorescence microscopy

Newborn pups were killed by decapitation and immediately immersed in Tissue Tek OCT embedding medium (Sakura Finetek, Torrance, CA), frozen in liquid nitrogen-cooled 2-methylbutane, and sectioned at 6 μm on a cryostat. Sections were blocked with 10% normal goat serum in 1% BSA/PBS, then incubated with primary antibody diluted in 1% BSA/PBS. Slides were washed with PBS, and incubated with secondary antibody diluted in 1% BSA/PBS. Slides were again washed with PBS and mounted in Vectashield (Vector Laboratories, Temecula, CA). For laminin α4 staining, sections were fixed in 4% paraformaldehyde for 10 min, washed in PBS, treated with 0.1 M glycine pH 7.4 for 10 min, washed in PBS, treated with 0.1% SDS at 55°C for 1 h, washed in PBS, blocked, and stained as with other antibodies. For PECAM staining, sections were fixed in 100% ethanol prior to addition of the PECAM antibody.

Electron microscopy

For transmission electron microscopy, lungs from newborn pups were fixed in 3% glutaraldehyde in 0.1 M sodium cacodylate buffer, postfixed with aqueous 1.25% osmium tetroxide, stained with 4% aqueous uranyl acetate, dehydrated through an ethanol series, embedded in polybed, sectioned on a Reichert-Jung Ultra Cut, post-stained in 4% uranyl acetate and lead citrate, and viewed on a Zeiss 902 electron microscope. All reagents for electron microscopy were purchased from Electron Microscopy Sciences (Ft. Washington, PA) except for Polybed (Polysciences, Warrington, PA).

Histology and immunohistochemistry

Thoraces of newborn pups were isolated by decapitation and transection at the level of the liver. Care was taken to leave the thoracic cavity undisturbed (dissection without puncturing the chest cavity or diaphragm) to minimize collapse of the lungs. Thoraces were fixed in 4% paraformaldehyde in PBS, dehydrated in graded ethanols, embedded in paraffin, sectioned, and stained with hematoxylin and eosin for light microscopy.

For staining with SP-C, Ki-67, TTF-1, and cleaved caspase-3 antibodies, 5-μm paraffin sections were immunostained using Vectastain Elite ABC staining kits (Vector Laboratories). Antigen unmasking was performed with a pressure cooker (Biocare Medical, Carlsbad, CA), according to the manufacturer's instructions, using citrate buffer for cleaved caspase-3 and SP-C antibodies, Reveal (Biocare Medical) for TTF-1 antibody, and Tris 50 mM pH 9.5 for Ki-67 antibody. Antibodies were developed with DAB or Vector Red substrates (Vector Laboratories) and counterstained with hematoxylin.

Quantification of proliferation and apoptosis

Total lung cell proliferation and apoptosis were quantified with Optimas 3 software (MediaCybernetics, San Diego, CA). After immunostaining with Ki-67 or cleaved caspase-3 antibodies, a minimum of 25 random lung fields per animal at 200× magnification were captured with the Axiocam digital camera and Openlab software. Immunodetection with the DAB substrate and counterstaining with hematoxylin resulted in positive brown nuclei and counterstained blue nuclei. With the Optimas 3 software, the proportion of brown and the proportion of blue per total area were determined for each image, the ratio of brown area to blue area was reported as the amount of proliferation or apoptosis. At least 3 animals were quantified per group. Statistical analysis was performed using the Student's *t* test for independent samples, 2-tailed analysis.

Western blot analysis

Lungs were snap frozen in liquid nitrogen, ground with mortar and pestle, and placed in RIPA buffer (Nguyen et al., 2002) for 30 min. Samples were centrifuged and the supernatants subjected to 12% SDS-PAGE. Proteins were transferred to PVDF, blocked with 5% milk in TBS-Tween overnight, incubated for 1 h with antibody against T1α and GAPDH, or VEGF and GAPDH, washed with TBS-Tween, incubated with HRP-conjugated secondary antibodies (107 + 035–142 Jackson ImmunoResearch, West Grove, PA) for 1 h, washed with TBS-Tween, and detected using enhanced chemiluminescence (ECL+ kit, Amersham, Piscataway, NJ).

RT-PCR

Total RNA was extracted from whole newborn mouse lungs with the Ambion ToTALLY RNA Isolation Kit and DNAse treated with the Ambion DNA-free Kit (Ambion, Inc., Austin, TX). One microgram of RNA was reverse transcribed into cDNA with the GeneAmp RNA PCR Core Kit (Applied Biosystems, Foster City, CA). Real-time PCR was performed using the Mx3000P instrument (Stratagene, La Jolla, CA) with the Brilliant SYBR Green Master Mix QPCR Kit (Stratagene). Primers for real-time PCR were VEGF sense 5′-TTACTGCTGTACCTCCACC-3′ and VEGF antisense5′-ACA GGACGGCTTGAAGATG-3′; HPRT sense 5′-GTTGGATACAGGCCAGACTT-3′ and HPRT antisense 5′-TTGGCTTTTCCA GTTTCACT-3′. Primers for integrin α3 PCR were intα3 sense 5′-ATTGACTCAGAG-CTGGT GGAGGAG-3′ and intα3 antisense 5′-TACTTGGG-CATAATCCG GTAGTAG-3′; GAPDH sense 5′-CCCCTTCATTGACCTCAACTACATGG-3′ and GAPDH antisense 5′-GCCTGCTTC ACCACC TTCTTGATGTC-3′.

Results

Lung epithelial cell-specific laminin α5 null mice

Lung epithelial cell-specific laminin α5 null (SP-C*Lama5*$^{fl/-}$) mice (with the SP-C-rtTA/tetO-cre/*Lama5fl/–* genotype) that were exposed to doxycycline from E6.5 survived to birth. However, soon after parturition, they exhibited labored respiration, cyanosis, and died within hours. On gross examination, SP-C*Lama5*$^{fl/-}$ lungs had normal lobar septation but were less inflated than the lungs of littermate controls that did not contain the SP-C-rtTA, tetO-cre, or floxed laminin α5 transgenes. No differences from normal were noted in body weight or gross anatomy of organs other than the lungs in SP-C*Lama5*$^{fl/-}$ mice.

Littermates without at least one of the SP-C-rtTA, the tetO-cre, or the floxed *Lama5* transgenes never displayed the lung phenotype observed in the SP-C*Lama5*$^{fl/-}$ mice. However, in some SP-C*Lama5*$^{fl/-}$ mice that were not exposed to doxycycline, "leak" of the transactivator, expression of Cre, and subsequent development of the lung phenotype occurred. "Leak" never occurred in mice lacking any of the required transgenes. "Leak" appeared to be stochastic; in one litter, for example, it was evident in one pup but not in another pup with the same genotype. Given this variability, we used as controls only those littermates that were administered doxycycline, but that lacked one or more of the SP-C-rtTA, the tetO-cre, or the floxed *Lama5* transgenes.

Confirmation of lung epithelial cell-specific laminin α5 ablation

SP-C*Lama5*$^{fl/-}$ mice were designed to lack laminin α5 expression only in the lung epithelial cells. This was achieved through use of the SP-C promoter, which is only expressed in lung epithelial cells during development, and in distal lung epithelium of the adult lung. In the lungs of controls, abundant laminin α5 was detected by immunofluorescence staining in the epithelial basement membrane of the lung parenchyma and in the visceral pleura basement membrane (Fig. 1B). In contrast, the SP-C*Lama5*$^{fl/-}$ mouse had a near complete absence of laminin α5 immunofluorescence in the lung parenchyma (Fig. 1D), but there was strong staining for laminin α5 in the visceral pleura basement membrane (Fig. 1D arrow). This was expected, because the mesothelial cells that are the source of laminin α5 in the pleura do not activate the SP-C promoter and therefore should not express Cre. Similarly, immunofluorescence for laminin α5 in the skin of SP-C*Lama5*$^{fl/-}$ and control mice showed no difference in staining intensity or pattern (Figs. 1C, E). These studies confirm that the deletion of laminin α5 expression was restricted to the lung epithelium.

Histology of SP-C$Lama5^{fl/-}$ *lungs*

By light microscopy, the lungs of newborn SP-C*Lama5*$^{fl/-}$ pups were markedly different from controls (Fig. 2). SP-C*Lama5*$^{fl/-}$ lungs had less total lung parenchyma, the proximal and distal airspaces were dilated, and the lung mesenchyme was thinner in many areas (Figs. 2A, B). At the periphery, SP-C*Lama5*$^{fl/-}$ lungs (Fig. 2D) had more airspaces lined with cuboidal epithelial cells close to the pleural surface compared to controls (Fig. 2C). This finding of "proximalization" of the distal airspace epithelium suggests lack of, or delay in, differentiation of distal airspace epithelium. Also, the intraluminal spaces of SP-C*Lama5*$^{fl/-}$ lungs (Fig. 2D) had more cellular debris and amorphous, proteinaceous material than controls (Fig. 2C).

Distribution of other laminin α chains

Deletion of a native laminin chain sometimes results in compensation by another laminin chain (Noakes et al.,

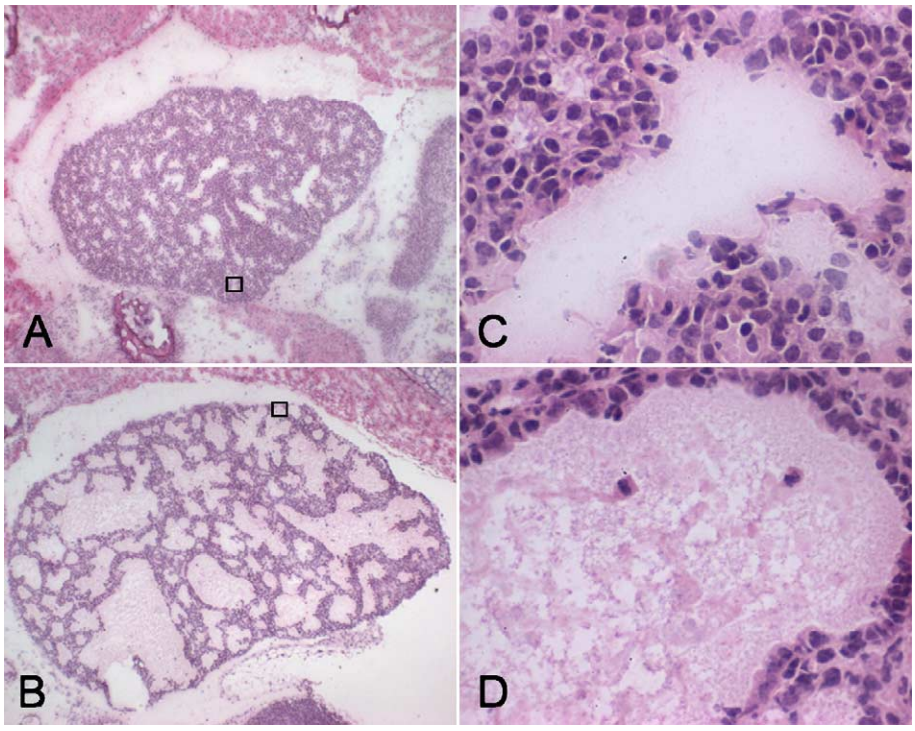


Fig. 2. Lung histology. Frozen sections of newborn control (A, C) and SP-C*Lama5*$^{fl/-}$ (B, D) lungs were stained with hematoxylin and eosin. The SP-C*Lama5*$^{fl/-}$ lung had enlarged proximal and distal airspaces, with less total lung tissue. On higher magnification, epithelial cells in the distal airspaces of SP-C*Lama5*$^{fl/-}$ lung (D) were more cuboidal when compared with control (C). In addition, the airspaces of SP-C*Lama5*$^{fl/-}$ lungs (D) were filled with large amounts of proteinaceous material and cellular debris not seen in controls (C). Original magnification 40× (A, B), and 400× (C, D).

1995b; Patton et al., 1997). By immunofluorescence, no differences in the level of expression or distribution of laminin α1–α3 chains were observed between SP-C*Lama5*$^{fl/-}$ and control mice (Fig. 3). No staining was visible for laminin α1 or α2 in either control (Figs. 3A, C) or SP-C*Lama5*$^{fl/-}$ (Figs. 3B, D) lungs at birth. Laminin α3B was evident in the epithelial airway basement membranes of both control (Fig. 3E) and SP-C*Lama5*$^{fl/-}$ (Fig. 3F) lungs, as was laminin α3A (data not shown). Theoretically, laminin α3B serves as the full-length α chain required to form polymeric laminin and maintain basement membrane structure (Colognato and Yurchenco, 1999). To confirm this, further studies will need to be performed. Laminin α4 was detected in the basement membrane of smooth muscle cells surrounding the airway epithelium in control lungs (Fig. 3G). However, in the SP-C*Lama5*$^{fl/-}$ lung (Fig. 3H), there was ectopic deposition of laminin α4 in airway epithelial basement membranes. Due to aberrant laminin α4 in airway basement membrane, double immunostaining with antibodies against laminin α4 and laminin γ1 revealed co-localization of laminin α4 and γ1 in airway epithelial basement membrane in the SP-C*Lama5*$^{fl/-}$ lung (J, arrows) but not in the control lung (I). This finding is consistent with our previous report of ectopic deposition of laminin α4 in the airway basement membrane in the *Lama5*$^{-/-}$ fetal lung (Nguyen et al., 2002).

Airway basement membrane ultrastructure

While basement membrane components can be detected by immunofluorescence, confirmation of basement membrane integrity is established by ultrastructural analysis. By transmission electron microscopy, a continuous electron dense basement membrane of uniform thickness was present in airways of both newborn control (Fig. 4A) and SP-C*Lama5*$^{fl/-}$ (Fig. 4B) mice. Consistent with this, laminin γ1 and entactin/nidogen-1 were present in both SP-C*Lama5*$^{fl/-}$ and control airway basement membranes (data not shown). Thus, lack of laminin α5 did not overtly affect airway basement membrane formation or ultrastructure.

Vascular development

Laminin α5 is present in basement membrane of murine lung vessels throughout development. Since our targeting of laminin α5 was confined to lung epithelium, we did not anticipate effects upon vascular development. However, PECAM staining showed a dramatic decrease in the density of the capillary network in SP-C*Lama5*$^{fl/-}$ lungs (Fig. 5B) compared to controls (Fig. 5A).

The difference in PECAM staining suggested an abnormality in vascular development and prompted us to explore expression of vascular endothelial growth factor (VEGF).

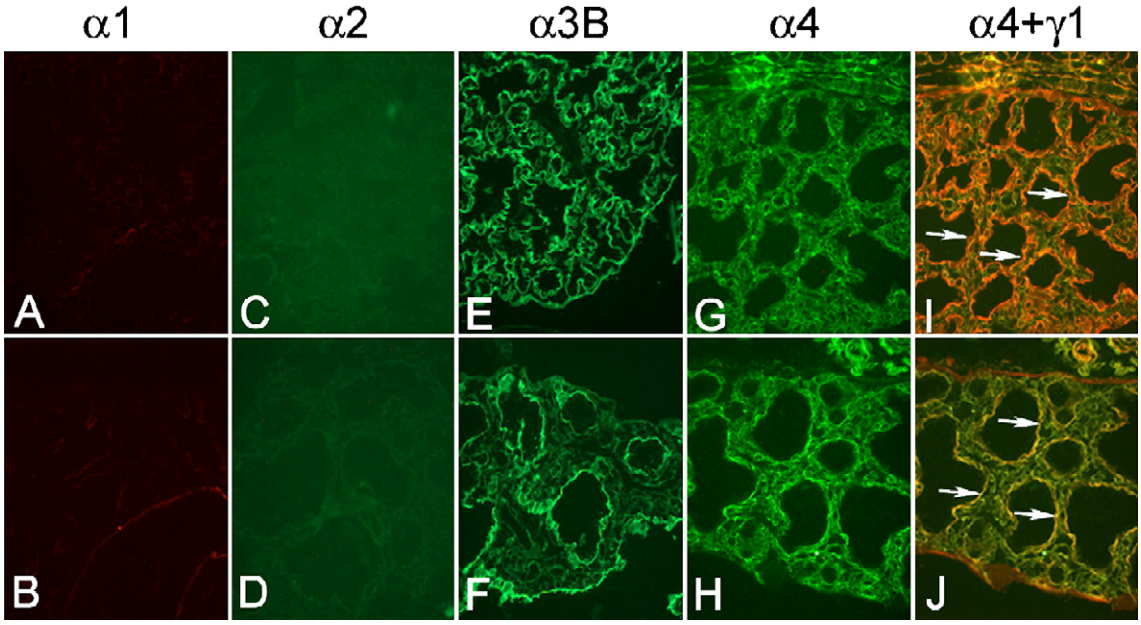


Fig. 3. Deposition of laminin α chains. Sections from newborn control and SP-C*Lama5*$^{fl/-}$ lungs were stained with antibodies to laminins α1–α4 and γ1. The laminin α1 (A, B) and α2 (C, D) chains were not detected at this stage. Laminin α3B was present in the airway basement membranes of both control (E) and SP-C*Lama5*$^{fl/-}$ (F) lungs. Laminin α4 was associated with smooth muscle cells around large airways in the control (G). The SP-C*Lama5*$^{fl/-}$ lung had laminin α4 in its usual distribution, as well as ectopically in airway basement membranes (H). Double staining for laminin α4 (green) and laminin γ1 (red) shows co-localization (yellow) of laminin α4 with laminin γ1 in the airway basement membrane in the SP-C*Lama5*$^{fl/-}$ lung (J, arrows) but not in control (I). Original magnification 200×.

VEGF is crucial for developmental vasculogenesis, acting as a mitogen and as a chemotactic factor for endothelial cells (Ferrara and Henzel, 1989; Gospodarowicz et al., 1989). VEGF is expressed by bronchial epithelium in early lung development and in alveolar type II (ATII) cells in late lung development (Acarregui et al., 1999; Bhatt et al., 2000). Immunohistochemical staining for VEGF showed expression of VEGF in all lung epithelial cells in the control (Fig. 5C). However, in the SP-C*Lama5*$^{fl/-}$ lung, the intensity of staining of VEGF was decreased in bronchiolar epithelial cells and difficult to detect in the distal lung parenchyma, where one would expect to find ATII cells (Fig. 5D). To corroborate these observations, we performed Western blotting for VEGF protein on whole lung homogenates from SP-C*Lama5*$^{fl/-}$ and control pups (Fig. 5E). Using Western blot for GAPDH to normalize loading, densitometric analysis of the ratio of VEGF to GAPDH revealed a reduction of nearly 50% in the SP-C*Lama5*$^{fl/-}$ lung (Fig. 5F). Given the decrease in VEGF protein, we next quantified VEGF mRNA expression in whole newborn lungs by real-time PCR analysis with primers for total VEGF (all isoforms) normalized to HPRT. This analysis showed an approximately 4-fold decrease in VEGF mRNA in SP-C*Lama5*$^{fl/-}$ lungs (Fig. 5G). Taken together, the immunofluorescence, the Western blot, and the real-time PCR analyses indicate a deficiency of VEGF in SP-C*Lama5*$^{fl/-}$ lungs, which could account for the reduced complexity of the vasculature.

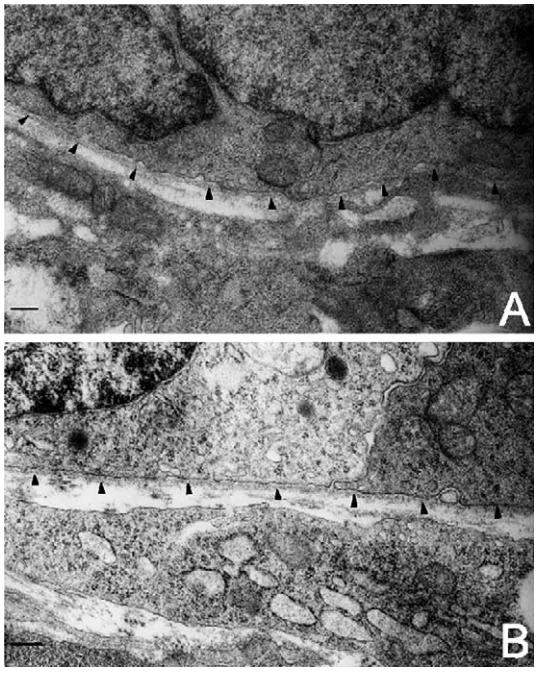
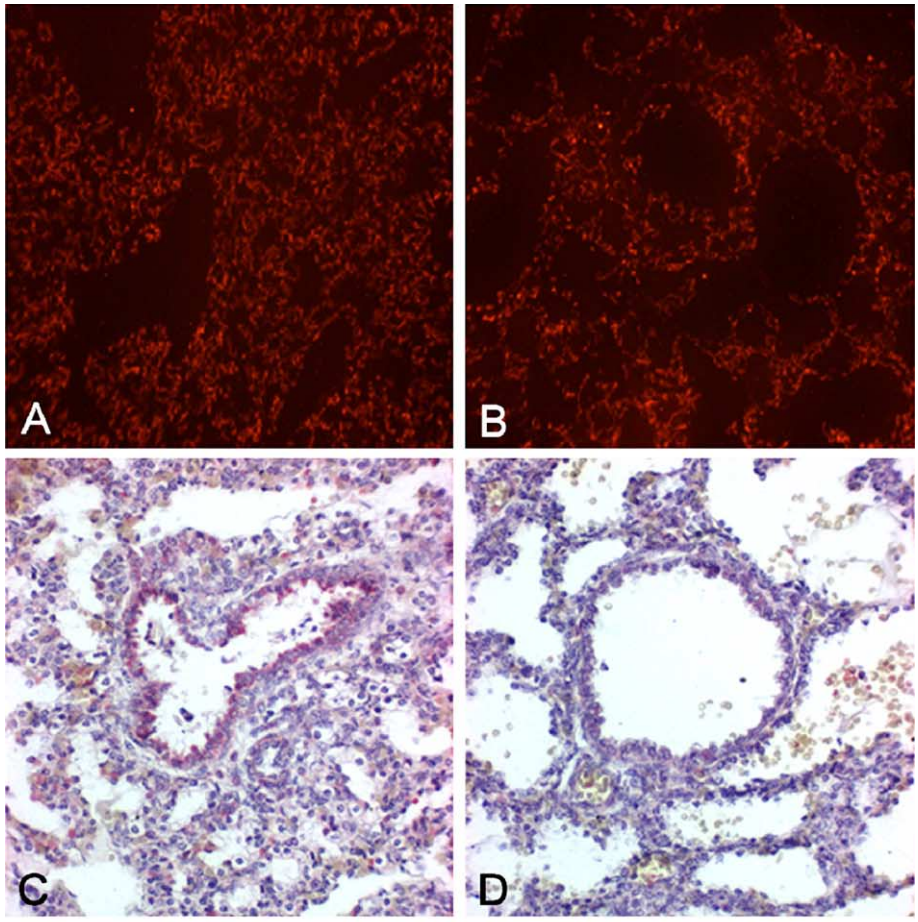
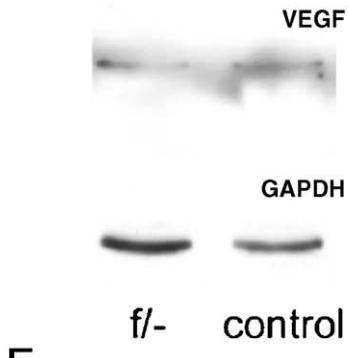


Fig. 4. Airway basement membrane ultrastructure. Transmission electron micrographs of airways in both newborn control (A) and SP-C*Lama5*$^{fl/-}$ (B) lungs revealed a continuous, electron-dense basement membrane (arrowheads) underlying airway epithelial cells. Scale bar = 280 nm.

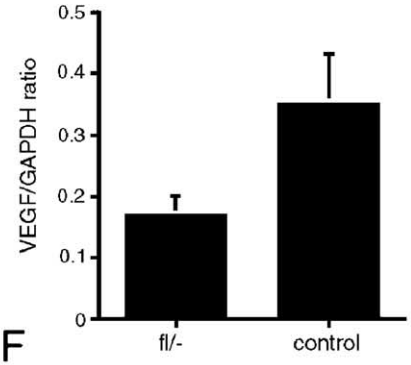
Epithelial cell differentiation

Because our gene targeting utilized the SP-C promoter, which is expressed predominantly by lung epithelial cells, we examined the epithelial cell characteristics of the SP-C*Lama5*$^{fl/-}$ lungs. By cytokeratin-8 staining, the cells lining


A
B
C
D


VEGF
GAPDH
f/-
control

E


VEGF/GAPDH ratio
0.5
0.4
0.3
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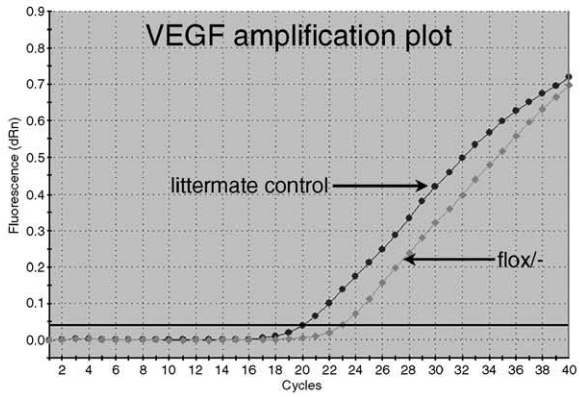
F


VEGF amplification plot
Fluorescence (dRn)
littermate control
flox/-
Cycles

G

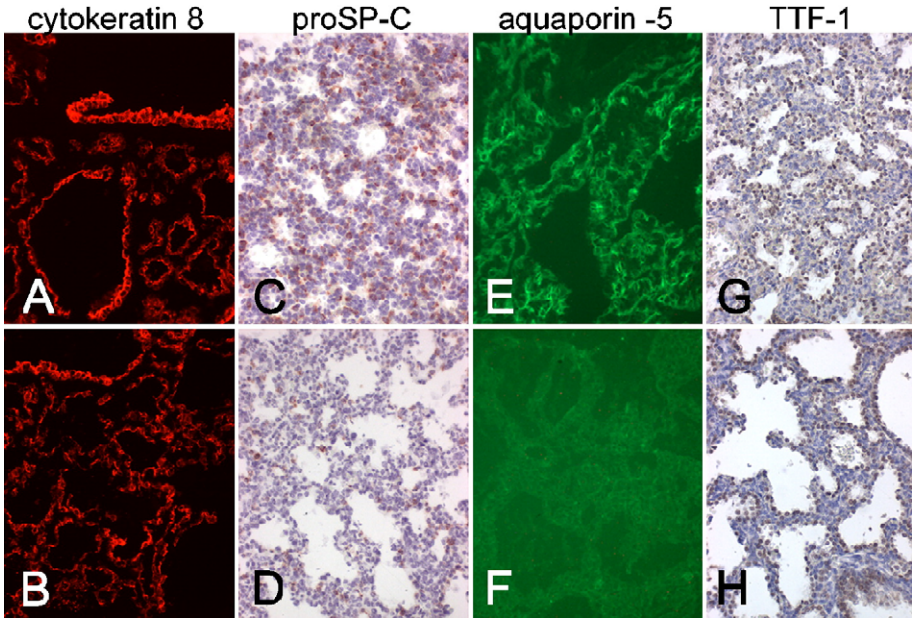


Fig. 6. Impaired epithelial cell differentiation. Sections of newborn control (A, C, E, G) and SP-C*Lama5*$^{fl/-}$ (B, D, F, H) lungs were stained with antibodies to epithelial cell markers. Staining with the cytokeratin-8 antibody revealed that airspaces of control (A) and SP-C*Lama5*$^{fl/-}$ (B) lungs were lined with epithelial cells. However, staining for the ATII cell-specific marker proSP-C revealed a significant decrease in proSP-C positive cells in the SP-C*Lama5*$^{fl/-}$ lung (D) compared with control (C). Immunofluorescence for the ATI cell-specific marker aquaporin-5 revealed a near absence of positive cells in the SP-C*Lama5*$^{fl/-}$ lung (F) compared with control (E). Expression of TTF-1 by epithelial cells was similar in control (G) and SP-C*Lama5*$^{fl/-}$ (H) lungs. Original magnification 200×.

the airspaces of the SP-C*Lama5*$^{fl/-}$ lungs (Fig. 6B) were clearly epithelial, as they were in the control (Fig. 6A). Examination of three markers of differentiated large/proximal airway epithelial cells, namely, CCSP for Clara cells, PAS staining for mucus-producing cells, and cilia by electron microscopy for ciliated cells, did not uncover any abnormalities (data not shown). However, we found a striking difference using markers for distal airway epithelial cells. Immunohistochemical staining with an antibody to the ATII cell-specific marker proSP-C showed a marked decrease in the number of proSP-C positive epithelial cells in SP-C*Lama5*$^{fl/-}$ lungs (Fig. 6D) compared to control (Fig. 6C), although, cells that were proSP-C positive in the SP-C*Lama5*$^{fl/-}$ lung appeared to be in the same location as in the control lung. ATII cells are the major producers of lung surfactant, which is visualized as tubular myelin by electron microscopy. In control lungs, an abundance of tubular myelin was seen in all airway lumina. In contrast, tubular myelin was only infrequently found in lungs of SP-C*Lama5*$^{fl/-}$ mice (data not shown). This decrease in tubular myelin is consistent with the decrement in ATII cells in SP-C*Lama5*$^{fl/-}$ lung.

Having found a decrease in the number of ATII cells, we next examined the alveolar type I (ATI) cell population. Immunofluorescence staining with antibody to the ATI cell-specific marker, aquaporin-5, showed a near absence of positive cells in the SP-C*Lama5*$^{fl/-}$ lung (Fig. 6F) unlike the control, in which many cells were stained (Fig 6E). To confirm this apparent decrease in ATI cells, we performed Western blot analysis of whole lung homogenate with the 8.1.1 antibody, which detects the ATI cell-specific marker T1α (Fig. 7A). With GAPDH as loading control, there was a marked decrease in T1α in lungs of SP-C*Lama5*$^{fl/-}$ mice (Fig. 7B). Thus, lack of laminin α5 expression by lung epithelial cells is associated with a striking impairment or delay in the differentiation of alveolar epithelium into ATII and ATI cells.

The homeodomain transcription factor *Nkx2.1* (also known as TTF-1 or T/EBP) is important for lung development, specifically for pulmonary epithelial cell differentiation. *Nkx2.1*$^{-/-}$ mice die at birth from respiratory insufficiency; proximal epithelial cell differentiation is preserved in these mice, but distal epithelial cell differentiation is defective, with no expression of SP-A, SP-B, or

Fig. 5. PECAM and VEGF expression. (A–D) Sections of newborn control and SP-C*Lama5*$^{fl/-}$ lungs were stained with antibodies against PECAM and VEGF. PECAM staining revealed diminished density of capillary networks in SP-C*Lama5*$^{fl/-}$ lung (B) compared with control (A). VEGF staining revealed decreased levels in large airway epithelial cells and barely detectable VEGF in the distal epithelial cells of SP-C*Lama5*$^{fl/-}$ lung (D) compared with control (C). (E–G) Total protein and RNA were extracted from lungs of newborn control and SP-C*Lama5*$^{fl/-}$ mice. (E, F) Western blot analyses for VEGF normalized to GAPDH revealed a nearly 50% reduction in VEGF protein in SP-C*Lama5*$^{fl/-}$ lungs. Experiments were performed in triplicate using three separate sets of SP-C*Lama5*$^{fl/-}$ and control lungs ($P < 0.05$, Student's t test). (G) Real-time PCR with primers for VEGF and HPRT was performed with reverse-transcribed mRNA from newborn control and SP-C*Lama5*$^{fl/-}$ lungs. The amplification plot revealed a C_T difference greater than 2, indicating an approximate 4-fold reduction of VEGF mRNA in SP-C*Lama5*$^{fl/-}$ lung. HPRT was utilized as an internal control. Original magnification 200×, A–D.

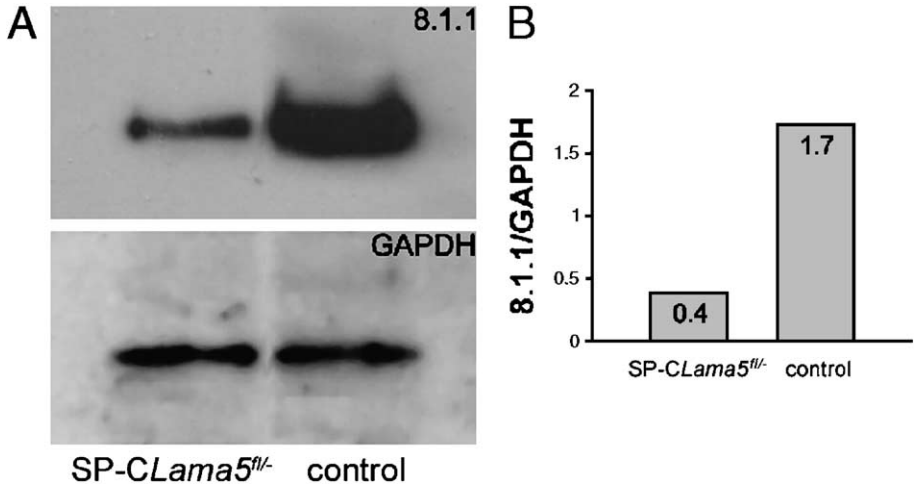


Fig. 7. T1α in alveolar epithelium. (A) Total protein was extracted from lungs of newborn control and SP-C*Lama5*$^{fl/-}$ mice and subjected to Western blot analyses for T1α (upper panel), a marker specific for ATI cells, and GAPDH (lower panel). (B) Densitometric analysis revealed a marked reduction in T1α protein in SP-C*Lama5*$^{fl/-}$ lung.

SP-C (Yuan et al., 2000). Since the defect in alveolar epithelial cell (AEC) differentiation in SP-C*Lama5*$^{fl/-}$ lungs was reminiscent of that observed in *Nkx2.1*$^{-/-}$ mice, we examined the expression of *Nkx2.1* in SP-C*Lama5*$^{fl/-}$ lungs with the TTF-1 antibody. The pattern and intensity of TTF-1 was similar in SP-C*Lama5*$^{fl/-}$ (Fig. 6H) and control lungs (Fig. 6G); both displayed staining of epithelial cells lining the airspaces, and in both, TTF-1 intensity was more pronounced in distal compared to proximal epithelium. Thus, the abnormality in epithelial cell differentiation in SP-C*Lama5*$^{fl/-}$ lungs is not due to reduced *Nkx2.1* expression.

Cell proliferation and apoptosis

Because SP-C*Lama5*$^{fl/-}$ lungs appeared to have fewer total cells than control lungs (Fig. 2), we evaluated cell proliferation and apoptosis using antibodies to Ki-67 and cleaved caspase-3, respectively. We found a significant decrease in cell proliferation (Fig. 8A) and a significant increase in apoptosis (Fig. 8B) in SP-C*Lama5*$^{fl/-}$ lungs. These studies were confirmed with BrdU labeling and TUNEL staining, respectively (data not shown).

Laminin α5 receptors

Abnormal SP-C*Lama5*$^{fl/-}$ lung development likely stems in part from a lack of interactions between epithelial cell receptors and laminin α5. Integrin α3β1 is one of the major receptors for laminin α5 on pulmonary epithelial cells and elsewhere, and signaling through integrin α3 can affect both cell growth and survival (Araki et al., 2002; Sheppard, 2003; Yoshimasu et al., 2004). Because the SP-C*Lama5*$^{fl/-}$ lungs showed abnormalities in proliferation and apoptosis and a phenotype with similarities to the integrin α3 knockout lung (Kreidberg et al., 1996), we examined expression of integrin α3. Control lungs showed pericellular

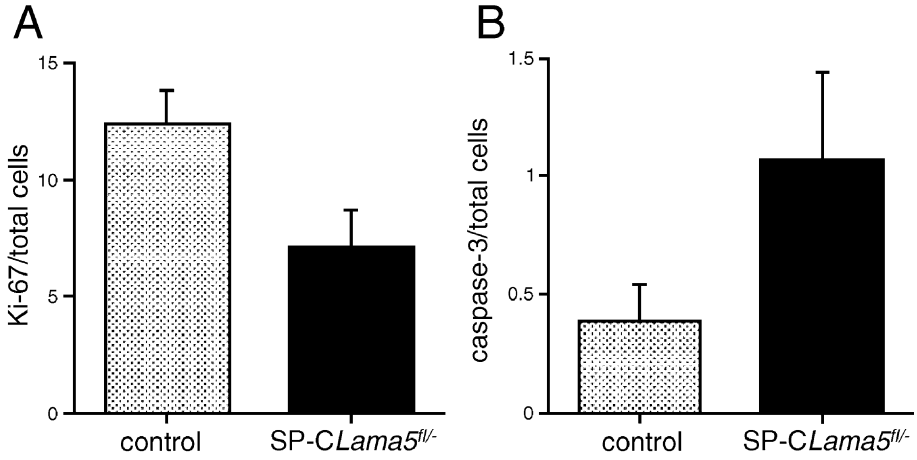


Fig. 8. Cell proliferation and apoptosis. Sections from newborn control and SP-C*Lama5*$^{fl/-}$ lungs were stained with antibodies against Ki-67 and cleaved caspase-3 to assess proliferation and apoptosis, respectively (see Materials and methods). Lungs of SP-C*Lama5*$^{fl/-}$ mice had a statistically significant decrease in total cell proliferation (A) and a statistically significant increase in total cell apoptosis (B). $N = 3$. $P < 0.05$, Student's t test.

staining for integrin α3 on all epithelial cells of the trachea, conducting airways, and distal epithelium (Figs. 9A, C, E). In the SP-C*Lama5*$^{fl/-}$ lung, integrin α3 immunostaining was present in these same cell types, but the distribution and intensity of staining were different (Figs. 9B, D, F). Epithelial cells of SP-C*Lama5*$^{fl/-}$ lungs exhibited more intense staining for integrin α3, and integrin α3 localized more to the basal side in tracheal and conducting airway epithelial cells of SP-C*Lama5*$^{fl/-}$ lungs. RT-PCR for integrin α3 from whole lung RNA showed increased expression in SP-C*Lama5*$^{fl/-}$ lungs compared to controls (Fig. 9G). Thus, it appears that lack of laminin α5 expression in lung epithelial basement membrane affects expression and localization of one of its main receptors, integrin α3. In contrast, no abnormality in expression or localization of integrin α6, another common receptor for

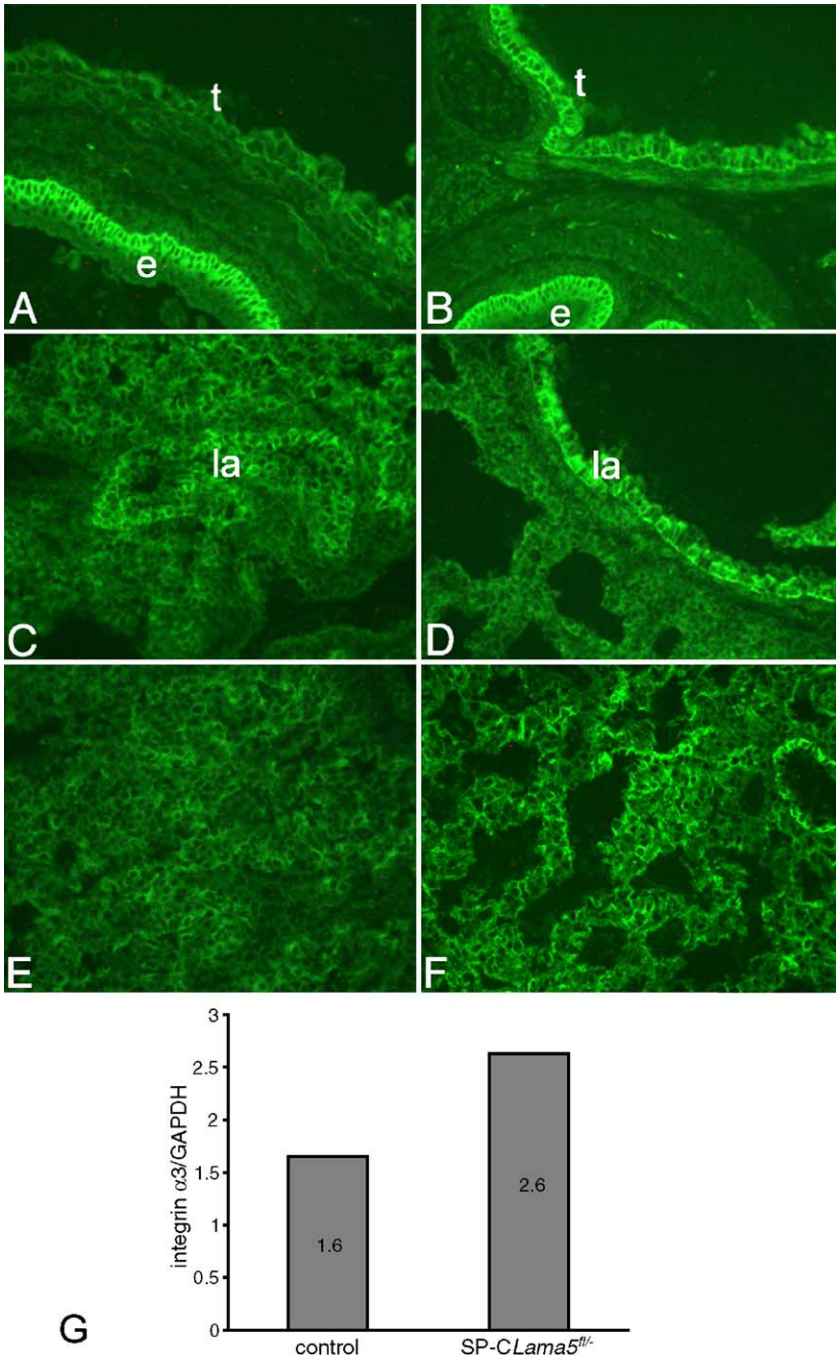


Fig. 9. Expression of integrin α3. (A–F) Sections from newborn control and SP-C*Lama5*$^{fl/-}$ lungs were stained with antibody to integrin α3. Compared with control (A, C, E), integrin α3 was increased in intensity in SP-C*Lama5*$^{fl/-}$ (B, D, F) tracheal (t), large airway (la), and distal (d) epithelial cells, while staining of the esophagus (e) was similar. (G) Total RNA was extracted from whole lungs of newborn control and SP-C*Lama5*$^{fl/-}$ mice. RT-PCR was performed with primers specific for integrin α3 and GAPDH. Densitometric analysis with GAPDH as loading control revealed less integrin α3 mRNA in the control, consistent with the immunostaining results. Original magnification 200×, A–F.

laminin α5 (Kikkawa et al., 1998; Tani et al., 1999), was observed in SP-C*Lama5*$^{fl/-}$ lungs (data not shown).

Discussion

Here, we show that laminin α5, the α chain of laminin-10/11, is essential for normal development of the lung parenchyma. The absence of laminin α5 in lung epithelium led to formation of giant distal airspaces lined by mostly undifferentiated epithelial cells. The lung-specific Cre system we used has previously served as a tool to define the roles of cytokines, growth factors, signaling molecules, and transcription factors in both lung development and response to injury/disease (Hokuto et al., 2004; Mucenski et al., 2003; Perl et al., 2003; Wan et al., 2004; Zheng et al., 2000). To our knowledge, however, there have been no prior reports of a lung-specific conditional mutation of an extracellular matrix gene.

The consequences of laminin α5 deficiency appear unique, as knockouts of other laminin chain genes do not cause as severe a lung phenotype. While redundancy and/or compensation to preserve physiologic function are commonly associated with gene ablation, this has generally not been the case with laminins (Miosge et al., 2002). In *Lamb2* mutant kidney, persistence of laminin β1 maintains glomerular basement membrane integrity, but there is a defective filtration barrier that leads to massive proteinuria (Noakes et al., 1995b). In skeletal muscle, loss of laminin α2 results in upregulation of laminin α4, but muscular dystrophy still occurs (Patton et al., 1997). In the *Lama5*$^{-/-}$ embryo, compensation by laminin α1, α2, and α4 was observed in ectodermal basement membrane, and compensation by laminin α2 and α4 was seen in intestinal epithelial basement membrane (Bolcato-Bellemin et al., 2003; Miner et al., 1998). As with the *Lama5*$^{-/-}$ embryonic lung, we found that the SP-C*Lama5*$^{fl/-}$ newborn lung had ectopic deposition of laminin α4, suggesting that α4 may be compensating for the deficiency of laminin α5. In addition, basement membranes of SP-C*Lama5*$^{fl/-}$ lungs appeared normal by electron microscopy. However, despite possible compensation by laminin α4 and formation of an ultrastructurally normal basement membrane, lungs of SP-C*Lama5*$^{fl/-}$ mice demonstrated abnormal morphology and function. Accordingly, the laminin α5 chain, specifically, is necessary for normal lung development; ectopic laminin α4, together with laminin α3A and α3B chains, are insufficient for normal alveolization. In addition, the lung contains an alternate source of laminin α5 from endothelial cells, and this source was not disturbed in our system. However, despite the presence of endothelium-derived laminin α5, epithelial differentiation was impaired, suggesting that the laminin α5 chain necessary for epithelial maturation must be of epithelial origin.

A key finding in the SP-C*Lama5*$^{fl/-}$ lung was defective maturation of the distal epithelium. Cells lining the distal epithelial airspaces were lung epithelial in origin, as shown by cytokeratin and TTF-1 staining, but there was a delay or inhibition of differentiation to terminal alveolar epithelial cells, as few cells displayed markers of ATII cells, and virtually none expressed markers of ATI cells. ATII cell formation in the fetal lung occurs at the late canalicular stage (Burri, 1984). Numerous factors have been associated with the transition from undifferentiated epithelium to ATII cells, including [D-Ala(2)]deltorphin I (DADTI), mechanical stretch, fibroblast growth factors, C/EBP delta, KGF, cAMP, and glucocorticoids (Ballard et al., 1991; Breed et al., 1997; Gonzales et al., 2002; Gutierrez et al., 1999; Sanchez-Esteban et al., 2001; Shannon et al., 1999; Shiratori et al., 1996; Sunday et al., 2001). Interestingly, although many studies implicate the extracellular matrix in stabilizing the ATII cell phenotype or influencing transition of ATII to type I cell, there is little data regarding the role of the extracellular matrix in the transition from undifferentiated epithelial to ATII cells. Our results are consistent with a requirement for laminin α5 in the differentiation of ATII cells and their transition into ATI cells.

ATII cells are the major producers of lung VEGF in late lung development (Compernolle et al., 2002; Maniscalco et al., 1995; Zeng et al., 1998), so the decrease in VEGF production (Fig. 5) may reflect the decrease in the number of ATII cells (Fig. 6). VEGF is crucial for vascular development, and a heterozygous null *Vegfa* mutation is embryonically lethal due to severe vascular defects (Carmeliet et al., 1996). Mice unable to express the VEGF164 and VEGF188 isoforms die by 2 weeks of age with hemorrhages and abnormal development of multiple organs, including the lungs (Carmeliet et al., 1999). Interestingly, their lungs were morphologically similar to the lungs of SP-C*Lama5*$^{fl/-}$ mice (Ng et al., 2001). Furthermore, inhibition of VEGF or VEGF receptor function in mice results in abnormal alveolization in developing lung and emphysema in adult lung (Compernolle et al., 2002; Kasahara et al., 2000; Tang et al., 2004). Abnormal alveogenesis with emphysematous-appearing lungs was also noted in knockouts where VEGF production was decreased (Kalinichenko et al., 2001; Yuan et al., 2000). Given these data, we propose that decreased VEGF production by ATII cells as a result of markedly fewer ATII cells, and perhaps defective ATII maturation, is critical to the pathogenesis of the SP-C*Lama5*$^{fl/-}$ lung phenotype. On the surface, the decrease in lung capillary density in newborn SP-C*Lama5*$^{fl/-}$ pups appears to contradict our finding in the E16.5 *Lama5*$^{-/-}$ lung, where vascular development appeared normal (Nguyen et al., 2002). However, because ATII cells are the major producers of VEGF in the newborn lung, differences in capillarity are expected to become more pronounced towards birth.

Our studies demonstrate that epithelial laminin α5 deficiency is associated with decreased cell proliferation and increased apoptosis in the developing lung. Decreased proliferation has also been observed in association with the absence of α5 in hair follicles and in intestinal mesenchymal cells (Bolcato-Bellemin et al., 2003; Li et al., 2003). While

no studies have examined either fetal or adult AEC growth on laminin-10/11 in vitro, extracellular matrices containing laminin clearly promote proliferation of primary adult AECs (Adamson and Young, 1996; Sugahara et al., 1998). Besides affecting proliferation, laminins also affect AEC apoptosis. Cultures of AECs with KGF and laminin-1 containing matrices had a protective effect on oxygen-induced apoptosis in AECs (Buckley et al., 1998, 1999). Rescue from apoptosis induced by serum removal occurred with A549 cells, an ATII-like cell line, when plated on a laminin-10/11-coated surface, but not when the cells were plated on poly-L-lysine- or fibronectin-coated surfaces (Gu et al., 2002).

In investigations of laminin and cell proliferation and apoptosis in vitro, the receptor mediating these effects was usually found to be an integrin. The predominant integrins for laminin-10/11 are α3β1, α6β1, and α6β4 (Kikkawa et al., 1998; Nishiuchi et al., 2003; Tani et al., 1999). We found increased integrin α3 expression in airway epithelial cells of SP-C*Lama5*$^{fl/-}$ mice. Because SP-C*Lama5*$^{fl/-}$ lung epithelial cells lack laminin α5, a ligand for integrin α3, we postulate that epithelial cells upregulate integrin α3 in an attempt to compensate for the lack of interaction with its ligand. Of note, mice lacking integrin α3 exhibit reduced airway branching and proximalization of distal epithelium (Kreidberg et al., 1996), demonstrating that the absence of either receptor (integrin α3) or ligand (laminin α5) can result in similar defects.

In summary, our analysis of SP-C*Lama5*$^{fl/-}$ mice reveals that expression of laminin α5 by lung epithelial cells is required for normal cell proliferation and apoptosis, distal epithelial cell differentiation, VEGF production, and alveolization. These results show that laminin α5 is important independent of its ability to maintain basement membrane integrity, suggesting that it is a signaling molecule necessary for proper late lung development.

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