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T1α, a lung type I cell differentiation gene, is required for normal lung cell proliferation and alveolus formation at birth

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Abstract

T1α, a differentiation gene of lung alveolar epithelial type I cells, is developmentally regulated and encodes an apical membrane protein of unknown function. Morphological differentiation of type I cells to form the air-blood barrier starts in the last few days of gestation and continues postnatally. Although T1α is expressed in the foregut endoderm before the lung buds, T1α mRNA and protein levels increase substantially in late fetuses when expression is restricted to alveolar type I cells. We generated *T1α* null mutant mice to study the role of T1α in lung development and differentiation and to gain insight into its potential function. Homozygous null mice die at birth of respiratory failure, and their lungs cannot be inflated to normal volumes. Distal lung morphology is altered. In the absence of T1α protein, type I cell differentiation is blocked, as indicated by smaller airspaces, many fewer attenuated type I cells, and reduced levels of aquaporin-5 mRNA and protein, a type I cell water channel. Abundant secreted surfactant in the narrowed airspaces, normal levels of surfactant protein mRNAs, and normal patterns and numbers of cells expressing surfactant protein-B suggest that differentiation of type II cells, also alveolar epithelial cells, is normal. Anomalous proliferation of the mesenchyme and epithelium at birth with unchanged numbers of apoptotic cells suggests that loss of *T1α* and/or abnormal morphogenesis of type I cells alter the proliferation rate of distal lung cells, probably by disruption of epithelial-mesenchymal signaling.

Keywords: Lung; Development; Epithelium; Alveoli; Type I cell; Knockout; Differentiation; Proliferation

Introduction

T1α is a differentiation gene highly expressed by type I epithelial cells lining most of the gas exchange surface of the lung (Dobbs et al., 1988). As one of the first marker genes for the alveolar type I cell to be identified at the molecular level, there is considerable information about regulation of its expression in normal and diseased lung, and about gene and protein structure (Cao et al., 2000; Koslowski et al., 1998; Ma et al., 1998; McElroy et al., 1995, 1997; Ramirez et al., 1997, 1999; Rishi et al., 1995; Vanderbilt and Dobbs, 1998; Williams et al., 1996). However, the function of the protein is unknown even though it is a major lung protein in terms of abundance. Sequence analysis indicates that T1α protein does not share common domains with other protein families of known function that could predict its function.

T1α gene expression is developmentally regulated (Rishi et al., 1995; Williams et al., 1996). T1α protein is detected in the mouse foregut endoderm before the lung buds (embryonic day E8.5–9.0) (unpublished results). During lung morphogenesis, its expression pattern changes from widespread in embryonic lung epithelium to one in which expression is restricted to type I cells of the distal epithelium. The levels of T1α mRNA and protein in the lung are highly up-regulated at the end of gestation, when morphogenesis of type I cells occurs. Although type II epithelial cells also

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reside in the alveolar epithelium, they do not express T1α (Cao et al., 2000; Dobbs et al., 1988; McElroy et al., 1995; Williams et al., 1996). T1α is also expressed in the developing nervous system. In contrast to lung, there are high levels of expression in the early neural tube but, by midgestation, the gene is down-regulated, being detected in the adult brain only in the choroid plexus (Ramirez et al., 1999; Wetterwald et al., 1996; Williams et al., 1996).

Although the adult lung and embryonic brain express the most T1α mRNA, lesser amounts are expressed in adult rodents in kidney, bone, lymphatic endothelium, and thymus (Breiteneder-Geleff et al., 1997, 1999; Farr et al., 1992; Nose et al., 1990; Ramirez et al., 1999; Rishi et al., 1995; Wetterwald et al., 1996; Williams et al., 1996) and in a subset of cells obtained from murine bone marrow (Kotton et al., 2001).

Computer analysis of the rodent *T1α* sequence shows that the protein has putative extracellular and transmembrane domains, consistent with its localization in the type I cell apical plasma membrane, and a short cytoplasmic tail with putative PKC and cAMP phosphorylation sites (Ma et al., 1998; Rishi et al., 1995). The protein is highly *O*-glycosylated and contains sialic acid (Gonzalez and Dobbs, 1998). Homologous genes have been cloned in several systems. The proteins RT140 (McElroy et al., 1995), rat podoplanin (Breiteneder-Geleff et al., 1997), and E11 antigen (Wetterwald et al., 1996) are identical to rat T1α; the partial cDNA OTS-8 (Nose et al., 1990), and proteins gp38 (Farr et al., 1992) and PA2.26 (Scholl et al., 1999), are identical to mouse T1α. In addition canine (Zimmer et al., 1997) and human (Ma et al., 1998; Zimmer et al., 1999) sequences with moderate levels of homology have been identified.

The physiological role of T1α has not yet been determined. Based on expression patterns, several functions can be hypothesized. Developmental expression patterns suggest that T1α might influence formation of the bronchial tree or the central nervous system. In the adult, since it is expressed by a number of tissues involved in fluid fluxes and is coexpressed with some aquaporins, T1α could be a regulator of fluid transport. Finally the location of T1α on the type I cell apical membrane, a surface area of large magnitude (>70 m^2 in the adult human), raises the possibility that it interacts with molecules or cells present in alveolar lumens.

We generated *T1α* null mice containing a targeted mutation in the *T1α* gene locus. Homozygous null mutant mice [*T1α* ($-/-$)] die at birth of respiratory failure. No gross morphological abnormalities of lung or brain are observed in newborns. However, histologic and molecular analyses of the lung at birth show an abnormally high expression of proliferation markers in the distal lung, many fewer attenuated type I cells, narrower and irregular air spaces, and defective formation of alveolar saccules in homozygous animals compared to wild-type littermates [*T1α* ($+/+$)]. This phenotype suggests that the loss of T1α affects the regulation of peripheral lung cell proliferation in the perinatal period and interferes with differentiation of type I cells that line the potential air spaces. Although a detailed analysis of the homozygous brain at term has not been completed, no obvious abnormalities have been observed.

Materials and methods

Targeting vector

A 12-kb *Eco*R1 fragment, spanning the 5′ flanking region, the 5′ untranslated region (5′UTR)-first exon, and part of the first intron, was characterized by restriction enzyme and Southern blot analyses of a BAC clone containing the mouse *T1α* gene (mouse *T1α* = *OTS-8* Accession No. M73748) (Incyte Genomics Inc.). This fragment was subcloned into p-BluescriptSK (Stratagene) and partially sequenced. To generate the targeting vector, a 5′ arm of homology ($\sim$2 kb fragment in the *T1α* promoter from -3.5 to -1.5 kb) was obtained by PCR (forward primer 5′ CTGACTCGAGTAGCAACAGCCACCAACG3′, reverse primer 5′CTGACTCGAGCCAATGAAGGTGACAATGAGC3′) and its sequence was confirmed by comparison to the original BAC clone. The 3′ arm of homology was a *Nar*1–*Ssp*1 fragment spanning 4 kb in the first intron. Arms were designed to replace $\sim$1.5 kb of the promoter, 210 bp of the UTR, the first exon (67 bp), and 181 bp of the first intron. Arms were cloned in a vector (pLNTK) containing pGK-Neo and pGK-HSVtk, positive and negative selection genes (kindly provided by Dr. G. Taccioli, Boston University School of Medicine, Boston, MA) (Taccioli et al., 1998).

T1α gene targeting

The linearized targeting vector was electroporated into 129/SvEv TC1 ES cells (Deng et al., 1994; Xu et al., 1999). Two of 102 clones that survived selection in G418 and FIAU had undergone homologous recombination as determined by Southern blot. Cells from these clones were injected into C57BL/6 blastocysts and implanted into foster mothers in the Boston University School of Medicine transgenic facility. Chimeric males were mated to 129/SvEv females for germline transmission of the mutated *T1α* allele. Southern blots of EcoR1-digested genomic DNA were used to identify mutant mice. Probes external to the 5′ and 3′ arms and a Neo probe were generated by PCR (oligonucleotides to amplify the 5′-probe were forward 5′AAGCCAGGTTCCCACTCATAG3′, reverse 5′ GTATCAGGTCATTCTCCCTTC3′; oligonucleotides to amplify the 3′-probe were forward 5′TGCCTACTGCCATGCTTCTACC3′, reverse 5′ CTACACCATAGTTATCGCTC3′). Heterozygous null mutant [*T1α*($+/-$)] mice were mated to generate *T1α*($-/-$) offspring. Term deliveries were directly observed to record survival period, respiratory behavior, body weight, and lung weight. Tissue samples were collected

immediately after birth for histologic and biochemical analyses. All experiments were performed in an A.A.A.L.A.C. approved animal facility under supervision of the Institutional Animal Care and Use Committee of Boston University Medical Center.

Lung wet/dry weight ratio and DNA content

Newborn lungs were dissected and weighed. To determine wet/dry weight ratios, lungs were dissected in two fragments and weighed. One fragment was dried to constant weight in a SpeedVac at room temperature. The second fragment was used to purify genomic DNA as described previously (Ramirez et al., 1999). DNA concentration was estimated by UV spectroscopy using A260/280 absorbances.

Lung histology

Newborn lungs were immersed in 2% glutaraldehyde, 1% paraformaldehyde, 0.15 M sodium cacodylate buffer for 16–18 h, dehydrated, and embedded in either JB-4 or Epon 812 substitute resins (Polysciences, Inc.). JB-4 sections (2 μm) were stained with hematoxylin and eosin (H&E) and Epon sections (0.5 μm) were stained with toluidine blue.

Lung inflation

E19.5 fetal lungs were inflated with 4% paraformaldehyde/PBS fixative under pressure (15 cm H_2O pressure) via a tracheal cannula of PE 20 tubing (Intramedic, Clay Adams) and 25-gauge blunt catheter. After inflation the thoracic cavity was filled with fixative. After 5 min, the lungs were removed, stored in fixative overnight at 4°C, and embedded in paraffin. Sections (6 μm) were stained with H&E.

Northern blots

Northern blots were performed by previously described methods (Ramirez et al., 1999) using total RNA (5 or 10 μg) from newborn lungs. Probes were ^{32}P-labeled. Mouse caveolin-1 (forward primer 5′TGTGATTGCAGAACCAGAAGGG3′, reverse primer 5′GGAACTTGAAATTGGCACCAGG3′) and carboxypeptidase-M (CP-M) (forward primer 5′GAGACTCTGGCAATCATGGAGTG3′, reverse primer 5′GCGAGGATATTTACAGCACGACAG3′) probes were generated by RT-PCR. VEGF probe, a 650-bp cDNA fragment of the 188-kDa isoform, was a gift of Dr. A. Healy, Boston University, Boston, MA. Other probes were described previously (Meneghetti et al., 1996; Ramirez et al., 2000). Gene expression levels were determined in three to four blots, using mRNAs from different litters. Densitometry was performed in a Bio-Rad Fluor-S MultiImager. β-Actin was used to normalize data. Statistical analysis was performed using StatView 5.12 (Brain-Power, Inc.). Data are expressed as mean ± SEM. Statistical significance was analyzed using Student's *t*-test.

Western blots

Protein was extracted from lung homogenates using RIPA buffer as described previously (Williams et al., 1996). Protein (10 μg) was electrophoresed on 12% polyacrylamide gels, transferred to PVDF membranes, blocked with 5% milk, and incubated overnight at 4°C with specific primary antibodies. T1α was detected with mAb 8.1.1 (dilution 1:200), a hamster monoclonal antibody (a gift of Dr. Andrew G. Farr, Department of Biological Structure, University of Washington School of Medicine, Seattle, WA) that recognizes the thymic protein gp38 identical to T1α. This antibody labels alveolar type I cells in adult mouse lung by immunohistochemistry (Cao et al., unpublished data). The mAb 8.1.1 is deposited in the Developmental Studies Hybridoma Bank (www.uiowa.edu/~dshbww/). HRP-labeled anti-hamster IgG (dilution 1:2500) (ICN) was used as a secondary antibody. CP-M was detected using an affinity-purified rabbit antibody to the N-terminal peptide of the human CP-M protein (dilution 1:2000) (a gift of Dr. Randal A. Skidgel, Department of Pharmacology, University of Illinois at Chicago); binding was detected with HRP-labeled anti-rabbit IgG (dilution 1:10,000). PCNA was detected with a murine monoclonal antibody NCL-PCNA (dilution 1:500) (Novocastra Laboratories) and an anti-mouse secondary antibody. HRP was developed using SuperSignal West Pico Chemiluminescent Substrate (Pierce). Total protein per well was used to normalize the data.

Protein for Aqp-5 Western blots was extracted from newborn lung homogenates by the methods of Korfhagen et al. in 500 μl buffer containing protease inhibitors (Korfhagen et al., 1996). Samples were centrifuged at 250*g*, 10 min at 2°C. Supernatants were ultracentrifuged at 135,000*g* for 4 h, and pellets were resuspended in homogenization buffer without sucrose (50 μl). Newborn lung protein extracts (30 μg) and adult lung protein extracts (10 μg) were electrophoresed on 16% polyacrylamide gels, transferred to nitrocellulose, blocked, and incubated overnight at 4°C with an affinity-purified rabbit polyclonal Aqp-5 antibody (dilution 1:1000) (a gift of Dr. Alfred N. Van Hoek, Renal Unit, Massachusetts General Hospital, Charlestown, MA). HRP-labeled anti-rabbit IgG (dilution 1:6000) (ICN) was used as a secondary antibody.

Immunohistochemistry

Newborn lungs were fixed overnight by immersion in 4% paraformaldehyde in PBS at 4°C and embedded in paraffin as described (Williams et al., 1996). Sections (6 μm) were deparaffinized and hydrated by standard methods. Endogenous peroxidase was quenched with 3% H_2O_2 in methanol for 15 min. Blocking was performed with 2% normal goat serum in PBS/0.2% Triton X-100 for 2 h at

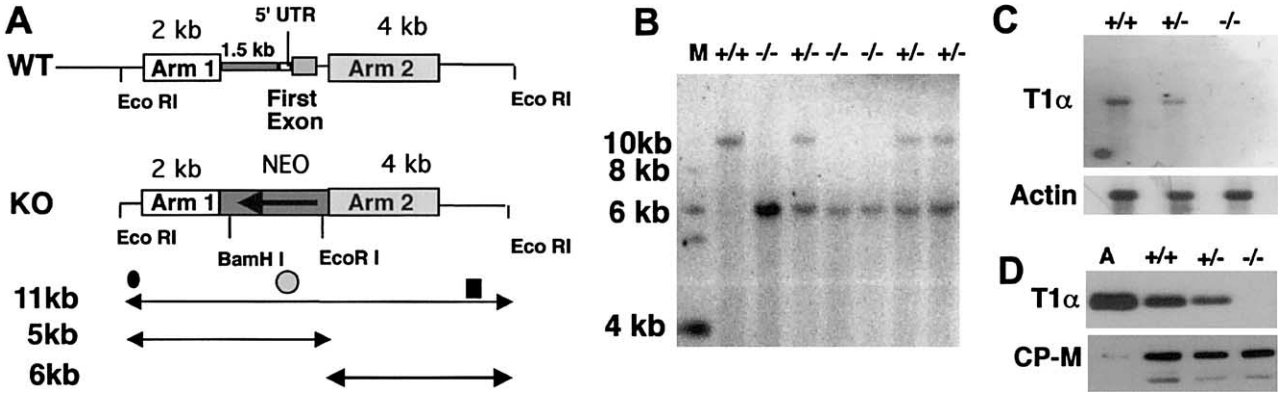


Fig. 1. T1α gene targeting. (A) Diagram of the targeting vector used to generate mutants showing arms of homology 1 and 2 that flank 1.5 kb of the T1α promoter and first exon as cloned in the Neo cassette-containing pLNTK vector. EcoR1 sites 5′ and 3′ to the region to be targeted and a third EcoR1 site introduced in the Neo sequence were used to digest genomic DNA and differentiate wild-type (WT) from targeted DNA (KO). The oval, circle, and square indicate 5′, Neo, and 3′ probes, respectively, used in Southern blots. (B) Southern blot of genomic DNA digested with EcoR1 and probed with the 3′ probe shows the wild-type allele as an 11-kb fragment. The 6-kb fragment is the targeted allele. (C) Northern analysis of newborn lung shows the absence of T1α mRNA in ($-/-$) animals with reduced expression in ($+/-$) animals. No transcripts of any size are noted in the ($-/-$) lungs. β-Actin expression shows equal loading of mRNA. (D) Western analysis for T1α protein expression shows absence of T1α protein in ($-/-$) lungs with reduced expression in ($+/-$) lungs. Western analysis shows that CP-M expression is not altered in the same protein extracts ($+/+$, wild-type; $+/-$, heterozygous; $-/-$, homozygous; A, adult lung; M, markers).

room temperature. Tissues were exposed to primary antibodies in PBS for 16 h, 4°C. Washes were performed with PBS containing 0.2% Triton X-100 except where noted.

The following antibodies were used: rabbit anti-human prosurfactant-B (C-terminal) polyclonal antibody (Chemicon International, Inc.) (1:1000 dilution); anti-mouse goat polyclonal antibody SP-C (M-20) (Santa Cruz Biotechnology Inc.) (1:1000 dilution) (omitting Triton X-100); rabbit anti-Aqp-5 antibody (above) (1:100 dilution); anti-PECAM-1 (M-20) antibody (Santa Cruz Biotechnology Inc.) (1:1000 dilution), and stained as described previously (Ramirez et al., 2002). For Aqp-5, antigen retrieval was performed for 20 min at 90°C using unmasking solution (Vector Laboratories, Inc.). Sections were washed with PBS/0.2% Triton X-100 and incubated 1 h at room temperature with a secondary antibody diluted in PBS/0.2% Triton X-100. Antibody binding was detected with the Vectastain Elite ABC kit using also PBS/0.2% Triton X-100 (Vector Laboratories, Inc.) and diaminobenzidine (DAB) as substrate. For some antibodies signals were enhanced using the TSA kit (NEN Life Science Products, Inc.). PECAM-1 staining was performed as described previously (Ramirez et al., 2002). Control slides omitting the primary antibody were run simultaneously. Sections were counterstained with methyl green (Vector Laboratories, Inc.). PCNA was detected with the PCNA staining kit (Zymed) using a biotinylated primary antibody, HRP-streptavidin, and DAB substrate. Some sections were counterstained with H&E.

Apoptosis analysis

Apoptotic nuclei were detected by TUNEL staining of paraffin sections using the TdT-FragEL DNA fragmentation detection kit (Oncogene Research Products). The percentage of TUNEL-positive cells in two wild-type and two knockout lungs was determined by counting TUNEL-positive nuclei and total nuclei stained with methyl green in 10 fields using the 100× objective lens and OpenLab software (Improvision, Inc.) with the density slice module and advanced measurement settings. Values were statistically analyzed as described above.

Results

Characterization of T1α null mutant mice

T1α($+/-$) mice containing the null *T1α* gene locus were generated by crossing the initial chimeras to 129/SvEv wild-type mice (Fig. 1A). *T1α*($+/-$) mice were mated and litters genotyped (Fig. 1B). The resultant litters have a Mendelian ratio of (1:1.8:0.75) (Table 1) at birth. The slightly lower number of *T1α*($-/-$) is not statistically significant ($\chi^2 = 0.23$), indicating that the lack of *T1α* gene is not lethal during embryogenesis. Northern and Western blot analyses of newborn lung RNA and protein extracts show no T1α mRNA or protein in *T1α*($-/-$) mice, and reduction in the levels of *T1α* in *T1α*($+/-$) mice (Fig. 1C and D). No bands of smaller molecular weight were detected in either Northern or Western blots. By RT-PCR, no T1α mRNA was detected in *T1α*($-/-$) brain and kidney (data not shown) as expected.

Neonatal respiratory failure

Homozygous null mutant mice die at birth. Mice make a respiratory effort but are cyanotic and lethargic and only

Table 1
Characterization of T1α knock out mice

Genotype	+/+	+/−	−/−
Mendelian ratio	1 (48)	1.81 (87)	0.75 (36)
Body weight (g)	1.42 ± 0.07 (6)	1.53 ± 0.02 (17)	1.69 ± 0.02[a] (9)
Lung weight (mg)	28 ± 2 (6)	32 ± 2 (11)	35 ± 5 (7)
Lung/body weight ratio	19.4 ± 0.7 (6)	21.3 ± 1.7 (11)	20.5 ± 2.8 (7)
Lung Wet/Dry weight ratio	1 ± 0.05 (12)	0.93 ± 0.07 (16)	0.66 ± 0.04[b] (5)
Lung DNA/Dry weight ratio	1 ± 0.08 (11)	0.70 ± 0.08 (13)	0.43 ± 0.01[c] (4)

Note. *P* value −/− to +/+
(n) number of mice
[a] $P \leq 0.01$
[b] $P \leq 0.02$
[c] $P \leq 0.07$

survive about 3–10 min, leading us to conclude that they die of respiratory failure. A few animals survived for up to 4 h before dying of apparent respiratory failure. The gross morphology, number of lobes, lung shape, and lung/body wet weight ratio of the *T1α*(−/−) mice are similar to *T1α*(+/+) littermates. On average, homozygous mice are slightly heavier (19%) than wild-type mice (Table 1). Although the difference is statistically significant ($P \leq 0.01$), the lung/body weight ratios are conserved. The lungs of mice that die a few minutes after birth are not inflated (Figs. 2 and 3), while those of longer survivors are partially inflated with small volumes of air. In these animals, air appears to be

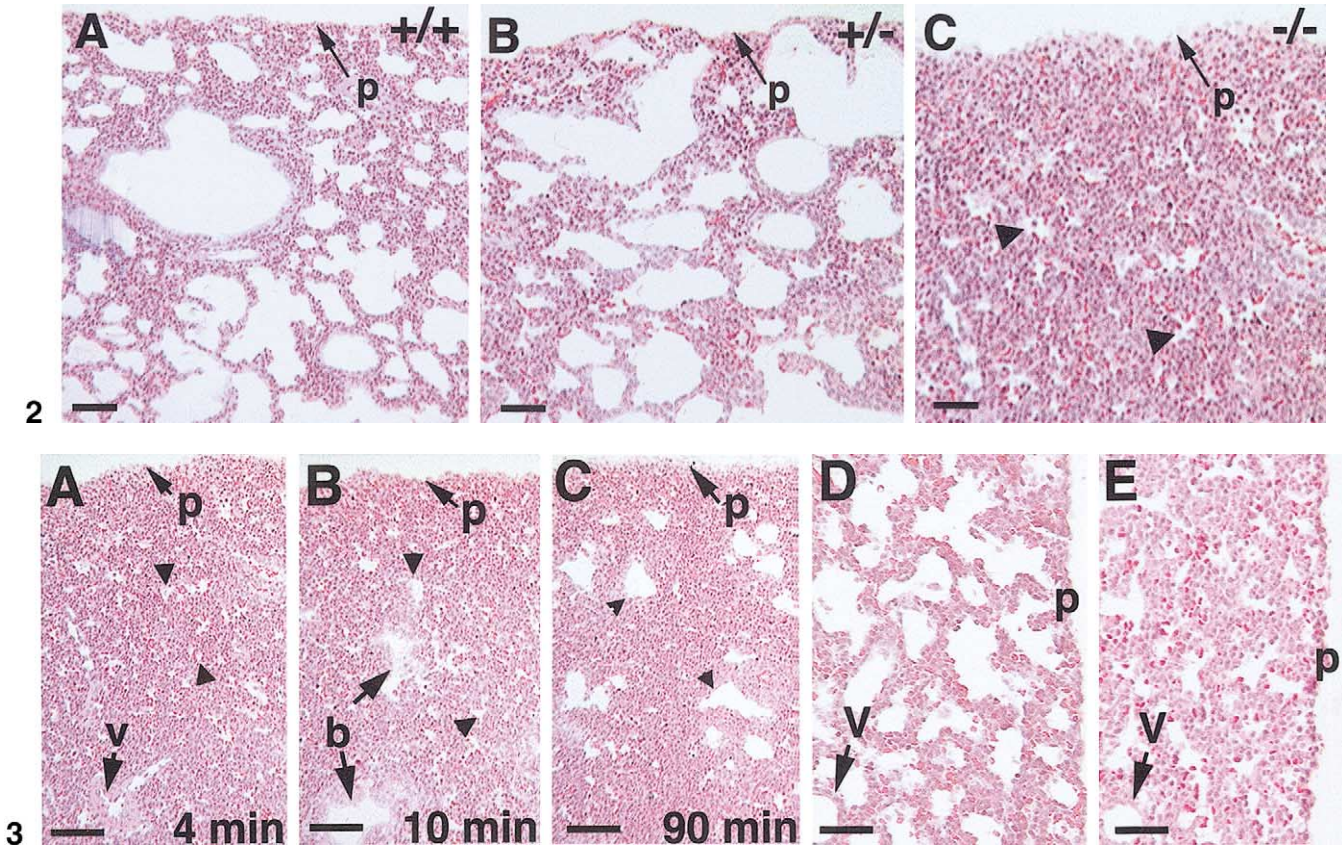


Fig. 2. Marked alterations in distal lung morphology are observed in (A) (+/+), (B) (+/−), and (C) (−/−) newborns. The (−/−) lungs characteristically have dense cellularity, narrow and tortuous air spaces (arrowheads), and thicker interalveolar septae compared to (+/+) or (+/−) lungs. p, pleural surface. Plastic sections (2 μm) stained with H&E. Bars = 75 μm.

Fig. 3. Newborn (−/−) mice that survive for longer than 2–3 min after birth have partially inflated airspaces as in mice surviving for (A) 4 min, (B) 10 min, and (C) 90 min, respectively. (D) E19.5 (+/+) and (E) (−/−) lungs inflated with 4% paraformaldehyde fixative at 15 cm H_2O pressure. (−/−) Lungs show small increases in alveolar volume but fail to reach inflation volumes of (+/+) lungs. p, pleura; v, blood vessels; b, bronchioles; arrowheads indicate partially inflated or distended airspaces. A–C, plastic sections (2 μm) and D–E, paraffin sections (6 μm) stained with H&E. Bars, A–C = 100 μm, D–E = 70 μm.

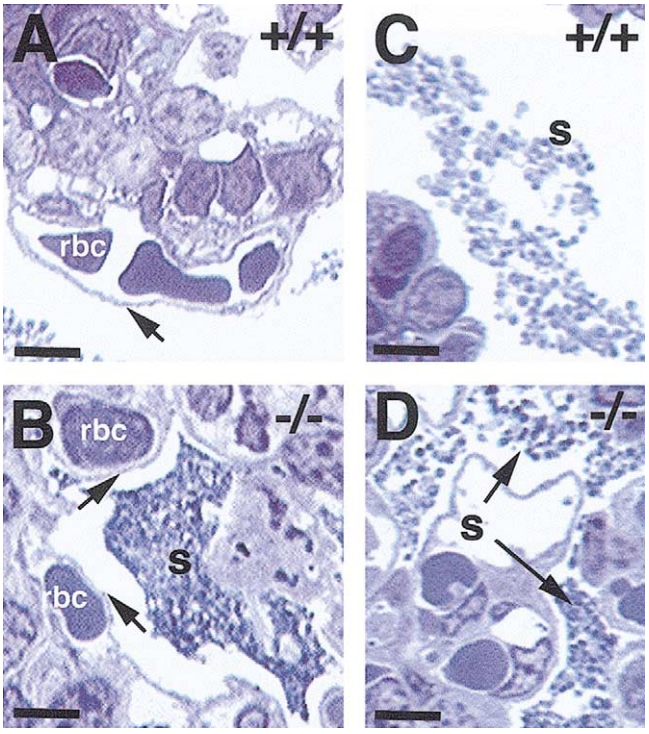


Fig. 4. Secreted surfactant and infrequent attenuated air blood barriers are observed in (−/−) T1α null mutant lungs. Lungs of (+/+) (A, C) and (−/−) (B, D) newborns show similar features in these selected fields. The presence of some flattened cells separating airspaces from capillaries (RBC, red blood cells) in (−/−) mice is indicated by arrows pointing to the air-blood barrier (A–B). Secreted surfactant can be observed in (+/+) (A, C) and (−/−) (B, D) mice. s, surfactant; rbc, red blood cells. Plastic sections (0.5 μm) stained with toluidine blue. Bars = 5 μm.

trapped in the airways rather than in the peripheral lung, making the airways look distended (Fig. 3C). Using the lectin *Lycopersicum esculentum* to delineate the contours of the potential airspaces confirms that the T1α(−/−) lungs have narrower, irregular airspaces compared to T1α(+/+) (data not shown). Heterozygous mice are viable, fertile, and have been maintained for more than a year in a pathogen-free area.

Altered distal lung morphology

Toluidine blue stained, 0.5-μm plastic sections (Fig. 4C and D) show abundant secreted surfactant in air spaces of both wild-type and *T1*α(−/−) animals. By EM, the secreted surfactant in both wild-type and *T1*α(−/−) lungs is composed of lamellar bodies and tubular myelin with normal structural features (data not shown). The use of 0.5 μm plastic sections also makes it possible to visualize very thin septae between airspaces and capillaries in the *T1*α(−/−) lung (Fig. 4A and B), indicating that a few attenuated type I cells are present although infrequent. The presence in *T1*α(−/−) lungs of a few type I cells with normal cellular characteristics has been confirmed by EM. Electron microscopy also demonstrates that the structure of type II cells is normal and that the cells contain lamellar bodies as expected.

We tested the distensibility of day E19.5 *T1*α(−/−) lungs by instilling fixative under pressure via a tracheal cannula. We noted a small increase in dimensions of the peripheral lung lumens but did not observe expansion approaching that seen in wild-type animals (Fig. 3D and E).

Expression of differentiation marker genes in distal lung cells

Expression of differentiation genes was compared on Northern blots of wild-type, heterozygous, and homozygous lungs (Fig. 5). We selected genes expressed by distal lung

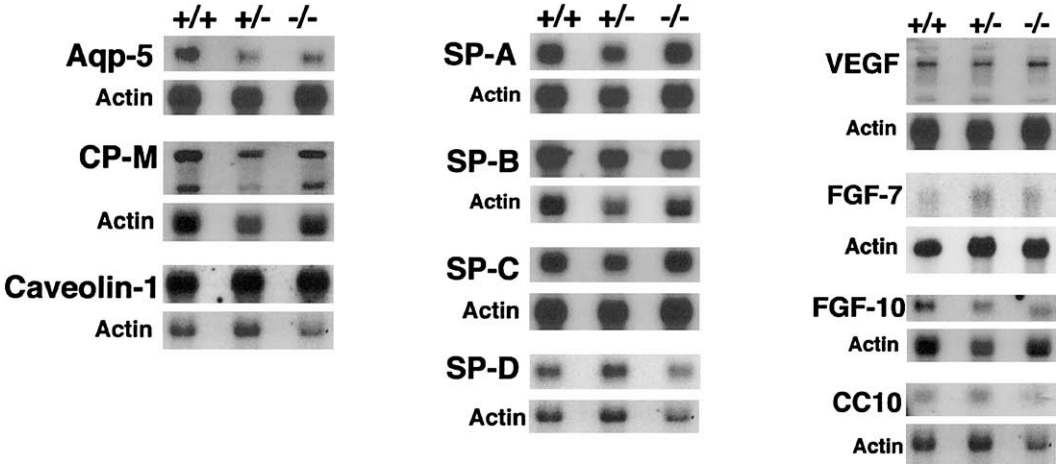


Fig. 5. Representative Northern blots of total RNA (5–10 μg) from (+/+), (+/−), and (−/−) newborn lungs (n = 3–4) show expression patterns of distal lung cell genes including type I cells (Aqp-5, CP-M, caveolin-1), type II cells, and/or Clara cells (SP-A, −B, −C, −D, VEGF, CC10), fibroblasts (FGF-7, FGF-10), and endothelial cells (caveolin-1). β-Actin expression indicates mRNA loading. Densitometry of Northern blots is shown in Table 2.

Table 2
Expression of distal lung genes analyzed by Northern blot (densitometry values)

Gene	+/+	+/−	−/−
Aqp-5	1 ± 0.01	0.64 ± 0.07[a]	0.45 ± 0.14[b,c]
CP-M	1 ± 0.02	0.83 ± 0.08	1.20 ± 0.45
Caveolin-1	1 ± 0.02	1.18 ± 0.11	1.48 ± 0.18
SP- A	1 ± 0.01	1.20 ± 0.09	1.24 ± 0.26
SP- B	1 ± 0.001	0.99 ± 0.07	0.97 ± 0.06
SP- C	1 ± 0.02	0.86 ± 0.12	0.93 ± 0.07
SP- D	1 ± 0.01	0.92 ± 0.13	0.89 ± 0.09
CC10	1 ± 0.02	0.84 ± 0.16	1.07 ± 0.11
VEGF	1 ± 0.02	0.81 ± 0.10	0.85 ± 0.12
FGF7	1 ± 0.03	1.26 ± 0.39	1.27 ± 0.32
FGF10	1 ± 0.02	0.97 ± 0.34	1.00 ± 0.12

[a] +/+ to +/− $P \leq 0.01$
[b] +/+ to −/− $P \leq 0.02$
[c] +/− to −/− $P \leq 0.05$

cells as follows: *Aqp-5* (Funaki et al., 1998) and *CP-M* (Nagae et al., 1993) expressed in type I cells; *SP-A, -B, -C,* and *-D,* and *VEGF* (Bhatt et al., 2000) expressed in type II cells and/or Clara cells in small bronchioles; *CC10* expressed by Clara cells; and *caveolin-1* expressed by alveolar type I cells and endothelial cells (Kasper et al., 1998). We also analyzed expression of the fibroblast growth factors *FGF-7* (Yano et al., 2000), a lung epithelial cell mitogen, and *FGF-10* (Bellusci et al., 1997), an important regulator of epithelial branching during lung development.

Densitometric analyses of three to four Northern blots containing RNA from different groups of animals (Table 2) indicate that only *Aqp-5* is significantly changed as indicated by a 2.3-fold decrease in *T1α*(−/−) lungs compared to wild-type. Western blots of Aqp-5 protein confirm this decrease (Fig. 6). The decrease in Aqp-5 mRNA and protein likely reflects the presence of many fewer attenuated type I cells in homozygous animals as shown histologically (Fig. 7A and B). Expression levels of other genes do not differ between wild-type and *T1α*(−/−) animals. There is a small although not significant increase in caveolin-1 mRNA in homozygous lungs; whether it is up-regulated in epithelial or endothelial cells is not known. The levels of SP-A, -B, -C, and -D mRNAs are the same in wild-type and *T1α*(−/−) animals, consistent with immunohistochemical findings with SP-B and SP-C antibodies showing that the patterns of expression of SP-B and SP-C are similar in wild-type and *T1α*(−/−) lungs (Fig. 7C–F). Likewise cell counts show that a similar percentage of total distal lung cells expresses SP-B protein in *T1α*(−/−) lungs (8.3 ± 0.83%) compared to wild-type (7.2 ± 0.89%). Western blot analysis also shows that CP-M protein levels are not altered (Fig. 1D). No differences are apparent in immunohistochemical expression patterns of PECAM-1 (Fig. 7G and H), suggesting that endothelial cell differentiation is not markedly altered.

Cell proliferation and apoptosis in homozygous lungs

Immunostaining shows that there is a significantly higher number of PCNA-positive cells in the distal lung of mutant mice than in wild-type (Fig. 8A–G). This indicates that cells in the *T1α*(−/−) have proliferated in the 24 to 30-h period before birth while cells of wild-type animals have ceased proliferation as expected. The altered pattern of PCNA-stained cells is reflected in Western blot analyses that show that PCNA protein levels are increased 1.8-fold in *T1α*(−/−) compared to wild-type lungs ($n = 4$, $P \leq 0.02$) (Fig. 6). Wet/dry lung weight ratio and DNA content per dry weight were determined to see if enhanced PCNA staining was accompanied by an increase in cell number; both values are 40–60% lower in *T1α*(−/−) compared to wild-type (Table 1). This suggests that, on average, cells in the *T1α*(−/−) lung may be bigger than in the wild-type, or there may be increased abundance of extracellular matrix proteins or a combination of these.

Increased apoptosis could compensate for increased cell proliferation and thus conserve the size of the lung. However, quantitation of TUNEL-positive nuclei shows no difference in the percentage of apoptotic nuclei between *T1α*(−/−) (1.5%) and wild-type (1.0%) lungs at birth (Fig. 8H and I).

Discussion

We generated *T1α* null mutant mice to explore the function of *T1α* and to see if it plays a critical role in formation and maintenance of the air-blood barrier. *T1α* null mice die at birth, likely due to lung abnormalities present in newborns that impair respiration.

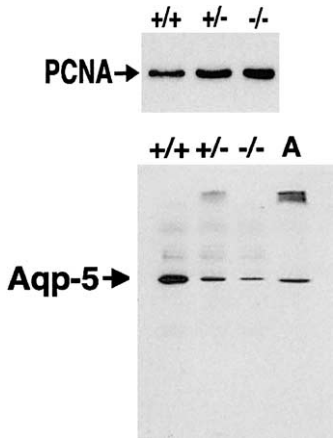


Fig. 6. Western blots of newborn lung protein extracts (30 μg) show reduced abundance of Aqp-5 protein ($n = 2$) in (−/−) lungs, consistent with decreased Aqp-5 mRNA. Adult protein extract control, 10 μg, lane A. (A). In contrast, PCNA protein expression is increased in (−/−) lungs ($n = 4$) compared to (+/−) and (+/+) lungs (10 μg newborn protein extract) (B). This increase is consistent with PCNA staining patterns in (−/−) lungs.

These mice reveal that expression of the *T1α* gene is associated with, although not absolutely required for, the timely morphogenesis and differentiation of type I cells in the distal lung before birth. The lack of type I cells would be expected to reduce the surface area for gas exchange to a level below that required for survival of the newborn. However this cannot be the only defect leading to neonatal death because, despite attempts to breathe, *T1α*(−/−) mice cannot inflate their lungs. Although the lungs look hypercellular, wet/dry and DNA/dry weight ratios argue against this and indicate that the homozygous null animals have a slightly reduced number of cells, a larger cell size, or both when compared to wild-type controls. These features could be a general sign of lung immaturity since it is known that the size of distal lung cells decreases in late gestation when cells lose glycogen and undergo morphogenesis to acquire their adult cellular characteristics (Adamson and Bowden, 1975; O'Hare and Townes, 1970).

There is abnormal regulation of peripheral lung cell proliferation in *T1α* null mutant animals. PCNA staining shows a large excess of proliferating cells in *T1α*(−/−) distal lung; PCNA protein levels are increased in homozygous null lungs analyzed by Western blot. In addition different cell populations are PCNA-positive in the knockout lung compared to the wild-type. Although the dense cellularity of the knockout lung makes identification of dividing cells problematic, proliferating cells can be recognized in both the distal epithelium and the adjacent connective tissues. There appear to be few PCNA-labeled endothelial cells. In the wild-type lungs PCNA-positive cells at term are almost completely restricted to blood vessels and airways, consistent with observations of a number of previous studies (Kauffman, 1975; Kauffman et al., 1974; O'Hare and Townes, 1970).

The increased proliferation of peripheral lung cells in the term *T1α*(−/−) differs markedly from the time course of the thymidine-labeling index (LI) reported for the normal lung (Kauffman, 1975; O'Hare and Townes, 1970). During normal development, there is rapid cell proliferation during early and mid-gestational lung organogenesis; on gestational day 15 the LI is 53%. However the LI falls to 14% on E18 and reaches a nadir of ~2% at term (rat). This trend has been documented in a number of studies in both rats and mice, although there is variability in the absolute numbers reported. By postnatal days 2–4 the labeling index increases substantially to support postnatal growth (Kauffman et al., 1974). That proliferation is increased in the *T1α*(−/−) newborn lungs indicates that a key regulatory pathway, not yet identified, influencing cell division in the peripheral lung has been interrupted. Analyses of proliferation patterns during *T1α*(−/−) lung development are planned to determine whether the increased PCNA expression observed at birth is a late event or whether there is sustained proliferation during lung development. We are not aware of other reports of this type of proliferation defect in lung at term although lungs of corticotropin-releasing-hormone (*CRH*) null mice also show increased PCNA staining in late fetuses (E18.5) (Muglia et al., 1999). Whether the CRH animals show the expected drop in mitotic index at term was not reported.

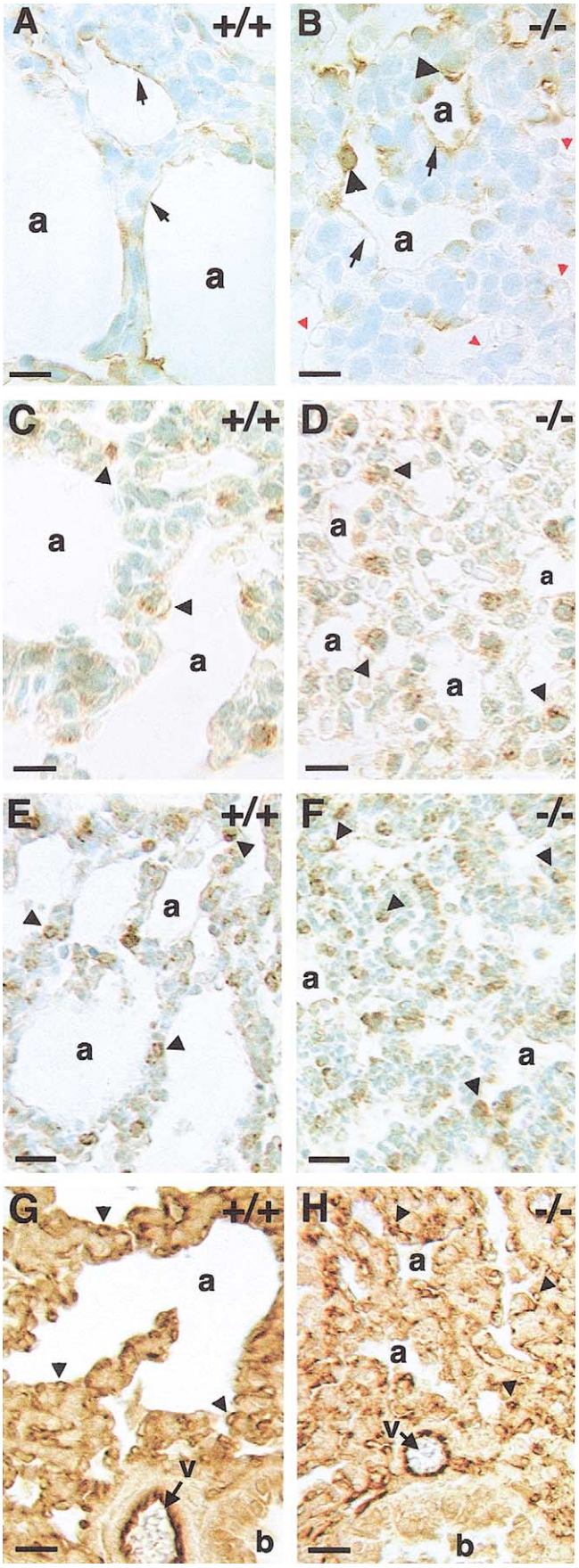
In addition there is abnormal development of alveolar saccules in the absence of T1α. The extremely reduced alveolar spaces, the marked decrease in flattened epithelial cells, and significantly reduced levels of Aqp-5 mRNA and protein are evidence of failure of morphogenesis of the normal number of type I cells, a process that normally occurs in the last 2–3 days of gestation (Adamson and Bowden, 1975; O'Hare and Townes, 1970).

Exactly how type I cells form and what their parental cell lineage is are uncertain. There are tentative data for their derivation from postproliferative type II cells (Adamson and Bowden, 1975), from a common precursor common to both type I and type II cells, and from different subpopulations committed early to type I or type II cell phenotypes (Joyce-Brady and Brody, 1990; Williams and Dobbs, 1990). Although debated, the developmental expression patterns of genes specific for adult type I and type II cells suggest that commitment to be type I or type II cells occurs early in organogenesis via separate cell populations. These interpretations are limited by the small number of type I cell-specific markers examined during development; only *T1α* has been studied in detail.

Overall the *T1α* null lung phenotype appears to be related to a general dysregulation of proliferation in the distal lung at the expense of correct differentiation. It is not clear whether proliferation is sustained because differentiation (due to loss of T1α protein and/or formation of very few type I cells) is blocked or whether differentiation is impaired because there is too much proliferation (epithelial cells continue to be produced but have no surface area available for formation of normal type I cells). Likewise current understanding of the overall null phenotype does not provide insight into which characteristics are due to the loss of T1α protein and which are due to failure of formation of type I cells.

Our findings indicate that *T1α* is not necessary for type II cell differentiation because their number and differentiation, as evaluated by surfactant gene expression, are normal in *T1α* knockout lungs. The cells contain lamellar bodies, express surfactant protein mRNAs at normal levels, express SP-B and SP-C proteins, and secrete abundant surfactant as evidenced by intraalveolar lamellar bodies and tubular myelin. Although SP-B or surfactant lipid deficiencies can result in respiratory failure (Clark et al., 1995), these appear unlikely to contribute to respiratory failure in term T1α null mice.

The lung phenotype of the T1α mutants is unique for type I cell genes since inactivation of other type I cell genes does not affect type I cell formation or lung development. *Aqp-5* (Ma et al., 2000; Song et al., 2000), *caveolin-1* (Drab et al., 2001; Razani et al., 2001), and *ICAM-1* knockout mice (Xu et al., 1994) are viable and appear to have normal



lungs at birth. Aqp-5 loss appears not to affect alveolar fluid clearance in neonates, making it unlikely that decreased Aqp-5 expression in *T1α* null mice contributes to their failure to breathe. Likewise *caveolin-1* null mice seem normal at birth. Postnatally (4–5 months), however, their lungs have thickened alveolar septae and endothelial cell hyperproliferation. The delay in this phenotype argues that the modest increase in caveolin-1 expression does not influence the *T1α* mutant phenotype. Similarly although null mutants of *ICAM-1*, expressed by type I cells, have reduced immune responses, they have no obvious developmental defects in the lung.

In contrast, glucocorticoid receptor (*GR*) (Cole et al., 1995), *CRH* (Muglia et al., 1999), and *Sp3* transcription factor knockouts (Bouwman et al., 2000), *p21/p57* double knockout (Zhang et al., 1999), and NF-1B (nuclear factor 1B) knockout (Grunder et al., 2002) produce a lung phenotype similar to the T1α knockout, although none is a type I cell differentiation gene. These mice fail to form normal distal airspaces and die of neonatal respiratory failure. The *p21/p57* double knockout lungs do not show increased proliferation or apoptosis and are therefore likely to have differentiation defects. GR knockout mice show severe atelectasis with unaltered surfactant protein expression. Glucocorticoid-deficient *CRH* knockout lungs are similar to the *T1α* knockout with thickened alveolar septae and increased PCNA-staining in epithelial and mesenchymal cells on E18.5. Apoptotic cell numbers and surfactant protein mRNA levels in late gestation are normal. Although similarities between the *CRH* and *T1α* knockout mice are striking, mutations show opposite effects on cell number (DNA/dry wet ratios). The *CRH* null animal is hypercellular, whereas the *T1α* mutant is slightly hypocellular. The morphology of the *CRH* knockout suggests that lungs may lack normal numbers of type I cells, raising the question of whether T1α expression is regulated by glucocorticoids. Low T1α expression could interfere with type I cell formation as in *T1α* null animals. Experiments are planned to test this possibility. Lungs of the T1α mutants also show significant similarities to lungs homozygous for the transcrip-

Fig. 7. Immunohistochemistry for selected marker proteins expressed in peripheral lung. Aqp-5 expression in (+/+) (A) and (−/−) (B) newborn lungs is patchy and is confined to flattened cells lining the alveolar spaces. Black arrows in (A) and (B) indicate flattened positive cells. Black arrowheads in (B) indicate cells that may have a cuboidal morphology; alternatively these may be the perinuclear region of attenuated type I cells. Field (B) clearly shows alveoli negative for Aqp-5 staining (red arrowheads) indicating the patchy pattern of Aqp-5 expression. Immunostaining for SP-B (C, D) and SP-C (E, F) shows similar patterns, locations, and numbers of positive cells in (+/+) and (−/−) newborn lungs. Likewise PECAM1 staining (G, H) shows no obvious difference between (+/+) and (−/−) lungs. Arrowheads indicate staining parallel to the alveolar surface that is characteristic of the newborn endothelium. a, alveolar spaces; b, bronchioles; v, blood vessels. Paraffin sections (6 μm) counterstained with methyl green. Bars, A–D = 10 μm, E–H = 15 μm.

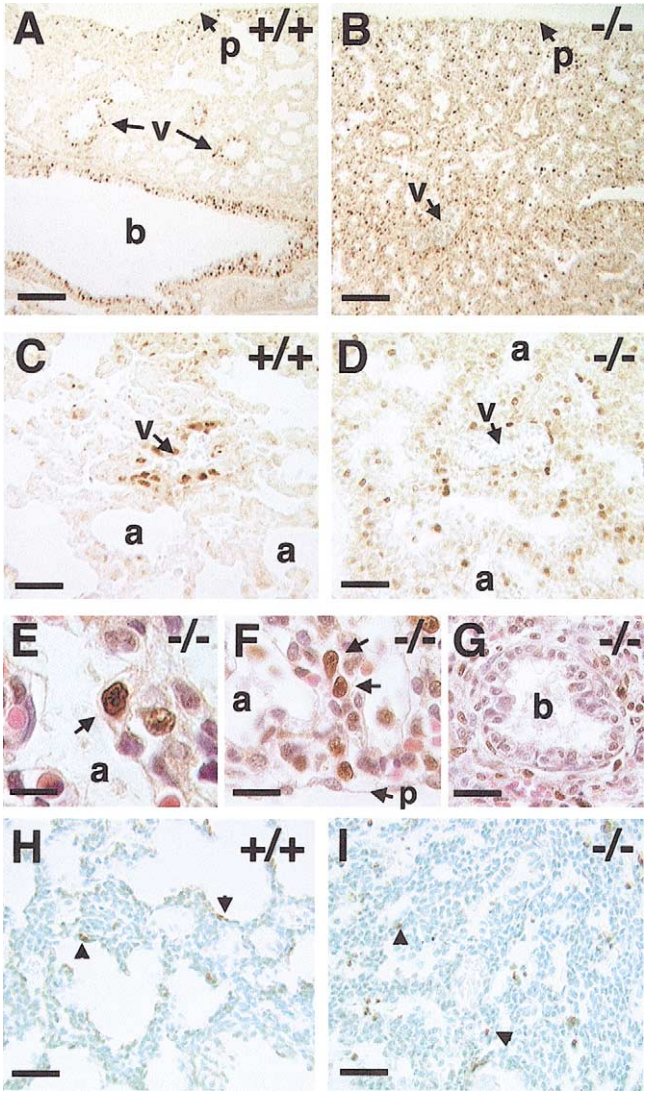


Fig. 8. Increased proliferation and normal apoptosis in T1α knockout lungs at birth. In (+/+) newborn lung PCNA staining is observed in upper airways, small bronchioles, and blood vessels excluding those in alveolar septae; distal lung cells are negative (A, C). In distal lungs of (−/−) newborns there is widespread, sustained proliferation of epithelial and mesenchymal cells shown by PCNA staining (B, D). At high magnification (E–G) PCNA staining of (−/−) lungs shows that both alveolar epithelial cells (E, arrow) and mesenchymal cells such as fibroblasts (F) express PCNA, while bronchiolar epithelial cells (G) do not. Tunel assays of newborn lung (H-I) indicate no differences in apoptotic cells between (+/+) and (−/−) newborns, a finding substantiated by nuclear counts. a, alveolar spaces; b, bronchioles; p, pleura; v, blood vessels. Paraffin sections (6 μm); (E–G) counterstained with H&E; (H, I) counterstained with methyl green. Bars, A–B = 100 μm, C–D and H–I = 30 μm, E = 10 μm, F = 15 μm, G = 25 μm.

tion factor NF-1B (nuclear factor 1B) (Grunder et al., 2002), raising the possibility that decreased NF-1B contributes to the *T1α* null phenotype. NF-1B null newborns die within minutes after birth, apparently of respiratory failure, presumably from reduced expression of surfactant protein B. This finding is consistent with studies showing that NF1-B increases surfactant protein expression (Bachurski et al., 1997). Preliminary microarray studies show that NF-1B2 is reduced (two-fold, $n = 5$) in the absence of T1α, although these data need to be validated. If loss of NF-1B contributes to the T1α phenotype, it likely does so by mechanisms other than interfering with surfactant protein expression.

We are currently using microarrays to obtain additional information on the genes whose expression is altered in lungs lacking T1α. We have analyzed five wild-type and five homozygous E18.5 day lungs. There are statistically significant changes in a number of genes that are implicated in peripheral lung cell biology that might contribute to the *T1α* null phenotype. Among the altered genes are OTS-8 (identical to mouse T1α) with decreased expression levels as anticipated, HFH-8 *[Foxf1]* (Costa et al., 2001) that is increased in *T1α*(−/−) lung, and prostaglandin synthase 1 (*Ptgs-1*, encoding cyclooxygenase 1 that produces PGE2) that is decreased. These changes have been confirmed by Northern blot (data not shown). OTS-8/T1α has the lowest ratio (T1α −/−)/(T1α +/+) in the microarray analysis and thus serves as an internal control. *HFH-8* haplo-insufficient animals have high perinatal lethality due to lung abnormalities (Kalinichenko et al., 2001; Lim et al., 2002; Mahlapuu et al., 2001), but the effects of increased HFH-8 are not known. Similarly we have confirmed by Northern blot a decrease in Ptgs1 mRNA in T1α(−/−). This is particularly interesting because PGE2 inhibits peripheral lung fibroblast proliferation (Pan et al., 2001) and may therefore account for the increase in fibroblast proliferation observed in the knockout lung. Ptgs1/Cox1 have been suggested to be type I rather than type II cell products (Lipchik et al., 1990), thus decreased Ptgs-1 may reflect failure of type I cell formation. Further studies on Ptgs1 gene, as well as other genes, will be helpful to determine whether the alterations observed in T1α(−/−) lungs are related to the impaired epithelial-mesenchymal communication resulting from abnormal differentiation of type I cells in the absence of T1α.

Of the genes known to be expressed by type I cells, *T1α* is the only one that seems to be essential for the timely development of alveoli and the air-blood barrier. We hypothesize that T1α could be directly involved in a pathway that induces cell-cycle exit and differentiation toward the type I cell phenotype of epithelial cells in late development. T1α could also be part of a type II–type I cell signaling pathway that inhibits proliferation in the distal lung once type I cells are differentiated. This cell–cell signaling could act via molecules present in the alveolar space, through intercellular junctions, or via mesenchymal–epithelial interactions. Whether T1α acts directly or is part of a more complex pathway is not known, nor are the molecules that may play important roles in the final proliferation vs differentiation equilibrium in the distal lung. *T1α* null mice are likely to be a helpful tool to understand the fate of the alveolar epithelial cells during lung organogenesis.

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References

Adamson, I.Y., Bowden, D.H., 1975. Derivation of type 1 epithelium from type 2 cells in the developing rat lung. Lab. Invest. 32, 736–745.

Bachurski, C.J., Kelly, S.E., Glasser, S.W., Currier, T.A., 1997. Nuclear factor I family members regulate the transcription of surfactant protein-C. J. Biol. Chem. 272, 32759–32766.

Bellusci, S., Grindley, J., Emoto, H., Itoh, N., Hogan, B.L., 1997. Fibroblast growth factor 10 (FGF10) and branching morphogenesis in the embryonic mouse lung. Development 124, 4867–4878.

Bhatt, A.J., Amin, S.B., Chess, P.R., Watkins, R.H., Maniscalco, W.M., 2000. Expression of vascular endothelial growth factor and Flk-1 in developing and glucocorticoid-treated mouse lung. Pediatr. Res. 47, 606–613.

Bouwman, P., Gollner, H., Elsasser, H.P., Eckhoff, G., Karis, A., Grosveld, F., Philipsen, S., Suske, G., 2000. Transcription factor Sp3 is essential for post-natal survival and late tooth development. EMBO J. 19, 655–661.

Breiteneder-Geleff, S., Matsui, K., Soleiman, A., Meraner, P., Poczewski, H., Kalt, R., Schaffner, G., Kerjaschki, D., 1997. Podoplanin, novel 43-kd membrane protein of glomerular epithelial cells, is down-regulated in puromycin nephrosis. Am. J. Pathol. 151, 1141–1152.

Breiteneder-Geleff, S., Soleiman, A., Horvat, R., Amann, G., Kowalski, H., Kerjaschki, D., 1999. [Podoplanin—A specific marker for lymphatic endothelium expressed in angiosarcoma]. Verh. Dtsch. Ges. Pathol. 83, 270–275.

Cao, Y.X., Jean, J.C., Williams, M.C., 2000. Cytosine methylation of an Sp1 site contributes to organ-specific and cell-specific regulation of expression of the lung epithelial gene t1alpha. Biochem. J. 350 (Pt. 3), 883–890.

Clark, J.C., Wert, S.E., Bachurski, C.J., Stahlman, M.T., Stripp, B.R., Weaver, T.E., Whitsett, J.A., 1995. Targeted disruption of the surfactant protein B gene disrupts surfactant homeostasis, causing respiratory failure in newborn mice. Proc. Natl. Acad. Sci. USA 92, 7794–7798.

Cole, T.J., Blendy, J.A., Monaghan, A.P., Krieglstein, K., Schmid, W., Aguzzi, A., Fantuzzi, G., Hummler, E., Unsicker, K., Schutz, G., 1995. Targeted disruption of the glucocorticoid receptor gene blocks adrenergic chromaffin cell development and severely retards lung maturation. Genes Dev. 9, 1608–1621.

Costa, R.H., Kalinichenko, V.V., Lim, L., 2001. Transcription factors in mouse lung development and function. Am. J. Physiol. Lung Cell. Mol. Physiol. 280, L823–L838.

Deng, C.X., Wynshaw-Boris, A., Shen, M.M., Daugherty, C., Ornitz, D.M., Leder, P., 1994. Murine FGFR-1 is required for early postimplantation growth and axial organization. Genes Dev. 8, 3045–3057.

Dobbs, L.G., Williams, M.C., Gonzalez, R., 1988. Monoclonal antibodies specific to apical surfaces of rat alveolar type I cells bind to surfaces of cultured, but not freshly isolated, type II cells. Biochim. Biophys. Acta 970, 146–156.

Drab, M., Verkade, P., Elger, M., Kasper, M., Lohn, M., Lauterbach, B., Menne, J., Lindschau, C., Mende, F., Luft, F.C., Schedl, A., Haller, H., Kurzchalia, T.V., 2001. Loss of caveolae, vascular dysfunction, and pulmonary defects in caveolin-1 gene-disrupted mice. Science 293, 2449–2452.

Farr, A.G., Berry, M.L., Kim, A., Nelson, A.J., Welch, M.P., Aruffo, A., 1992. Characterization and cloning of a novel glycoprotein expressed by stromal cells in T-dependent areas of peripheral lymphoid tissues. J. Exp. Med. 176, 1477–1482.

Funaki, H., Yamamoto, T., Koyama, Y., Kondo, D., Yaoita, E., Kawasaki, K., Kobayashi, H., Sawaguchi, S., Abe, H., Kihara, I., 1998. Localization and expression of AQP5 in cornea, serous salivary glands, and pulmonary epithelial cells. Am. J. Physiol. 275, C1151–C1157.

Gonzalez, R.F., Dobbs, L.G., 1998. Purification and analysis of RT140, a type I alveolar epithelial cell apical membrane protein. Biochim. Biophys. Acta 1429, 208–216.

Grunder, A., Ebel, T.T., Mallo, M., Schwarzkopf, G., Shimizu, T., Sippel, A.E., Schrewe, H., 2002. Nuclear factor I-B (Nfib) deficient mice have severe lung hypoplasia. Mech. Dev. 112, 69–77.

Joyce-Brady, M.F., Brody, J.S., 1990. Ontogeny of pulmonary alveolar epithelial markers of differentiation. Dev. Biol. 137, 331–348.

Kalinichenko, V.V., Lim, L., Stolz, D.B., Shin, B., Rausa, F.M., Clark, J., Whitsett, J.A., Watkins, S.C., Costa, R.H., 2001. Defects in pulmonary vasculature and perinatal lung hemorrhage in mice heterozygous null for the Forkhead Box f1 transcription factor. Dev. Biol. 235, 489–506.

Kasper, M., Reimann, T., Hempel, U., Wenzel, K.W., Bierhaus, A., Schuh, D., Dimmer, V., Haroske, G., Muller, M., 1998. Loss of caveolin expression in type I pneumocytes as an indicator of subcellular alterations during lung fibrogenesis. Histochem. Cell Biol. 109, 41–48.

Kauffman, S.L., 1975. Kinetics of pulmonary epithelial proliferation during prenatal growth of the mouse lung. Anat. Rec. 183, 393–403.

Kauffman, S.L., Burri, P.H., Weibel, E.R., 1974. The postnatal growth of the rat lung. II. Autoradiography. Anat. Rec. 180, 63–76.

Korfhagen, T.R., Bruno, M.D., Ross, G.F., Huelsman, K.M., Ikegami, M., Jobe, A.H., Wert, S.E., Stripp, B.R., Morris, R.E., Glasser, S.W., Bachurski, C.J., Iwamoto, H.S., Whitsett, J.A., 1996. Altered surfactant function and structure in SP-A gene targeted mice. Proc. Natl. Acad. Sci. USA 93, 9594–9599.

Koslowski, R., Dobbs, L.G., Wenzel, K.W., Schuh, D., Muller, M., Kasper, M., 1998. Loss of immunoreactivity for RT140, a type I cell-specific protein in the alveolar epithelium of rat lungs with bleomycin-induced fibrosis. Eur. Respir. J. 12, 1397–1403.

Kotton, D.N., Ma, B.Y., Cardoso, W.V., Sanderson, E.A., Summer, R.S., Williams, M.C., Fine, A., 2001. Bone marrow-derived cells as progenitors of lung alveolar epithelium. Development 128, 5181–5188.

Lim, L., Kalinichenko, V.V., Whitsett, J.A., Costa, R.H., 2002. Fusion of lung lobes and vessels in mouse embryos heterozygous for the forkhead box f1 targeted allele. Am. J. Physiol. Lung Cell. Mol. Physiol. 282, L1012–L1022.

Lipchik, R.J., Chauncey, J.B., Paine, R., Simon, R.H., Peters-Golden, M., 1990. Arachidonate metabolism increases as rat alveolar type II cells differentiate in vitro. Am. J. Physiol. 259, L73–L80.

Ma, T., Fukuda, N., Song, Y., Matthay, M.A., Verkman, A.S., 2000. Lung fluid transport in aquaporin-5 knockout mice. J. Clin. Invest. 105, 93–100.

Ma, T., Yang, B., Matthay, M.A., Verkman, A.S., 1998. Evidence against a role of mouse, rat, and two cloned human t1alpha isoforms as a water channel or a regulator of aquaporin-type water channels. Am. J. Respir. Cell Mol. Biol. 19, 143–149.

Mahlapuu, M., Enerback, S., Carlsson, P., 2001. Haploinsufficiency of the forkhead gene Foxf1, a target for sonic hedgehog signaling, causes lung and foregut malformations. Development 128, 2397–2406.

McElroy, M.C., Pittet, J.F., Allen, L., Wiener-Kronish, J.P., Dobbs, L.G., 1997. Biochemical detection of type I cell damage after nitrogen

dioxide-induced lung injury in rats. Am. J. Physiol. 273, L1228–L1234.

McElroy, M.C., Pittet, J.F., Hashimoto, S., Allen, L., Wiener-Kronish, J.P., Dobbs, L.G., 1995. A type I cell-specific protein is a biochemical marker of epithelial injury in a rat model of pneumonia. Am. J. Physiol. 268, L181–L186.

Meneghetti, A., Cardoso, W.V., Brody, J.S., Williams, M.C., 1996. Epithelial marker genes are expressed in cultured embryonic rat lung and in vivo with similar spatial and temporal patterns. J. Histochem. Cytochem. 44, 1173–1182.

Muglia, L.J., Bae, D.S., Brown, T.T., Vogt, S.K., Alvarez, J.G., Sunday, M.E., Majzoub, J.A., 1999. Proliferation and differentiation defects during lung development in corticotropin-releasing hormone-deficient mice. Am. J. Respir. Cell Mol. Biol. 20, 181–188.

Nagae, A., Abe, M., Becker, R.P., Deddish, P.A., Skidgel, R.A., Erdos, E.G., 1993. High concentration of carboxypeptidase M in lungs: presence of the enzyme in alveolar type I cells. Am. J. Respir. Cell Mol. Biol. 9, 221–229.

Nose, K., Saito, H., Kuroki, T., 1990. Isolation of a gene sequence induced later by tumor-promoting 12-*O*-tetradecanoylphorbol-13-acetate in mouse osteoblastic cells (MC3T3-E1) and expressed constitutively in ras-transformed cells. Cell Growth Differ. 1, 511–518.

O'Hare, K.H., Townes, P.L., 1970. Morphogenesis of albino rat lung: an autoradiographic analysis of the embryological origin of the type I and II pulmonary epithelial cells. J. Morphol. 132, 69–87.

Pan, T., Mason, R.J., Westcott, J.Y., Shannon, J.M., 2001. Rat alveolar type II cells inhibit lung fibroblast proliferation in vitro. Am. J. Respir. Cell Mol. Biol. 25, 353–361.

Ramirez, M.I., Cao, Y.X., Williams, M.C., 1999. 1.3 kilobases of the lung type I cell T1alpha gene promoter mimics endogenous gene expression patterns during development but lacks sequences to enhance expression in perinatal and adult lung. Dev. Dyn. 215, 319–331.

Ramirez, M.I., Chung, U.I., Williams, M.C., 2000. Aquaporin-5 expression, but not other peripheral lung marker genes, is reduced in PTH/PTHrP receptor null mutant fetal mice. Am. J. Respir. Cell Mol. Biol. 22, 367–372.

Ramirez, M.I., Pollack, L., Millien, G., Cao, Y.X., Hinds, A., Williams, M.C., 2002. The alpha-Isoform of caveolin-1 is a marker of vasculogenesis in early lung development. J. Histochem. Cytochem. 50, 33–42.

Ramirez, M.I., Rishi, A.K., Cao, Y.X., Williams, M.C., 1997. TGT3, thyroid transcription factor I, and Sp1 elements regulate transcriptional activity of the 1.3-kilobase pair promoter of T1alpha, a lung alveolar type I cell gene. J. Biol. Chem. 272, 26285–26294.

Razani, B., Engelman, J.A., Wang, X.B., Schubert, W., Zhang, X.L., Marks, C.B., Macaluso, F., Russell, R.G., Li, M., Pestell, R.G., Di Vizio, D., Hou Jr., H., Kneitz, B., Lagaud, G., Christ, G.J., Edelmann, W., Lisanti, M.P., 2001. Caveolin-1 null mice are viable but show evidence of hyperproliferative and vascular abnormalities. J. Biol. Chem. 276, 38121–38128.

Rishi, A.K., Joyce-Brady, M., Fisher, J., Dobbs, L.G., Floros, J., VanderSpek, J., Brody, J.S., Williams, M.C., 1995. Cloning, characterization, and development expression of a rat lung alveolar type I cell gene in embryonic endodermal and neural derivatives. Dev. Biol. 167, 294–306.

Scholl, F.G., Gamallo, C., Vilar, S., Quintanilla, M., 1999. Identification of PA2.26 antigen as a novel cell-surface mucin-type glycoprotein that induces plasma membrane extensions and increased motility in keratinocytes. J. Cell Sci. 112, 4601–4613.

Song, Y., Fukuda, N., Bai, C., Ma, T., Matthay, M.A., Verkman, A.S., 2000. Role of aquaporins in alveolar fluid clearance in neonatal and adult lung, and in oedema formation following acute lung injury: studies in transgenic aquaporin null mice. J. Physiol. 525 (Pt. 3), 771–779.

Taccioli, G.E., Amatucci, A.G., Beamish, H.J., Gell, D., Xiang, X.H., Torres Arzayus, M.I., Priestley, A., Jackson, S.P., Marshak Rothstein, A., Jeggo, P.A., Herrera, V.L., 1998. Targeted disruption of the catalytic subunit of the DNA-PK gene in mice confers severe combined immunodeficiency and radiosensitivity. Immunity 9, 355–366.

Vanderbilt, J.N., Dobbs, L.G., 1998. Characterization of the gene and promoter for RT140, a differentiation marker of type I alveolar epithelial cells. Am. J. Respir. Cell Mol. Biol. 19, 662–671.

Wetterwald, A., Hoffstetter, W., Cecchini, M.G., Lanske, B., Wagner, C., Fleisch, H., Atkinson, M., 1996. Characterization and cloning of the E11 antigen, a marker expressed by rat osteoblasts and osteocytes. Bone 18, 125–132.

Williams, M.C., Cao, Y., Hinds, A., Rishi, A.K., Wetterwald, A., 1996. T1 alpha protein is developmentally regulated and expressed by alveolar type I cells, choroid plexus, and ciliary epithelia of adult rats. Am. J. Respir. Cell Mol. Biol. 14, 577–585.

Williams, M.C., Dobbs, L.G., 1990. Expression of cell-specific markers for alveolar epithelium in fetal rat lung. Am. J. Respir. Cell Mol. Biol. 2, 533–542.

Xu, H., Gonzalo, J.A., St Pierre, Y., Williams, I.R., Kupper, T.S., Cotran, R.S., Springer, T.A., Gutierrez-Ramos, J.C., 1994. Leukocytosis and resistance to septic shock in intercellular adhesion molecule 1-deficient mice. J. Exp. Med. 180, 95–109.

Xu, X., Toselli, P.A., Russell, L.D., Seldin, D.C., 1999. Globozoospermia in mice lacking the casein kinase II alpha' catalytic subunit. Nat. Genet. 23, 118–121.

Yano, T., Mason, R.J., Pan, T., Deterding, R.R., Nielsen, L.D., Shannon, J.M., 2000. KGF regulates pulmonary epithelial proliferation and surfactant protein gene expression in adult rat lung. Am. J. Physiol. Lung. Cell Mol. Physiol. 279, L1146–L1158.

Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J.W., Elledge, S.J., 1999. p21(CIP1) and p57(KIP2) control muscle differentiation at the myogenin step. Genes Dev. 13, 213–224.

Zimmer, G., Lottspeich, F., Maisner, A., Klenk, H.D., Herrler, G., 1997. Molecular characterization of gp40, a mucin-type glycoprotein from the apical plasma membrane of Madin-Darby canine kidney cells (type I). Biochem. J. 326, 99–108.

Zimmer, G., Oeffner, F., Von Messling, V., Tschernig, T., Groness, H.J., Klenk, H.D., Herrler, G., 1999. Cloning and characterization of gp36, a human mucin-type glycoprotein preferentially expressed in vascular endothelium. Biochem. J. 341, 277–284.