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Lung Kruppel-like Factor, a Zinc Finger Transcription Factor, Is Essential for Normal Lung Development*

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Lung Kruppel-like factor (LKLF) is a member of the Kruppel-like factor family of transcription factors and is highly expressed in lung with limited distribution in other tissues. Mice lacking LKLF due to inactivation of *LKLF* by gene targeting die *in utero* at midgestation around day 12.5 due to severe hemorrhage, making it difficult to study the role of this transcription factor in lung development and function. However, *in vitro* organ culture of lung buds removed from 11.5-day-old LKLF$^{-/-}$ embryos show normal tracheobronchial tree formation. To examine later stages of lung development, the embryonic lethality due to germ line *LKLF* null mutation was circumvented by constructing *LKLF* homozygous null mouse embryonic stem cells, using a two-step gene targeting procedure, and determining whether these cells give rise to lung tissue. The targeted cells were used to produce chimeric animals, and the contribution of LKLF-deficient cells to the formation of various internal organs was analyzed. In chimeric mice that survived after birth, null embryonic stem cells contributed significantly to all of the major organs except the lungs. On the other hand, some highly chimeric animals died at birth, and histopathological examination of their lungs suggested abnormalities in their lung development. These studies show that LKLF plays an important role in normal lung development.

During mouse embryogenesis, normal lung development is initiated at 9.5 days post coitus (pc)[1] in the form of two endodermally derived epithelial buds that arise from the primitive foregut (1, 2). As the epithelial buds invade the surrounding mesenchyme, they undergo a defined pattern of branching morphogenesis and differentiate into conducting and respiratory airways of the adult lung, whereas the surrounding mesenchyme forms the lung stroma. Although cells in these compartments appear to be undifferentiated, they express lung-specific genes at this early time. Initial patterning of the lung occurs during the pseudoglandular stage of lung development (9.5–16 days pc) due to the rapid growth and branching of the primitive lung epithelium to form the tracheobronchial tree and terminal acinar buds. These primitive acini dilate during the canalicular (16–17 days pc) and saccular stages (17 days pc to birth) of lung development to form the respiratory epithelium. Subsequent maturation of the lung parenchyma continues after birth during the process of alveolization (birth to 14 days postnatal) (2).

Transcription factors, in conjunction with growth factors, play important roles in development by regulating cell growth and differentiation. Establishment of cell type-specific patterns of gene expression resulting from the combinatorial action of transcription factors (3) is critical during normal embryogenesis. During lung morphogenesis, a number of transcription factors (4–7), as well as regulatory molecules such as growth factors (8–11) and growth factor receptors (12–15), exert a positive or a negative effect on gene activation and are implicated in branching morphogenesis and cellular differentiation. Genes that are normally expressed during lung development are ideal candidates for regulation of its development. LKLF is one transcription factor that is highly expressed in lung with limited distribution in other organs (16). Expression of LKLF during lung development suggests that it has a role in the development of this vital organ. We earlier developed mice with targeted disruption of the *LKLF* gene with the goal of discerning its role in lung development and function (17). Interestingly, the mice homozygous for *LKLF* deletion die *in utero* around 12.5 days of gestation due to severe hemorrhage (17, 18). This makes it difficult to study the effects of a null mutation on normal lung development beyond the stage of embryonic lethality. One of the strategies to overcome embryonic lethality due to null mutations includes tracking the contribution of embryonic stem (ES) cells in chimeric animals (19–23). *LKLF*$^{-/-}$ ES cells were therefore generated, and their contribution to different tissues in chimeric mice was determined. In chimeric mice that survived birth, mutant ES cell contribution was found in all the major organs examined, except for the lung. Histological examination of the chimeric mice that died at birth revealed abnormalities in their lung development. These result suggest that LKLF expression is important for normal lung development.

EXPERIMENTAL PROCEDURES

Creation of LKLF$^{-/-}$ ES Cells—Embryonic stem cells with one LKLF gene knocked out (17) were electroporated with an *LKLF* targeting vector containing a neomycin resistance gene in place of the HPRT minigene used during initial targeting as described earlier (17). The neomycin gene was under the control of *PGK* promoter and contained polyadenylation sequences. The ES cells with one *LKLF* gene knocked out were electroporated in the second round of targeting under similar conditions as described (17). Following electroporation, the cells were plated on neomycin-resistant feeder cells and were selected in hypoxanthine/aminopterin/thymidine medium containing 300 μg/ml G418 and 2 μM gancyclovir. The resistant colonies were screened for homologous recombination by PCR. The double targeted cells were expanded

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[1] The abbreviations used are: pc, post coitus; PCR, polymerase chain reaction; LKLF, Lung Kruppel-like factor; ES, embryonic stem; GPI, glucose phosphate isomerase; TTF, thyroid transcription factor; TGF, transforming growth factor.

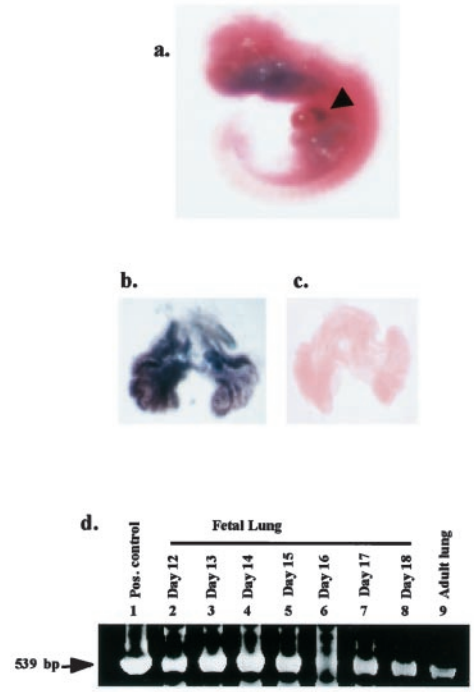

FIG. 1. **LKLF expression.** Whole mount *in situ* hybridization of an 11.5-day-old mouse embryo (*a*) (*arrowhead* shows hybridization in lung bud), lung bud dissected from a 12.5-day-old embryo after hybridization (*b*), and a negative control (*c*). *d,* reverse transcription-PCR using RNA isolated from mouse lung during different days of gestation.

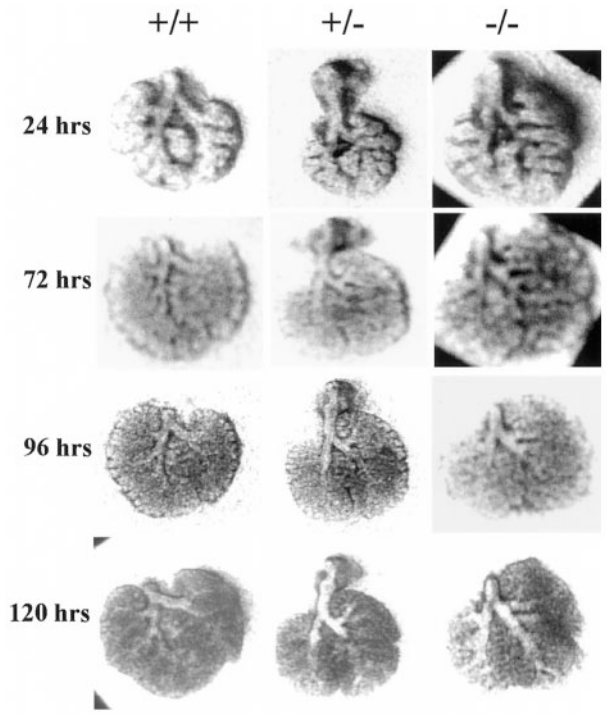

FIG. 2. **Branching morphogenesis of mouse embryonic lung buds in culture.** Lung buds were dissected from 11.5-day-old embryos after timed matings of $LKLF^{+/-}$ animals and were cultured in chemically defined medium for 5 days.

for DNA extraction for Southern analysis and subsequent blastocyst injection. DNA extraction and Southern analysis were performed as described (17) using ^{32}P-labeled *LKLF* probes as shown in Fig. 3.

Northern Blotting—The double targeted ES cells were grown to confluency, trypsinized, and replated for 2 h, during which time, only feeders attached to the plates. The medium containing the ES cells was collected and pelleted. Total RNA was extracted from ES cells by RNAzol (24) using the manufacturer's instructions. Twenty μg of total RNA were used for Northern analysis, transferred to nylon membrane, and probed with ^{32}P-labeled *LKLF* cDNA.

Glucose Phosphate Isomerase (GPI) Assay—The glucose phosphate isoenzymes were separated and detected as described (25). Briefly, Titan III Zip Zone cellulose acetate plates (Helena Laboratories) were soaked in Tris-glycine buffer (25 mM Tris, 200 mM glycine, pH 8.5) for 20 min before application of the samples. The samples were prepared by homogenizing tissues in 50 mM Tris-HCl, pH 8.5. The homogenized tissues were lysed by three rounds of freezing and thawing and centrifuged. The supernatants were electrophoresed in a Zip Zone chamber (Helena Laboratories) for 1.5 h at 150 V and 4 °C. Following electrophoresis, the membranes were stained with 10 ml of 1% agarose solution that contained 80 mM Tris (pH 8.0), 5 mM magnesium acetate, 15 mg of fructose 6-phosphate, 2 mg of methylthiazolium tetrazolium, 0.36 mg of phenazine methosulfate, 2 mg of nicotinamide adenine dinucleotide phosphate, and 10 units of glucose-6-phosphate dehydrogenase.

Histology and Immunohistochemistry—Tissues from neonates were surgically removed, fixed in 4% paraformaldehyde in phosphate-buffered saline, dehydrated in graded alcohol solutions, embedded in paraffin, and sectioned at 5 μm. The sections were stained with hematoxylin and eosin for histological analysis. Immunohistochemistry for PECAM (CD31), surfactant proprotein C, surfactant protein B, and thyroid transcription factor-1 (TTF-1), a marker for the developing pulmonary epithelium, was performed as described previously using a biotinylated secondary antibody and an avidin-biotin-peroxidase detection system (26, 27). Rabbit polyclonal antibody to rat TTF-1 was provided by Dr. Roberto Di Laura (Stazionl Zoological "Anton Dohrn," Naples, Italy).

Lung Bud Organ Culture—$LKLF^{+/-}$ mice were mated, and the morning of the appearance of the vaginal plug was counted as 0.5 days pc. Lung buds were isolated from 11.5 days pc embryos under a dissecting microscope and cultured on 8-μm pore size nucleopore membranes (Thomas Scientific) in a chemically defined medium. After the lung bud was isolated from an embryo, its remaining tissue was used for DNA extraction to genotype the embryo for *LKLF*. The membranes with lung buds were placed in tissue culture dishes containing 1:1 mixture of Ham's F-12 and Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 50 μg/ml gentamicin. Lung buds were grown at 37 °C in a humidified, 5% CO_2 incubator. Lung buds grown on membranes were photographed every 24 h for documentation using a dissecting microscope under bright field illumination.

Whole Mount in Situ Hybridization—The embryos were dissected from the pregnant mother after timed matings and used for whole mount *in situ* hybridization as described (28). The RNA probe was synthesized from *LKLF* cDNA labeled by *in vitro* transcription using fluoresceinated UTP. The detection of hybridization was done using antifluorescein antibody conjugated to alkaline phosphatase and subsequent staining with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Reverse Transcription-PCR Analysis—RNA was isolated from embryonic lungs using RNAzol (24) and used as a template for reverse transcription primed by oligo(dT). The synthesized cDNA was used for PCR amplification using *LKLF* primers 5′-CAAGGGGCTGGGTGTGGAAT-3′ and 5′-GGGAGGGAGGGAGGGAAAGA-3′. These primers amplify a 539-base pair *LKLF*-cDNA fragment.

RESULTS

Expression Analysis—In previous studies, we showed that *LKLF* is expressed at high levels in the adult lung and during embryogenesis (16). To show that *LKLF* is expressed during lung development, we used whole mount *in situ* hybridization of mouse embryos at different stages of gestation. Expression of *LKLF* in the lung bud was observed as early as day 11.5 of mouse development (Fig. 1*a*). Reverse transcription-PCR using RNA from lungs at various stages of development showed that *LKLF* is expressed throughout lung development and in the adult lung (Fig. 1*d*).

The expression pattern in developing, as well as adult, lungs suggests a critical role for LKLF in lung development and function. Interestingly, mice homozygous for *LKLF* deletion die around midgestation, when lung development has just started (17, 18). However, in LKLF mutant animals, the lung bud forms normally until the animals die. These lungs show primitive respiratory tubules with dichotomous branching (Fig. 2).

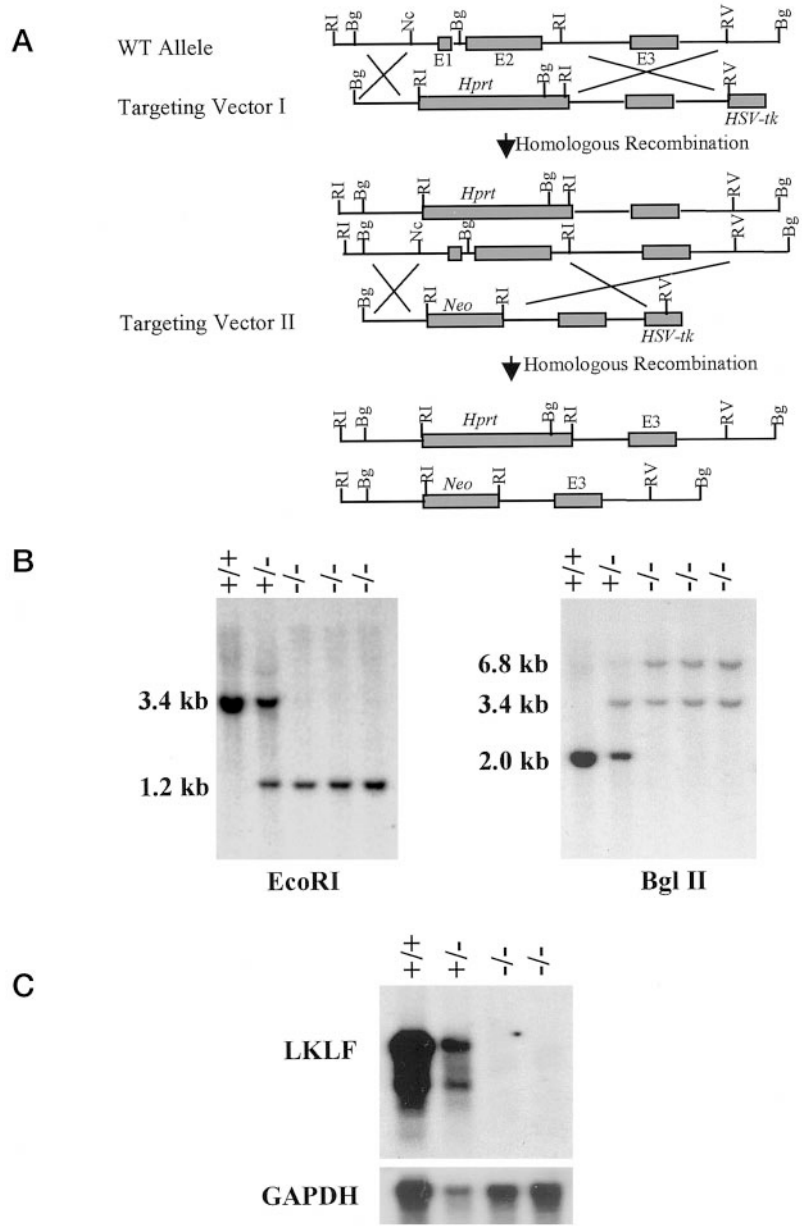

FIG. 3. **Isolation and characterization of *LKLF*$^{-/-}$ ES cells.** *A,* gene targeting strategy to create *LKLF*$^{-/-}$ ES cells. One allele of the *LKLF* gene was mutated in wild type (+/+) E14TG2a ES cells in the first round of targeting (17) using a vector carrying the *Hprt* minigene as a selectable marker and the homologous sequences from the *LKLF* gene. The remaining wild type allele in the heterozygous (+/−) ES cells was then subjected to a second round of gene targeting with a vector identical to the first one, in which the *Hprt* gene was replaced by a neomycin selectable marker, giving rise to LKLF-deficient (−/−) ES cells. Both targeting vectors contain herpes simplex virus thymidine kinase gene (*HSV-TK*) to provide negative selection with gancyclovir. *RI, Eco*RI; *Bg, Bgl*II; *RV, Eco*RV; *Nc, Nco*I. *B,* Southern blots of LKLF-deficient ES cells. The genomic DNA from wild type, heterozygous and several double gene-targeted ES cell clones was digested with either *Eco*RI or *Bgl*II and hybridized to a 0.875-kilobase *Bgl*II-*Nco*I DNA fragment located upstream of first exon. The sizes of the wild type and targeted alleles are indicated in kilobases (*kb*). *C,* Northern blot of LKLF-deficient ES cells. Twenty μg of total RNA isolated from wild type, heterozygous, and homozygous ES cells was blotted and probed with either *LKLF* cDNA or GAPDH.

Lung development beyond this point, however, cannot be studied, as the embryos do not survive. In order to follow lung development beyond the time of embryonic lethality, lung buds from mutant embryos were removed at 11.5 days of gestation and were grown in organ culture. Lung cultures were performed in defined medium, and their growth was followed for 4–5 days. Lung buds from wild type, heterozygous, and homozygous mutant embryos showed comparable branching morphogenesis (Fig. 2), suggesting that LKLF expression is not required for development of tracheobronchial tree. Histological analysis of the lung buds grown in culture also showed that the bronchial epithelial growth progressed normally in the mutant lungs buds (not shown), suggesting that LKLF expression may not be required during the pseudoglandular period of lung development.

Construction of LKLF$^{-/-}$ *ES Cells and Generation of Chimeric Animals*—To overcome embryonic lethality and, therefore, study the role of LKLF in lung development, chimeric animals were generated from *LKLF*$^{-/-}$ ES cells. ES cells bearing disruptions in both *LKLF* alleles were examined for their contribution to the lung in chimeric animals. *LKLF* homozygous mutant ES cells were generated using a two-step targeting procedure (29). The heterozygous E14TG2a, an Hprt-deficient ES cell line derived from 129/sv mouse strain, was constructed using *LKLF* targeting vector with *Hprt* minigene as a selectable marker (17). A single heterozygous ES cell clone was then used for a second round of targeting with another targeting vector to delete the remaining wild type allele. The procedure was identical to the first targeting step except that the *Hprt* selectable marker was replaced with a neomycin resistance gene (Fig. 3*A*). Following selection in hypoxanthine/aminopterin/thymidine medium containing G418 and gancyclovir, *LKLF*$^{-/-}$ ES cell clones were identified by PCR (not shown) for correct targeting of the second allele, which were subsequently confirmed by Southern blotting using *LKLF*-specific probes (Fig. 3*B*). The correctly targeted cells lack a band that corresponds to the wild type allele, and these cells instead contain an additional band showing homologous recombination between the wild type allele and the LKLF-neomycin targeting vector (Fig. 3*B*). Using this strategy, we were able to obtain several *LKLF*$^{-/-}$ ES cell clones. The two clones that were used for blastocyst injection were analyzed by Northern blot analy-

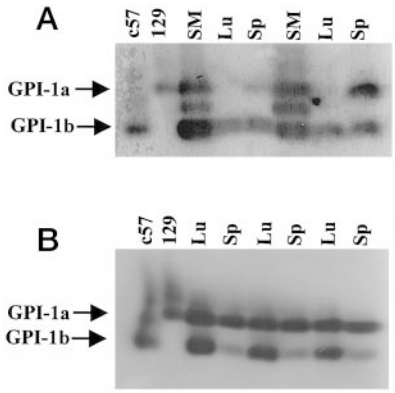

FIG. 4. **GPI analysis of tissues from chimeric animals derived from *LKLF*$^{-/-}$ (*A*) or *LKLF*$^{+/+}$ ES cells (*B*).** Lysates from several tissues were separated by cellulose acetate electrophoresis and stained as described under "Experimental Procedures." Tissues shown are skeletal muscle (*SM*), lung (*Lu*), spleen (*Sp*). ES cells (derived from strain 129/Sv) express GPI-1A, and the host C57Bl/6 cells express GPI-1B. The migration of the two isoforms is shown in control lanes as indicated.

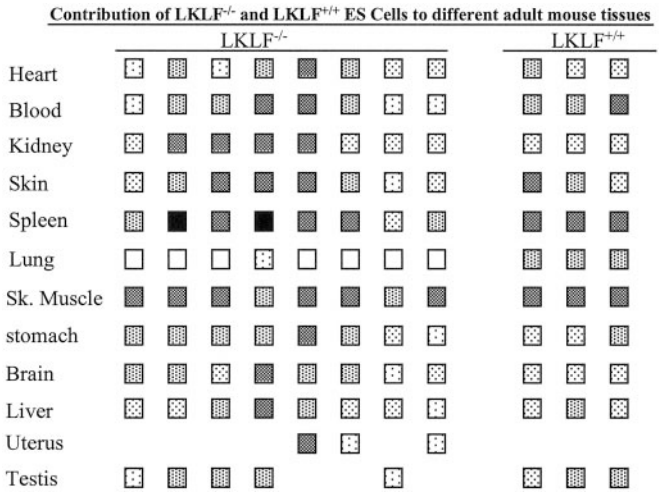

FIG. 5. **Contribution of *LKLF*$^{-/-}$ and *LKLF*$^{+/+}$ ES cells to various internal organs.** The extent of *shading* in the boxes indicates the extent of chimerism, where *open boxes* indicate no chimerism, and the *shaded boxes* indicate relative levels of chimerism.

sis to confirm the lack of LKLF expression. Whereas LKLF expression was seen both in wild type and heterozygous cells, it was not detected in either of the *LKLF*$^{-/-}$ ES cell clones (Fig. 3*C*).

Analysis of Chimeric Animals—The embryonic stem cells derived from mouse strain 129/sv were injected into blastocysts collected from C57Bl/6 mice. The contribution of LKLF-deficient ES cells in the chimeric animals can be estimated by the extent of agouti coat color. Several highly chimeric animals were obtained from the LKLF-deficient cells, and in these, the extent of contribution by LKLF-deficient ES cells was determined in various internal organs. The LKLF-deficient cells are homozygous for the *a* allele of glucose phosphate isomerase (*GPI-1s*^{a}), which encodes GPI-1A, whereas the cells from C57Bl/6 host embryo are homozygous for *GPI-1s*^{b}, encoding GPI-1B (30). Using cellulose acetate electrophoresis, the two isoforms were separated, and the specific enzyme activity was estimated after staining (25) (Fig. 4). The amount of these activities was used to determine the extent of the contribution by the two different cell types to the development of various tissues. Whereas LKLF-deficient ES cells were able to contribute significantly to various internal organs examined, including skeletal muscle, spleen, heart, liver, kidney, stomach,

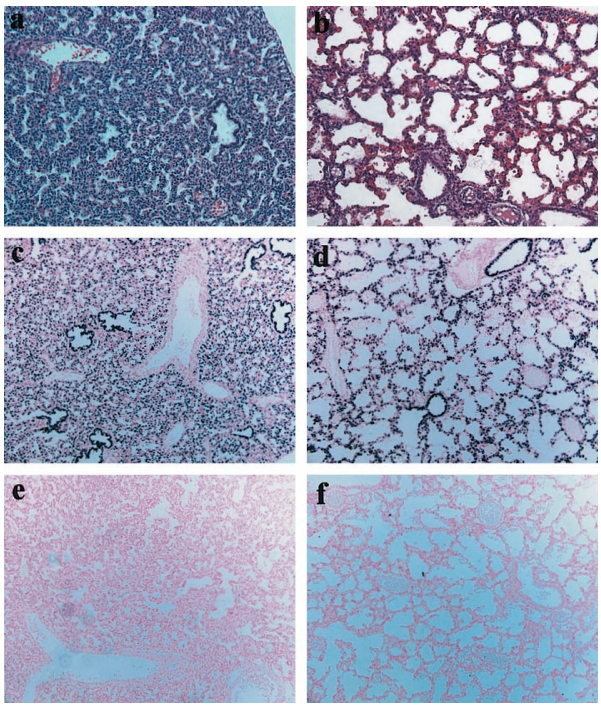

FIG. 6. **Histopathology of neonatal lungs.** Shown are hematoxylin and eosin-stained sections from lung of chimeric animal that died at birth (*a*) and wild type lung (*b*) and immunostaining for TTF-1 on chimeric lung (*c*) and wild type lung (*d*). Immunostaining in the absence of primary antibody (*e* and *f*) was used as a negative control.

brain, uterus, testis, skin, and brain, they were not able to contribute to the formation of adult lung in the chimeric mice that survived (Fig. 5). Histopathological examination of mice with high chimerism, as determined by Southern analysis (not shown), that died at birth revealed abnormalities in lung development and/or function. The lungs in these animals were not expanded and appeared to be arrested in the late canalicular stage of lung development with undilated acinar tubules and buds in peripheral regions of the lung (Fig. 6*a*). In contrast, wild type controls were in the saccular stage of lung development at birth, with well expanded saccular alveoli (Fig. 6*b*). This is consistent with the observation that in *LKLF*$^{-/-}$ embryos, lung development up to pseudoglandular stage is comparable with their wild type littermates when their lung buds are grown in culture. The effect observed on later stages of lung development *in vivo*, however, could not be detected in organ culture. Immunohistochemistry for surfactant proprotein C, surfactant protein B, and TTF-1, as epithelial cell markers, demonstrated normal type II epithelial cell differentiation (Fig. 6, *c* and *d,* and Fig. 7, *a–d*). Furthermore, immunohistochemistry for PECAM, an endothelial cell marker, demonstrated normal vascularization of the tissue (Fig. 7, *e* and *f*). The lack of contribution of LKLF-deficient cells to the adult lung of live chimeras in conjunction with delayed lung development in the highly chimeric mice that died at birth suggests that LKLF is an important transcription factor required for later stages of lung development.

DISCUSSION

LKLF belongs to a multigene family known as the Kruppel-like family of transcription factors (16), which include LKLF (16), EKLF (31), GKLF (32, 33), BKLF (34), UKLF (35), and IKLF (36). Members of this family encode zinc finger proteins with specific biological roles, through positive (37, 38) or negative regulation (39) of their target genes, in the tissues in which they are expressed. LKLF is highly expressed in lung,

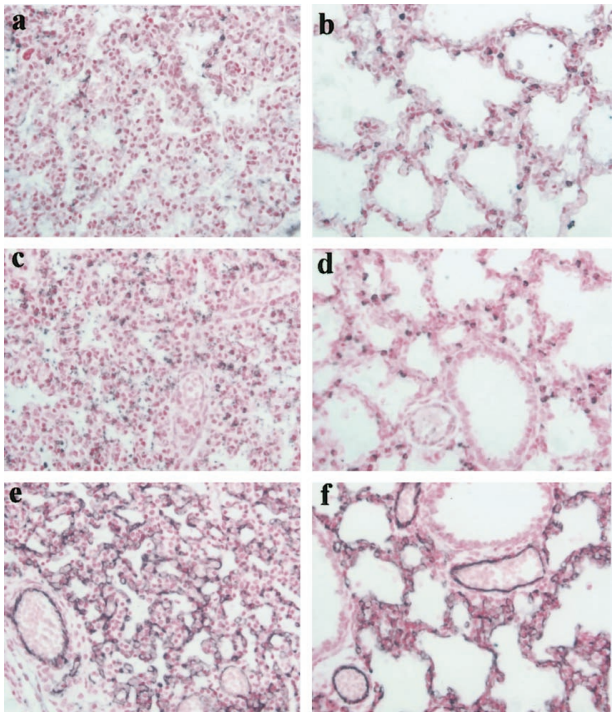

FIG. 7. **Immunohistochemical staining for surfactant protein B (*a* and *b*), surfactant proprotein C (*c* and *d*), and PECAM-1 (*e* and *f*) in chimeric (*a*, *c*, and *e*) and wild type (*b*, *d*, and *f*) neonatal lungs.**

with low level expression observed in other tissues (16). Interestingly, ES cells also express LKLF at very high levels, but these cells apparently maintain their growth and differentiation properties in the absence of LKLF expression. Furthermore, mice lacking LKLF develop normally up to 11.5 days of gestation, implying that LKLF expression is dispensable for ES cell growth, as well as during early stages of organogenesis. In order to determine the biological functions of LKLF, the gene was altered in mouse embryonic stem cells to generate mice with homozygous deletion of the *LKLF* gene. These mice die during development at midgestation due to severe hemorrhage (17, 18) associated with defects in blood vessel formation (18). In $LKLF^{-/-}$ embryos, lung buds are formed, and furthermore, when the lung buds from wild type and mutant embryos are grown in culture, they show comparable branching morphogenesis (Fig. 2). *In vitro* organ culture of lung buds has been useful in understanding the role of certain growth factors (9) and oncoproteins (40) in lung development. However, lung explants grown in culture do not undergo sacculation or vascular maturation and generally do not undergo differentiation beyond the pseudoglandular stage of lung development, limiting their use in studying LKLF effects on later stages of lung development. Because of the attendant embryonic lethality in $LKLF^{-/-}$ animals, it is extremely difficult to study *in vivo* effects of *LKLF* mutation on lung development beyond midgestation in these animals. The present work describes the creation of chimeric animals with LKLF-deficient ES cells made by two steps of gene targeting. Due to the contribution of the wild type cells from host embryo, some of the animals escaped midgestational lethality caused by germ line *LKLF* homozygosity and developed to birth. The strategy of tracking LKLF-deficient ES cells, with both alleles of *LKLF* inactivated, in live chimeric animals revealed that these cells did not contribute to lung formation in these animals. The chimeric animals that died at birth exhibited abnormal lung development. The lungs in these animals were unexpanded and appeared to be arrested at the canalicular stage of lung development, indicating that LKLF expression is important during later stages of lung development and maturation. This is expected because $LKLF^{-/-}$ embryos, generated from heterozygous crosses, survived up to 11.5–12.5 days of gestation and showed normal onset of lung development that progresses through the pseudoglandular stage in culture.

Lung development begins at 9.5 days of gestation as an endodermally derived epithelial bud originating from the pharyngeal region of the gut, which extends into the mesenchyme (1, 2). The epithelial buds undergo extensive proliferation and branching in a controlled pattern to form the bronchial tree and the respiratory surface of the lung, whereas the mesenchyme makes up the lung stroma composed of connective tissue, smooth muscle, and vasculature of the lung. The signaling molecules involved in the control of vasculogenesis and angiogenesis in lung vascular development are not fully known. Growth factors, as well as growth factor receptors, which are known to have a role in vasculogenesis and angiogenesis, are normally expressed in $LKLF^{-/-}$ animals (18). Normal vasculogenesis in $LKLF^{-/-}$ chimeric animals, as determined by the expression of PECAM-1, an endothelial cell marker, excludes the basis for abnormal lung phenotype due to lack of LKLF expression. Grafting experiments have demonstrated that inductive signals relayed through cell-cell interactions between the epithelial and mesenchymal compartment are essential in the proper patterning, differentiation, and development of the lung (6). The regulatory molecules that control branching, growth, differentiation, and maturation of the lung include transcription factors and growth factors, as well as circulating hormones. Experiments using transgenic and gene targeting strategies have suggested important biological roles for various transcription and growth factors during lung development. For example, targeted disruption of the TTF-1 gene results in a complete absence of branching morphogenesis (4), whereas the lack of both N-Myc and the EGF receptor results in defects during early lung development (7, 12, 13, 40–43) due to abnormalities in cell proliferation. Similarly, treatment of lung buds with TGF-β in organ culture has been shown to result in a failure of branching morphogenesis due to down-regulation of N-Myc expression (9). N-Myc acts as a transcription factor and probably is involved in activation of genes necessary for proliferation of lung epithelium. Because expression of TGF-β down-regulates N-Myc and causes arrested lung development, it becomes necessary to repress the expression of TGF-β and other genes that might have a growth inhibitory effect during normal embryogenesis. Therefore, LKLF could act as a negative regulator of gene expression to shut down the expression of genes, the products of which could otherwise result in effects like that of TGF-β. In this respect, it is interesting to note that LKLF acts as a negative regulator of gene expression during development of T-cells in the thymus (39). Normal expression of TGF-β in $LKLF^{-/-}$ animals (18), however, suggests that LKLF does not act through TGF-β signal pathway. The identification of target genes for LKLF in the lung will help in understanding the signaling pathway through which LKLF works in addition to its role in development and differentiation of specific cell types during normal lung development and function.

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