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

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

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) 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 alveologenesis (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 died 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 results 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 μg/ml gancyclovir. The resistant colonies were screened for homologous recombination by PCR. The double targeted cells were expanded...
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 confluence, 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 nitroblue tetrazolium, 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 Lauro (Stazional Zoologico “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 gentamycin. Lung buds were grown at 37 °C in a humidified, 5% CO₂ 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 (26). The RNA probe was synthesized from LKLF cDNA labeled by in vitro transcription using fluorescein-labeled 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'-CAAGGGGCTGGGGTGTG-GAAT-3' and 5'-GGGGAGGGAGGGGAAAGA-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. 1a). 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. 1d).

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).
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 (not shown), suggesting that LKLF expression may not be required during the pseudoglandular period of lung development.

A

**Fig. 2.** Isolation and characterization of LKLF<sup>−/−</sup> ES cells. A, gene targeting strategy to create LKLF<sup>−/−</sup> 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 mini-gene 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, EcoRI; Bg, BglII; RV, EcoRV; Nc, NcoI. 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 EcoRI or BglII and hybridized to a 0.875-kilobase BglII-NcoI 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.

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To overcome embryonic lethality and, therefore, study the role of LKLF in lung development, chimeric animals were generated from LKLF<sup>−/−</sup> 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 mini-gene 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. 3A). Following selection in hypoxanthine/aminopterin/thymidine medium containing G418 and gancyclovir, LKLF<sup>−/−</sup> 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. 3B). 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. 3B). Using this strategy, we were able to obtain several LKLF<sup>−/−</sup> ES cell clones. The two clones that were used for blastocyst injection were analyzed by Northern blot analy-
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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 C57B/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.

**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,
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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<sup>−/−</sup> 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<sup>−/−</sup> animals (18). Normal vasculogenesis in LKLF<sup>−/−</sup>-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<sup>−/−</sup> 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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