LGL1, a novel branching morphogen in developing kidney, is induced by retinoic acid

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Quinlan J, Kaplan F, Sweezy N, Goodyer P. LGL1, a novel branching morphogen in developing kidney, is induced by retinoic acid. Am J Physiol Renal Physiol 293:F987–F993, 2007. First published August 1, 2007; doi:10.1152/ajprenal.00098.2007.—Late-gestation lung protein 1 (LGL1) is a glycoprotein secreted by fetal lung mesenchyme that stimulates branching morphogenesis of the developing lung bud. We show that Lgl1 mRNA and protein are also expressed in mesenchymally derived lineages of fetal kidney. Although Lgl1 expression is stimulated by glucocorticoids in kidney cells, cortisol (10–7 M) actually suppresses ureteric bud branching of fetal kidneys from HoxB7/GFP mice in explant culture. However, early branching morphogenesis in the lung and kidney is stimulated by retinoic acid, and we identified putative retinoic acid response elements in the Lgl1 promoter. All-trans-retinoic acid (10–6 M) stimulated Lgl1 promoter activity and endogenous Lgl1 mRNA expression in vitro. Branching of cultured fetal kidney explants was increased in the presence of all-trans retinoic acid (10–6 M). Heterozygous Lgl1 knockout mice were crossed to HoxB7/GFP mice to visualize the extent of ureteric bud branching at fetal stages. At embryonic (E) days E12.5–E13.0, mutant Lgl1+/− embryos showed a 20% reduction in ureteric bud branching compared with wild-type litters. We propose a model in which retinoic acid stimulates branching morphogenesis by activating Lgl1 early in development. The prominent effects of glucocorticoids on Lgl1 expression in late lung development suggest a second role for LGL1 in alveolar maturation.

Branching morphogenesis; kidney development; glucocorticoids

DEVELOPMENT OF THE METANEPHRIC kidney begins when the ureteric bud (UB) emerges from the nephric duct and grows into the adjacent metanephric mesenchyme (MM). Signals from the tips of the UB induce the metanephric mesenchyme (MM) to condense and form nephrons, which fuse to the collecting system (22). Consecutive dichotomous branching of the UB occurs ~20 times during human gestation, generating between 300,000 and 1 million nephrons in each human kidney (17). The number of embryonic branching events determines the final number of nephrons an individual will have for life (8), and it has been proposed that suboptimal nephron number at birth increases susceptibility to acquired renal disease and essential hypertension later in life (4–6, 10).

Vitamin A is critical for kidney development (24). Offspring of vitamin A-deficient (VAD) rodents may have genitourinary tract anomalies or renal agenesis (12). These defects can be reversed with maternal vitamin A supplementation at the onset of renal organogenesis (29). The active physiological form of vitamin A is all-trans retinoic acid (atRA) (30–32). When fetal rat kidneys were cultured ex vivo in the presence of atRA (0.1–1 μM), new nephron formation was accelerated two- to threefold (28).

Branching morphogenesis is not unique to the kidney. Development of the lung also involves repeated branching of the primary lung bud (21). Lung development can be roughly divided into early and late events (26). In early lung development, the epithelial lung bud undergoes repetitive, dichotomous branching, beginning at ~week 3 of gestation. In later stages of development (~36 wk of gestation), the lung begins to mature and form the terminal gas-exchange units, the alveoli. At this time, airway branching comes to completion, air spaces widen, and surfactant is produced in preparation for postnatal life (25). Two important molecules that regulate lung development are retinoids and glucocorticoids (GC). RA stimulates initial lung branching of the primary lung bud (16, 29), while GC stimulates terminal maturation and differentiation of the alveoli (7).

In 1999, a novel molecule, late-gestation lung protein 1 (Lgll), was identified in lung fibroblasts as a GC-induced gene (9). Lgl1 mRNA was detected in fetal lung mesenchyme, and pulse-chase experiments determined that LGL1 was secreted as a 52-kDa glycoprotein that acted on the epithelium (20). When antisense oligodeoxynucleotides were directed against Lgl1, branching of fetal lung explants was inhibited (19). These results suggest that Lgl1 is a molecule secreted by mesenchymal cells that affect epithelial development in the lung. Screening of various tissues for Lgl1 expression by Northern blot analysis identified the transcript in adult kidney (9). Thus we hypothesized that LGL1 might function as a branching morphogen in the developing kidney, paralleling its role in the lung.

MATERIALS AND METHODS

RT-PCR analysis. Total RNA was prepared from fetal mice kidney tissue following directions for the Qiagen RNeasy Mini Column extraction kit (Qiagen, Germantown, MD). The research was conducted with approval of the McGill University Health Center Research Institute Animal Care Committee. Briefly, whole mouse kidneys were dissected using RNase-free techniques with RNAlater. The kidneys were homogenized using a mortar and pestle, and the extracted RNA was dissolved in RNase-free water. The RNA was then treated with RNase-free DNase 1 (Ambion). One microgram of total RNA was used for RT-PCR. RT-PCR was performed using 30 min at 50°C, 15 min at 95°C, 35 cycles each consisting of denaturation (94°C for 1 min), annealing (55°C for 1 min), and extension (68°C for 1 min). Synthetic oligodeoxynucleotide pairs were designed by aligning

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conserved sequences of murine Lgl1 cDNA in diencephalon to the Lgl1 cDNA sequence in the pancreas, yielding a 490-bp product. The following sets of PCR primers spanned exons 4 and 5:

Lgl1 forward primer: 5’-ATGCTTCCACAAAGCTGC-3’;

Lgl1 reverse primer: 5’-GCTGGATGGACACTCAGAGC-3’.

RT-PCR products were separated on a 1.5% (wt/vol) agarose gel and visualized by ethidium bromide staining.

Cell culture. Murine inner medullary collecting duct (mIMCD) (23), mK3 (murine mesenchymal cells), and mK4 (murine mesenchymal cells in epithelial transition) (kind gift from Dr. S. S. Potter) (27) cells were maintained in DMEM containing 10% (vol/vol) FBS/FCS, 1% penicillin, and streptomycin at 37°C in humidified 5% CO2 chamber in air. Cells were transfected at 50% confluency using Lipofectamine 2000 (Invitrogen) with 10 μg Lgl1 cDNA/10-cm-diameter dish. Cells were grown for 48 h and harvested for Western blotting experiments.

Electrophoresis and Western blot immunoblot analysis. At 48 h after transfection, HEK 293 cells grown in 10-cm-diameter dishes were washed twice with cold PBS. Cells were scraped in 500 μl of cold PBS and spun down to pellet cells. Postnatal (P) day 1 C3H/HeN mouse kidneys (Charles River Laboratories) were dissected and washed in cold PBS. Cell pellets and kidneys were lysed in hypotonic buffer [10 mM HEPES (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, aprotinin, pepstatin, leupeptin] and incubated for 15 min on ice. NP-40 was added to the lysates (10%) and vigorously vortexed for 10 s. The supernatant was collected by centrifugation at maximal speed for 3 min at 4°C.

The total protein concentration of lysates was determined according to the BCA Protein Assay (Pierce). Proteins (50 μg) diluted in sample buffer were boiled for 5 min and loaded in each well on a SDS-10% (wt/vol) polyacrylamide gel and transferred on a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). Membranes were blocked by incubation with 5% (wt/vol) nonfat dry milk in PBS-Tween [PBS/0.05% (vol/vol) Tween] at room temperature (25°C) for 1 h to prevent nonspecific binding. The membrane was incubated with rabbit anti-LGL1 antibody [1:500 dilution in 2% bovine serum albumin/TBS-Tween (TBS)/0.05% (vol/vol) Tween] at 4°C overnight, washed three times for 10 min with TBS-Tween, and incubated with HRP (horseradish peroxidase)-conjugated goat anti-rabbit IgG (Cell Signaling, 1:1,000 dilution) in PBS-Tween containing 5% (wt/vol) nonfat dry milk. After 3 × TBS-Tween washes, blots were detected with the enhanced chemiluminescence detection system

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Table 1. PCR program

<table>
<thead>
<tr>
<th>Initial Denaturation</th>
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<td>Denaturation</td>
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<td>Extension</td>
<td>72°C</td>
<td>3 min</td>
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<tr>
<td>Final extension</td>
<td>72°C</td>
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Fig. 1. Lgl1 mRNA expression. A: total RNA was extracted from embryonic (E) mouse kidney on days E12, E15, E18, and postnatal (P) day P1. The expected 490-bp Lgl1 RT-PCR product is seen at all time points but is absent in the no-RT and water controls. B: in similar experiments, the Lgl1 RT-PCR product was detected in mouse kidney cell lines derived from mesenchyme (mK3 and mK4) but not in inner medullary collecting duct (IMCD) cells derived from murine collecting duct or in controls.
Lgl1 and renal branching morphogenesis

...stained according to Oyewumi et al. (19) with minor changes. In short, dehydrated, and embedded in paraffin. Tissue sections were immuno-C

E18 P1 (Sigma) in DMEM/1% FBS/FCS for 48 h at 37°C in a humidified cold 1 was also determined in the same manner, using the same 20-...supernatant was used for both firefly luciferase and firefly buffer (Promega). The cell extract was centrifuged, and the cleared supernatant aliquot after addition of 100...Renilla ciency based on the firefly/...activity.

Lgl1 promoter region: putative binding site detection. The 872-bp fragment upstream of the Lgl1 start site that is expected to be the Lgl1 promoter region (unpublished observations) was analyzed for potential transcription factor binding sites using Genomatix (http://www.genomatix.de).

Lgl1 transfection and reporter gene assays with RA and GC. Functional promoter assays using the Lgl1 reporter gene construct were performed by transient transfections into mK3 cells. Cells in 24-well plates were seeded at 70% confluence. The next day, cells were transfected in serum- and antibiotic-free DMEM using Lipofectamine 2000 transfection reagent at the following concentrations: 0.8 μg Lgl1-pGL3Basic or empty pGL3Basic, 16 ng pRL-1K, and 1.0 μl Lipofectamine 2000. After 6 h, the media was changed to antibiotic-free DMEM/10% FBS, and the cells were incubated with 10−6 M atRA (Sigma) in DMEM/1% FBS/FCS, 10−6 M 9-cis retinoic acid (9cisRA; Sigma) in DMEM/1% FBS/FCS, or 10−7 M cortisol (Sigma) in DMEM/1% FBS/FCS for 48 h at 37°C in a humidified 20% O2-5% CO2 chamber. After 48 h, the cells were washed with cold 1× PBS and lysed by scraping in 100 μl of 1× passive lysis buffer (Promega). The cell extract was centrifuged, and the cleared supernatant was used for both firefly luciferase and firefly Renilla assays. Luciferase activity was determined in 20 μl of supernatant at room temperature in 100 μl of luciferase reagent (Promega) for 10 s after a 2-s delay in a Monolight 3010 luminometer. Renilla activity was also determined in the same manner, using the same 20-μl supernatant aliquot after addition of 100 μl of Stop and Glow buffer (Promega). Reporter assays were normalized for transfection efficiency based on the firefly/Renilla activity.

RA and GC stimulation of Lgl1 mRNA measured by real-time RT-PCR. mK3 and mK4 cells were grown in media with minimal serum (DMEM/1% FBS/FCS/1% Pen/Strep) in the presence or absence of atRA (10−6 M), 9cisRA (10−6 M), or GC (10−7 M) for 48 h. After 48 h, cells were washed with PBS, and total RNA was isolated using the RNeasy kit (Qiagen) according to the manufacturer’s recommendations. Samples were resuspended in 40 μl of RNase-free water and treated with RNase-free DNaseI (Ambion) as per manufacturer’s recommendations.

RNA samples were analyzed for levels of Lgl1 by real-time RT-PCR using a One-Step RT-PCR SYBR Green kit (Qiagen) as per the manufacturer’s recommendations on an ABI Prism 7000. Mouse Lgl1 primers were designed to span an intron and mouse β2-microglobulin (β2M) primers were used as a normalizing control. The sequences are as follows:

Lgl1 forward: 5'-GACCAAGAGACCCCGATCA-3'; Lgl1 reverse: 5'-CATCAGTAGACCCGATTTGG-3'; RT-PCR product size: 206 bp.

β2M forward: 5'-TGCGAGTAAACGCTTACGATTG-3'; β2M reverse: 5'-TGATGCTGATACAGTCTCG-3'; RT-PCR product size: 75 bp.

One hundred nanograms of total RNA were used per reaction, and RT-PCR conditions were as follows; a 30-min RT step at 50°C proceeded by 95°C for 15 min. A total of 35 cycles were performed at 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. Melt curves for each amplicon showed a single peak, indicating the absence of primer dimerization or nonspecific PCR products. Samples were run on a 1% agarose gel. Each sample was run in duplicate. The comparative CT method was used for relative quantification between treated and nontreated cells after standardization using the housekeeping gene β2M (14).

Fig. 2. Lgl1 mRNA expression pattern. In situ hybridization in E12.5 mouse embryos, using an antisense digoxigenin-labeled Lgl1 riboprobe. A: Lgl1 signal (blue staining) is seen in lung, intestine, and kidney (×4 magnification). B: higher power magnification (×10) of the kidney shows that the Lgl1 signal is predominantly in renal mesenchymal (MM) cells.
5% CO2/air for 96 h in the dark. Images were captured using a Leica microscope under fluorescent light after 0, 24, and 48 h in culture. The number of UB tips at each specified hour was calculated for each condition. The change in the number of UB tips was calculated by subtracting the baseline UB tip number.

Quantifying UB tip number in Lgl+/−/Hoxb7-Gfp embryos. Lgl1+/− mice (unpublished observations) were mated to Hoxb7-Gfp mice to allow visualization of the 7UB. E12.5−E13 embryos were obtained from timed mating, and the kidneys were microdissected and photographed under fluorescent light using a Leica microscope. Images were captured at ×32 magnification. The number of branching events was obtained by counting the number of UB tips. A section of each embryo was used for genotyping. Genomic DNA was isolated using a Wizard Genomic DNA Purification Kit (Promega), and genotyping was performed using the following PCR primers and conditions:

Lgl1 forward (5′PCR-F3): 5′- CACTGCTCCGTTGATCAAGCATCAC-3′;
Lgl1 reverse (5′PCR-R3): 5′- CAGGTCCTGCTGAGTTCCTTGGCA-3′;
Lgl1 Neo primer (Neo-1): 5′- GACAATCGGCTGCTCTGATG-3′;
Lgl1 Neo primer (Neo-2): 5′- GACAATCGGCTGCTCTGATG-3′.

The PCR reactions were performed with an initial denaturation step for 5 min at 94°C followed by three cycles consisting of 1 min at 94°C and 3 min at 72°C, three cycles consisting of denaturation (1 min at 94°C), annealing (1 min at 66°C), and extension (3 min at 72°C), which was repeated with each block of three cycles, with a decrease in the annealing temperature by 2°C until 56°C was reached. The final extension was performed at 72°C for 7 min. Table 1 represents repeating the block 3× with a decrease in the annealing temperature by 2°C until 56°C is reached.

PCR products were run on a 1.5% agarose gel and visualized with ethidium bromide staining. The Lgl1+/− mice have a Neo cassette inserted in their allele disrupting the LGL1 protein. The mutant allele is observed as a 1-kb band, and the wild-type allele is a 0.8-kb band (unpublished observations).

Data presentation and statistical analysis. All data are presented as means ± SD or means ± SE. Statistical significance was determined using Microsoft Excell software. Comparisons between two groups were made using Student’s t-test.

RESULTS

Lgl1 mRNA is expressed in murine kidney. The expression of Lgl1 mRNA was assessed by RT-PCR in C3H/HeN mouse kidneys at various stages of development. Specific primers were designed from the reported cDNA sequence for mouse Lgl1 (NCBI no. AK019034) to amplify a 490-bp fragment of the transcript spanning exon 4 to exon 5. Lgl1 mRNA was detected as early as E12 and was identified at E13, E14, and P1 (Fig. 1A). We also examined Lgl1 mRNA expression in cultured cells derived from mouse kidney mesenchyme (mK3 and mK4) (27) and from mIMCD (23). The Lgl1 transcript was present in murine mesenchymal cells (mK3 and mK4) but was conspicuously absent from mIMCD cells (Fig. 1B).

To localize the site of Lgl1 mRNA expression, we performed in situ hybridization using a 1.4-kb Lgl1 digoxigenin-labeled RNA to probe sections of E12.5 embryos. Endogenous Lgl1 mRNA was detected in mesenchymal structures surrounding epithelial branches of the developing kidney, lung, and midgut epithelium (Fig. 2).

LGL1 protein is present in the developing mouse kidney. Western blots of P1 mouse kidney extracts (40 µg protein/lane) were probed with rabbit antiserum raised against murine LGL1 peptide (19). The expected 52-kDa LGL1 protein band (Fig. 3A) was comparable to the band seen in human embryonic kidney (HEK293) cells transiently transfected with murine Lgl1 cDNA (data not shown).

To localize LGL1 protein, immunohistochemistry was performed on sections of fetal (E13.5−E18.5) and postnatal (adult) mouse kidney with the same antibody (Fig. 3, B–F). At E13.5 (Fig. 3C), LGL1 protein was localized to mesenchymal cells in the nephrogenic zone and in stromal cells surrounding the UB trunk. At E15.5 (Fig. 3D), LGL1 protein was seen in stromal cells; LGL1 was not evident in nephrogenic mesenchyme at the S-shaped body stage but was clearly seen in more differentiated tubules derived from nephrogenic mesenchyme. At E18.5 (Fig. 3E) and in adult mice (Fig. 3F), LGL1 protein was evident in maturing proximal tubules but not in glomeruli.

Lgl1 is induced by retinoic acid. Since branching morphogenesis is stimulated by retinoids in both fetal lung and fetal kidney (16, 28, 29), we reasoned that this effect might be mediated by stimulation of LGL1. We analyzed the 872-bp 5′-flanking sequence of the rat Lgl1 gene for potential regulatory motifs and identified several putative retinoic acid response elements (Fig. 4C). When the 872-bp Lgl1 5′-flanking sequence luciferase reporter was transiently transfected into mK3 cells with 9cisRA (10−6 M) or atRA (10−6 M), reporter activity was stimulated (means ± SD) sixfold (0.61 ± 0.08 luciferase/Renilla units, P < 0.0006) and sevenfold (0.69 ±
stimulate expression of endogenous controls, respectively (Fig. 4).

The effect of atRA on UB branching was examined by exposing E11.5 Hoxb7-Gfp explants to atRA for 48 h (Fig. 5A). In the presence of atRA (10^{-6} M) the mean percent change (means ± SE) in the number of UB tips compared with baseline (t = 0) was 107.7 ± 7.02% after 24 h (P = 0.18) and 130.8 ± 6.7% after 48 h (P = 0.0005). The stimulatory effect of atRA is plotted in Fig. 5B as a percentage of control explant branching at the same time.

E12.5–E13 Lgl1^{+/−}/Hoxb7-Gfp embryonic mouse kidneys have fewer UB branch tips than wild-type littermates. To determine whether LGL1 is critical for optimal branching morphogenesis of the fetal kidney, we examined arborization of the UB in heterozygous Lgl1^{+/−} knockout mice crossed with Hoxb7-Gfp mice to visualize the UB. E12.5–E13 embryonic mouse kidneys (5 litters) were isolated under fluorescent light, and the number of UB tips was counted (Fig. 6). Lgl1^{+/−} heterozygous mice (n = 17) had (means ± SD) 20 ± 14.11% fewer UB branch tips than their wild-type littermates (n = 10) (P = 0.028). [All supplemental material (including supplementary figures and supplementary results) is available in the online version of this article at the journal website.]

DISCUSSION

In 1999, Kaplan et al. (9, 19) identified a novel GC-inducible gene in late gestational lung mesenchyme (Lgl1) which encodes a secreted glycoprotein. Inhibition of Lgl1 expression in fetal rat lung explants suppressed pulmonary airway branching morphogenesis (19). In this report, we show that LGL1 expression is not confined to the lung but is identified in mesenchymal cells from the developing kidney and midgut as well. In the developing kidney, we detected Lgl1 mRNA at E12 and showed by in situ hybridization that it is expressed primarily in the metanephric mesenchyme. As renal development progresses, LGL1 protein is evident both in stromal cells and in proximal tubules derived from metanephric mesenchyme, but...
is consistently absent in the branching UB. In heterozygous Lgl1 mice bearing the Hoxb7-Gfp transgene to mark the UB lineage, we found reduced branching morphogenesis compared with their wild-type littermates. Taken together, these observations suggest that Lgl1 secreted from metanephric mesenchymal cells enhances branching morphogenesis of the renal collecting systems, paralleling its putative effects on the early stages of lung bud arborization.

During renal development, the rate of UB branching is highly dependent on retinoic acid synthesized locally from circulating retinol (3, 11). Severe maternal vitamin A deficiency causes renal agenesis (24), and pups born to rats with moderate vitamin A deficiency exhibit renal hypoplasia (12). In fetal rat kidney explants, addition of atRA to the culture medium stimulates branching nephrogenesis and new nephron formation by two- to threefold over 48 h (28). Retinoic acid appears to stimulate UB branching indirectly since mice with homozygous inactivation of retinoic acid receptor-α (RARα) and β2 (RARβ2) genes have arrested development of stromal compartments and renal hypoplasia with down-regulation of c-ret expression in UB tips (1). Although RARα is ubiquitous in the developing kidney, RARβ2 expression is restricted to renal mesenchyme (2). These observations suggested a model in which retinoic acid stimulates UB branching by inducing mesenchymal cells to secrete a paracrine branching morphogen.

We screened the Lgl1 gene 5′-flanking sequence for potential retinoic acid response elements and found several putative RXR binding sites and one putative RAR orphan receptor-related sequence (~190 bp upstream of the transcriptional start site). Our experiments do not prove that the Lgl1 gene transcription is directly activated by retinoic acid, but we observed an about sevenfold stimulation of LGL1 promoter activity and threefold stimulation of endogenous Lgl1 mRNA by atRA in cultured cells (mK4) derived from mouse metanephric mesenchyme. We hypothesize that the effects of retinoic acid on UB branching might be mediated, in part, by stimulation of renal mesenchymal cell Lgl1 synthesis in accordance with the model proposed by Batourina et al. (2).

Interestingly, retinoic acid has also been reported to stimulate branching of the lung bud at early stages of development. If maternal retinol is reduced, lung agenesis or hypoplasia results (29). Homozygous RARα/RARβ2 knockout mice have pulmonary agenesis and tracheoesophageal fistula in addition to renal dysplasia (15). Thus mesenchymal Lgl1 may mediate the effects of retinoic acid on early lung bud branching as well.

Lgl1 was originally observed as a GC-induced gene in late embryonic stages of lung development (9). We confirm that Lgl1 mRNA expression is stimulated by GC in kidney cells as it is in the lung. This is, at first, puzzling since glucocorticoids stimulate alveolarization of lung bud branches but are reported to inhibit lung bud branching (7, 18). Similarly, we noted reduced UB branching in fetal kidney explants exposed to cortisol (10 −7 M). However, fetal GC levels rise sharply toward the end of gestation, coinciding with stimulation of surfactant synthesis (7). At an earlier stage, both the lung and kidney undergo a phase of rapid branching morphogenesis,
which is stimulated by retinoic acid. We propose a model in which optimal arborization is driven by retinoic acid induction of LGL1 in both tissues. On the other hand, the prominent effects of GC on LGL1 in late lung development suggest a second role for LGL1 in alveolar maturation (Fig. 7).

GRANTS

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