LGL1 modulates proliferation, apoptosis, and migration of human fetal lung fibroblasts

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1Departments of Human Genetics and Pediatrics, McGill University, Montreal, Quebec, Canada; 2Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada; 3Hospital for Sick Children Research Institute, Toronto, Ontario, Canada; 4Departments of Pediatrics and Physiology, University of Toronto, Toronto, Ontario, Canada

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Zhang H, Sweezey NB, Kaplan F. LGL1 modulates proliferation, apoptosis, and migration of human fetal lung fibroblasts. Am J Physiol Lung Cell Mol Physiol 308: L391–L402, 2015. First published December 5, 2014; doi:10.1152/ajplung.00119.2014.—Rapid growth and formation of new gas exchange units (alveogenesis) are hallmarks of the perinatal lung. Bronchopulmonary dysplasia (BPD), common in very premature infants, is characterized by premature arrest of alveogenesis. Mesenchymal cells (fibroblasts) regulate both lung branching and alveogenesis through mesenchymal-epithelial interactions. Temporal or spatial deficiency of late-gestation lung 1/cysteine-rich secretory protein LD2 (LGL1/CRISPLD2), expressed in and secreted by lung fibroblasts, can impair both lung branching and alveogenesis (LGL1 denotes late gestation lung 1 protein; LGL1 denotes the human gene; Lgl1 denotes the mouse/rat gene). Absence of Lgl1 is embryonic lethal. Lgl1 levels are dramatically reduced in oxygen toxicity rat models of BPD, and heterozygous Lgl1+/− mice exhibit features resembling human BPD. To explore the role of LGL1 in mesenchymal-epithelial interactions in developing lung, we developed a doxycycline (DOX)-inducible RNA-mediated LGL1 knockdown cellular model in human fetal lung fibroblasts (MRC5LGL1KD). We assessed the impact of LGL1 on cell proliferation, cell migration, apoptosis, and wound healing. DOX-induced MRC5LGL1KD cells exhibited growth arrest and increased apoptosis of annexin V staining cells and caspase 3/7 activity. LGL1-conditioned medium increased migration of fetal rat primary lung epithelial cells and human airway epithelial cells. Impaired healing by MRC5LGL1KD cells of a wound model was attenuated by addition of LGL1-conditioned medium. Suppression of LGL1 was associated with dysregulation of extracellular matrix genes (downregulated MMP1, ColXVa1, and ELASTIN) and proapoptosis genes (upregulated BAD, BAK, CASP2, and TNFRSF1B) and inhibition of 44/42MAPK phosphorylation. Our findings define a role for LGL1 in fibroblast expansion and migration, epithelial cell migration, and mesenchymal-epithelial signaling, key processes in fetal lung development.

Lgl1(Crispdl2); human fetal lung fibroblast; proliferation/migration/apoptosis; mesenchymal-epithelial interaction; bronchopulmonary dysplasia

IN HEALTH, PERINATAL AND INFANT LUNGS exhibit rapid growth in weight and linear dimensions and rapid formation of new gas exchange units (alveogenesis). The most common chronic lung disease of infancy is bronchopulmonary dysplasia (BPD), a condition especially common among the very prematurely born that is characterized by developmental arrest of alveogenesis at an immature stage (15). The pathogenesis of BPD involves multiple factors, including intrauterine inflammation, placental insufficiency (28), and postnatal oxygen toxicity (37). Although the majority of alveogenesis is completed during the first few years of life, there is increasing evidence from animal models and some human data that it may continue to much later ages and that it may be modifiable (12, 30). This suggests the possibility that components of the underlying pathogenesis of BPD may be targetable postinfancy. Given the current absence of effective treatment for this condition, the identification of agents capable of stimulating alveogenesis is essential.

Lung development is determined morphologically and physiologically by complex interactions between mesenchymal and epithelial cells. Mesenchymal-epithelial interactions regulate fetal airway-branching morphogenesis. Alveogenesis involves cooperative interactions between mesenchymal and epithelial cells and the vascular compartments of the lung. The proliferation, migration, and differentiation of myofibroblasts are essential to secondary septation and the subdivision of terminal air sacs into alveoli (4). Disruption of this finely controlled mechanism leads to abnormal alveogenesis, the hallmark of modern-day BPD. We reported previously on the cloning and characterization of the secreted mesenchymal protein late-gestation lung 1/cysteine-rich secretory protein LD2 (LGL1/CRISPLD2) (17, 21, 26). Lgl1 is expressed in fetal rat lung as early as gestational day 12, and deficiency of Lgl1 impaired lung-branching morphogenesis (25). Maximal fetal glucocorticoid (GC)-responsive Lgl1 expression occurs in late gestation at the onset of alveogenesis (17). At this time, LGL1 is secreted by smooth muscle α-actin-positive myofibroblasts located adjacent to the epithelium and is concentrated at the tips of budding alveoli (24). The spatial localization of LGL1-secreted myofibroblasts pointed to a role for LGL1 in myofibroblast-epithelial communication that regulates alveogenesis (25, 26). Additional support for a key role for Lgl1 in alveogenesis came from our findings that dramatically reduced Lgl1 levels in oxygen toxicity models of BPD normalized with recovery (24). Absence of Lgl1 is embryonic lethal. Heterozygous Lgl1+/− mice exhibited features resembling human BPD, including distal airspace enlargement, disruption of elastin, and mucus cell hyperplasia (21).

In the current study, we developed a cellular model of doxycycline (DOX)-inducible RNA-mediated LGL1 knockdown in human fetal lung fibroblasts (MRC5LGL1KD) and investigated the role of LGL1 action in vitro. Suppression of LGL1 in MRC5LGL1KD cells reduced cell growth, suppressed cellular migration, and increased apoptosis. Notably, the addition of medium enriched with LGL1 partially compensated for impaired wound healing in LGL1-deficient cells. Medium conditioned with secreted LGL1 also promoted lung epithelial cell migration, suggesting the potential of LGL1 to regulate
key epithelial cell functions in lung airway-branching morphogenesis and alveogenesis. Our present findings provide the first direct analysis of LGL1 function at the cellular level in human fetal lung cells and point to potential roles for LGL1 in human fetal lung development. The profound effects of anomalous LGL1 levels on basic cellular function may explain the embryonic lethality of Lgl1 knockout (KO) mice.

**MATERIALS AND METHODS**

*Lentiviral transduction of shRNA LGL1 in MRC5 cells.* Human fetal lung fibroblasts, MRC5 (ATCC, Manassas, VA), grown in minimal essential medium (MEM) (Wisent, Montreal, Quebec, Canada) with 10% (vol/vol) FBS and 1% penicillin/streptomycin, were used between passages 5 and 15, such that studies were completed before the MRC5 cells enter senescence. LGL1 shRNA constructs and a scrambled shRNA used as a negative control were cloned into a pTRIPZ lentiviral DOX-inducible vector (Thermo, Ottawa, Ontario, Canada). Lentivirus was produced by transfecting 293T cells with shRNA constructs, pVSV-G and psPAX2, using Lipofectamine 2000 (Life Technology, Burlington, Ontario, Canada). MRC5 cells were infected with lentivirus, and puromycin (0.5 μg/ml) (Wisent) was added after 48 h to select stable clones. For characterization of MRC5LGL1KD, three stable DOX-inducible MRC5LGL1KD clones with three different LGL1 shRNA constructs were evaluated for LGL1 expression levels. A vector containing a scrambled shRNA (MRC5control) was used as control. DOX induction for 4 days of MRC5LGL1KD clones 1 and 3 (but not clone 2) led to the reduction of endogenous LGL1 mRNA expression to 30% and 25%, respectively (Fig. 1A). Therefore, both LGL1 shRNA constructs for clone 1 and 3 could be used to generate LGL1 suppression in MRC5. Suppression of LGL1 protein level in MRC5LGL1KD cells after 4-day DOX induction was confirmed by Western blot analysis. LGL1 was suppressed in MRC5LGL1KD cells only in the presence of DOX induction. LGL1

![Graph A](image1)

![Graph C](image2)

![Graph D](image3)

*Fig. 1.* Characterization of MRC5LGL1KD and HEK293LGL1ORF stable clones. *A:* qRT-PCR analysis showing late gestation lung 1 (LGL1) mRNA levels in 3 different MRC5LGL1KD clones (containing LGL1 shRNA) after 4-day doxycycline (DOX) induction. *B:* LGL1 protein expression level in MRC5control (containing scrambled shRNA) and MRC5LGL1KD clone 3 with/without 4-day DOX induction was detected by Western blot with anti-LGL1 antibody. There was reduced LGL1 expression in MRC5LGL1KD cells following DOX induction when normalized to β-actin. *C:* qRT-PCR showing LGL1 mRNA expression after DOX induction at 1, 5, and 10 μg/ml in MRC5LGL1KD clone 1 and 3 for 2 days. *D:* DOX induction at 0, 2, 4, and 10 days resulted in a time-dependent suppression of LGL1 mRNA expression in MRC5LGL1KD clone 3 by qRT-PCR. *E:* LGL1 was present in HEK293LGL1ORF cell lysate and supernatant but not in HEK293control lysate or supernatant as assessed by Western blot using the anti-V5 antibody (control, HEK293control; LGL1, HEK293LGL1ORF).
was not suppressed in the presence of either MRC5\textsuperscript{control} cells with DOX or MRC5\textsuperscript{LGL1KD} cells without DOX (Fig. 1B). To evaluate the dose response of LGL1 to DOX induction, MRC5\textsuperscript{LGL1KD} clone 1 and clone 3 cells were exposed to 0, 1, 5, or 10 \mu g/ml of DOX for 2 days; 1 \mu g/ml of DOX induced 50% suppression of LGL1, which was not further reduced in the presence of 5 or 10 \mu g/ml (Fig. 1C). No cell toxic effect was observed. We next evaluated LGL1 expression under DOX induction for different durations. DOX induced LGL1 mRNA suppression in a time-dependent manner, with a 50% reduction after 2 days, 70% reduction after 4 days, and a 90% reduction after 10 days (Fig. 1D). All further experiments were carried out using 2 \mu g/ml DOX induction for 4–5 days in MRC5\textsuperscript{LGL1KD} clone 3 cells unless otherwise specified. The suppression of LGL1 mRNA in MRC5\textsuperscript{LGL1KD} cultures was repeatedly confirmed before each set of experiments.

Cloning and recombinant expression of human LGL1 in HEK293 cells. The human LGL1 open-reading-frame (ORF) cDNA was cloned into the vector pIREs2 (Clontech, Palo Alto, CA) with the green fluorescent protein (GFP) and a V5 tag. GFP not fused to the inserted protein allows monitoring of transfection efficiency. V5 fused to the inserted protein before the stop codon allows for identification of the inserted protein. HEK 293 cells were transfected with human LGL1 ORF plasmid or empty vector using Lipofectamine 2000. After 48 h, G418 600 \mu g/ml was added for selection. After 2 wk, stable cell clones from single cells were used. Protein expression of LGL1 in HEK293\textsuperscript{control} (containing empty vector) and HEK293\textsuperscript{LGL1ORF} cell lysates and medium were examined by Western blot using the V5 antibody. The plasmid contained a 14-amino-acid V5 tag fused to the COOH-terminal region of the LGL1 ORF before the stop codon. The V5 tag allows for detection of the LGL1 protein ORF. Western blot analysis using the V5 antibody shows no band in HEK293 control cells. By contrast, the HEK293\textsuperscript{LGL1ORF} cells display a band corresponding to the molecular weight of LGL1 protein (Fig. 1E).

Collection of conditioned medium. To collect conditioned medium, HEK293\textsuperscript{LGL1ORF} and HEK293\textsuperscript{control} stable cells were grown in 15-cm plates until they were 90% confluent. The medium was centrifuged at 120 \times g for 3 min, and the supernatant was discarded to remove the contaminating fibroblasts. This step was repeated four times. After the cell pellet was resuspended in growth medium, cells were transferred to a 24-well plate, filled with conditioned medium with/without LGL1 collected from HEK293\textsuperscript{LGL1ORF} and HEK293\textsuperscript{control} cells. Serum-free medium containing 5 \times 10^5 MRC5 cells, 1.25 \times 10^4 IHAEO- cells, or 1 \times 10^4 rat primary cells was placed on Transwell membranes (8.0 \mu m) and filled with conditioned medium with/without LGL1 for 18 h.

Cells that passed through the membranes were fixed, stained, and assessed by light microscopy. Migrated cells were counted using light microscopy. Each condition was tested in three separate wells of three individual experiments. For each experiment, cell viability was assessed by Trypan blue exclusion before use in assays.

Wound-healing assay. A linear defect was produced in a confluent cell monolayer by scraping with a pipette tip. Closure of the defect was monitored by light microscopy. The distance between the edges of defect was measured, and the average value was determined as an indicator of wound healing.

Cell proliferation study. This study included four aspects. 1) cell proliferation measured by 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay, according to the manufacturer (Sigma-Aldrich, Oakville, Ontario, Canada). Human fetal lung fibroblast (MRC5) cells, human airway epithelial (1HAEo-) cells, and rat primary fetal lung epithelial cells were used. The 1HAEo- tracheal epithelial cell line was a kind gift from Dr. Dieter Gruenert (University of California at San Francisco) (5). 1HAEo- cells were grown in MEM medium containing 2 mM L-glutamine with 10% (vol/vol) FBS, and 1% penicillin/streptomycin was used. For the migration assay, Transwell inserts were placed in a 24-well plate, filled with conditioned medium with/without LGL1 collected from HEK293\textsuperscript{LGL1ORF} and HEK293\textsuperscript{control} cells. Serum-free medium containing 5 \times 10^5 MRC5 cells, 1.25 \times 10^4 IHAEO- cells, or 1 \times 10^4 rat primary cells was placed on Transwell membranes (8.0 \mu m) and filled with conditioned medium with/without LGL1 for 18 h.

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Annexin V-FITC/SYTOX red staining. Cells were gently collected and washed with PBS. Cells (3 \times 10^5) were resuspended in 300 \mu l of PBS containing 10 \mu g/ml RNaseA and 15 \mu l (1:100) SYTOX red (Life Technology) for 30 min at 37°C. All further experiments were carried out using 2 \times 10^5 cells/ml. To each well, 200 \mu l of propidium iodide (0.05 \mu g/ml) was added. Cells were analyzed by FlowJo software (Tree Star, Ashland, OR). A total of 10,000 gated single events were recorded each time, and the percentage of cells in G1, G2, and S phases was calculated. The experiments were repeated at least three times for each variable. 4) MAPK activity involved in cell proliferation was assessed by Western blotting comparing phosphorylated 44/42 MAPK level normalized to total 44/42 MAPK.

Migration assay. Transwell migration assays were performed according to the manufacturer (BD Bioscience, Mississauga, Ontario, Canada). Human fetal lung fibroblast (MRC5) cells, human airway epithelial (1HAEo-) cells, and rat primary fetal lung epithelial cells were used. The 1HAEo- tracheal epithelial cell line was a kind gift from Dr. Dieter Gruenert (University of California at San Francisco) (5). 1HAEo- cells were grown in MEM medium containing 2 mM L-glutamine with 10% (vol/vol) FBS, and 1% penicillin/streptomycin was used. For the migration assay, Transwell inserts were placed in a 24-well plate, filled with conditioned medium with/without LGL1 collected from HEK293\textsuperscript{LGL1ORF} and HEK293\textsuperscript{control} cells. Serum-free medium containing 5 \times 10^5 MRC5 cells, 1.25 \times 10^4 IHAEO- cells, or 1 \times 10^4 rat primary cells was placed on Transwell membranes (8.0 \mu m) and filled with conditioned medium with/without LGL1 for 18 h.

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sion levels were analyzed using the ΔΔ cycle threshold method (n ≥ 4 for all assays).

**Immunoblotting.** Proteins in cell lysate and supernatants (50 μg) were separated by SDS-PAGE and transferred to PVDF membranes. Primary antibodies mouse anti-V5 (1:6,000, Life Technology), rabbit anti-44/42MAPK (1:1,000), and rabbit anti-phospho44/42MAPK (1:1,000) (Cell Signaling Technology, Danvers, MA) were used and evaluated as previously described (25).

**Immunohistochemistry.** Cells (1 × 10^6) of MRC5control (containing scrambled shRNA) or MRC5LGL1KD cells were placed on coverslips. The next day, cells reached confluence and were then exposed to DOX for 4 days. Cells were fixed with 100% methanol at −20°C for 30 min. After being blocked with PBS containing 5% normal goat serum for 1 h at room temperature, cells were incubated with primary antibodies antitumorpoetasin (1:100), Elastin Products, Owensville, MO overnight at 4°C. The following day, goat anti-rabbit Alexa Fluor 488 secondary antibody was added (1:300, Life Technology) for 1 h at room temperature. Cells on coverslips were mounted with DAPI-containing mounting media (Vector Laboratories, Burlingame, CA), and images were taken using fluorescence microscopy.

**ELISA.** Pro-matrix metalloproteinase-1 (MMP-1) secretion in cell-free supernatants was assessed with a commercial human pro-MMP-1 ELISA kit (R&D Systems, Minneapolis, MN) according to the manufacturer’s instructions.

**Statistical analysis.** All results are presented as means ± SE. Statistical significance of differences between group averages was assessed by Student’s t-test or one-way ANOVA, using Prism (GraphPad, La Jolla, CA), with significance defined as P ≤ 0.05.

**RESULTS**

**Suppression of LGL1 reduces cell growth in human fetal lung cell cultures.** MRC5LGL1KD cells showed significantly reduced cell growth compared with MRC5control cells after 4 days of DOX induction, as assessed by counting cell numbers (n = 3, *P < 0.05, **P < 0.01, Fig. 2A). The reduction of cell growth in DOX-induced MRC5LGL1KD cells was also confirmed by MTT cell growth assay. A significant reduction of cell growth was observed in MRC5LGL1KD cells induced with DOX for 3–4 days (n = 4, *P < 0.05, Fig. 2B). To further evaluate the role of LGL1 in cell growth, we used flow cytometry-based DNA content analysis for cell cycle. As illustrated in Fig. 2C and Table 2, MRC5LGL1KD cells showed a significantly lower proportion of cells in S phase (DNA synthesis) than MRC5control cells (n = 3, *P < 0.05, Table 2), suggesting that LGL1 is required for DNA synthesis. In addition, we evaluated MAP kinases as a potential intracellular signaling pathway regulating cell proliferation (29). Significantly reduced phospho-44/42MAPK was evident in cytosolic extracts of MRC5LGL1KD with DOX, compared with MRC5control with/without DOX induction or MRC5LGL1KD without DOX (n = 4, *P < 0.05, Fig. 2D).

**Table 1. List of primers**

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<th>Forward</th>
<th>Sequence</th>
<th>Reverse</th>
<th>Sequence</th>
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<td>CCAATTTTGGCTCGAACCAAG</td>
<td>Human TNFRSF1B-R</td>
<td>TGCCGACTGTACTTCCTTTACA</td>
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<tr>
<td>Human MMP1-F</td>
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<td>Human MMP1-R</td>
<td>GTCACAAGAAAGATGCGCGGATT</td>
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<tr>
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<td>Human COL15α1-R</td>
<td>TGGAGGCTATGGCGAATTGACT</td>
</tr>
<tr>
<td>Human ELASTIN-F</td>
<td>CTGAGAGAGATTAGCAAG</td>
<td>Human ELASTIN-R</td>
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<tr>
<td>Human BAK1-F</td>
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<tr>
<td>Human GAPDH-F</td>
<td>GAATGCTGATCGGCTTCTGA</td>
<td>Human GAPDH-R</td>
<td>GGTTGCTTAAAGCAGTTG</td>
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**Suppression of LGL1 induces apoptosis in human fetal MRC5 cultures.** Having demonstrated a significant reduction in cell number in DOX-induced MRC5LGL1KD cells, we next tested whether this diminution of cell number was also attributable to increased cell apoptosis using the Annexin V/SYTOX red staining assay. Annexin V binds to phosphatidylserine, present only on the outer membrane of apoptotic cells, and SYTOX red binds to DNA (19). Annexin V+/SYTOX red−staining indicates early-stage apoptotic cells, whereas Annexin V+/SYTOX red−/stained cells are representative of late-stage apoptosis. The percentage of early-stage and late-stage apoptosis in total indicates the extent of apoptosis. MRC5LGL1KD and MRC5control cells were stained and analyzed by flow cytometry following 5 days of DOX induction. MRC5control cells treated with TNF-α (10 ng/ml) for 2 h were used as a positive control, and unstained MRC5control cells were used as negative control to confirm the gating strategy (Fig. 3A). After quantification, there were more apoptotic cells in MRC5LGL1KD cultures than in MRC5control cultures, demonstrating an increase in apoptosis (n = 3, *P < 0.05, Fig. 3B) and a trend toward increase in early apoptosis in MRC5LGL1KD cells (Fig. 3B). We also screened for apoptosis-related genes using the PCR array method (data not shown). Several candidates were confirmed by qRT-PCR. As shown in Fig. 3C, there was increased proapoptotic gene expression in MRC5LGL1KD cells, including the BCL2-associated agonist of cell death, BCL2-antagonist/killer 1, caspase 2, and tumor necrosis factor receptor superfamily, member 1B (n = 4, *P < 0.05) (8, 13, 20, 31). Activation of caspase 3 is important for the initiation of apoptosis. Caspase 3/7 activity in MRC5LGL1KD cells was significantly increased compared with MRC5control cells. MRC5control cells treated with TNF-α (10 ng/ml, 2 h) were used as a positive control; unstained MRC5control cells were used as a negative control to confirm the gating strategy (n = 3, *P < 0.05, Fig. 3D).

**LGL1 regulates migration of lung mesenchymal and epithelial cells.** To test whether secreted mesenchymal LGL1 regulates cell migration, we used an in vitro Transwell migration assay. We investigated the impact of LGL1 levels on migration of both human fetal lung fibroblasts (MRC5) and human airway epithelial (1HAEo-) cells. The number of migrated cells was significantly reduced in MRC5LGL1KD cells compared with control (n = 3, *P < 0.01, Fig. 4A). By contrast, LGL1-conditioned medium promoted 1HAEo- cell migration through the Transwell membrane (n = 3, *P < 0.05, Fig. 4B). We next validated this result on primary lung epithelial cells by determining the effect of LGL1-conditioned medium on E20 fetal rat lung epithelial cells. Incubation in the presence of LGL1-conditioned medium promoted migration of E20 rat epithelial cells.
lung cells \((n = 3, *P < 0.05, \text{Fig. 4C})\). Isolated cultures of epithelial cells were confirmed with the epithelial marker pan-keratin by Western blot (not shown). The extracellular matrix (ECM) has a major role in modulating cell migration. We screened for ECM genes using the PCR array method (data not shown). Several gene candidates were then confirmed by qRT-PCR as shown in Fig. 4D. At the molecular level, suppressed \(LGL1\) expression in MRC5\(^{LGL1KD}\) cells led to down-regulation of MMP-1, collagen type XV \(\alpha 1\) (COL15\(\alpha 1\)), and elastin \((n = 4, *P < 0.05, **P < 0.01, \text{Fig. 4D})\) (16, 23, 35). These effects were also observed at the protein level as illustrated in Fig. 4E. Expression of elastin was reduced in MRC5\(^{LGL1KD}\) cells as determined by immunofluorescence staining (Fig. 4E). Secretion of pro-MMP-1 in supernatant from MRC5\(^{LGL1KD}\) cells was significantly reduced compared with supernatant from MRC5\(^{control}\) cells, as assessed by ELISA assay \((n = 4, *P < 0.05, \text{Fig. 4F})\).

\(LGL1\) promotes wound healing in MRC5 cultures. Fibroblast growth and migration are critical to the wound-healing process. We examined the effect of \(LGL1\) on a model of wound healing in MRC5 cells. Suppression of \(LGL1\) in MRC5\(^{LGL1KD}\) cells

![Fig. 2. Suppression of \(LGL1\) reduces cell growth of MRC5 cells. A: \(3 \times 10^5\) cells were plated in each dish. Cell numbers were significantly reduced in MRC5\(^{LGL1KD}\) cultures compared with MRC5\(^{control}\) at each time point from day 4 to day 8 under DOX induction \((n = 3, *P < 0.05, **P < 0.01)\). B: cells were plated in 96-well plates \((10^4/well)\) for 3 or 4 days under DOX induction and processed with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Absorbance was observed at 540 nm \((n = 4, *P < 0.05)\). C: cytometry-based DNA content assessment. Cells were incubated with SYTOX red DNA stain and analyzed by flow cytometry. Representative flow cytometry images are shown. Populations in the G0/G1, S, and G2/M phases are shaded in green, pink, and blue, respectively. D: p44/42MAPK activity in MRC5\(^{LGL1KD}\) cells. Representative Western blots probed with phospho-44/42MAPK antibody and with MAPK antibody are shown. Activity of phospho44/42MAPK was normalized to total 44/42MAPK, and blots were quantified \((n = 4, *P < 0.05)\).

Table 2. Cell-cycle analysis of MRC5\(^{LGL1KD}\) cells

<table>
<thead>
<tr>
<th>Percent Cells</th>
<th>G1/M (\pm SE)</th>
<th>S (\pm SE)</th>
<th>G2 (\pm SE)</th>
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<tbody>
<tr>
<td>MRC5(^{control})</td>
<td>65 ± 0.98</td>
<td>22.5 ± 2.2</td>
<td>12.5 ± 3.3</td>
</tr>
<tr>
<td>MRC5(^{LGL1KD})</td>
<td>65.2 ± 1.9</td>
<td>9.9 ± 1.4*</td>
<td>24.9 ± 0.45</td>
</tr>
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Values are means \(\pm SE, n = 3, *P < 0.05\).
was associated with wound-healing retardation and corrected by addition of LGL1-enriched medium at 40 h (Fig. 5A). The distance between the edges of a wound was measured at 0 and 40 h after injury. There was a significant healing defect in MRC5LGL1KD cells compared with MRC5control cells 40 h after injury (n = 4, *P < 0.05, Fig. 5B). Impaired wound healing of MRC5LGL1KD cells was attenuated by addition of LGL1-enriched medium (Fig. 5, A and B).

DISCUSSION

The postnatal lung represents a period of explosive growth and expansion of air space. The units of this growth are the alveoli that populate the terminal lung. The process of alveogenesis, like that of primary fetal lung-branching morphogenesis, depends on the exquisitely regulated process of epithelial-mesenchymal signaling that defines lung development before and after birth. In humans, the process of alveogenesis is maximal from late in the third trimester of fetal development (gestational week 36) until 2 to 3 yr of postnatal age. In rodents, maximal alveogenesis is exclusive to the first 3 wk of life. As noted earlier, there is evidence for a continuous window for increases in the numbers of alveoli well beyond this period (1). Early respiratory distress and its sequelae, including the current form of BPD, are leading causes of neonatal morbidity in the Western world. Molecules that may trigger new alveolar formation in the postnatal period would be of great interest.

In early work, we described the cloning and characterization of CRISPLD2, which we initially called Lgl1 (there has since been some confusion as to the referencing of this gene) (17). The properties of rodent Lgl1 are consistent with a role in epithelial-mesenchymal interactions during both the periods of airway branching and alveogenesis (24–26). Whereas Lgl1 synthesis is almost exclusively restricted to the mesenchyme, Lgl1 protein is associated with lung epithelial cells from the late canalicular period onward (24, 26). Antisense inhibition of Lgl1 impaired lung-branching morphogenesis, whereas Lgl1 protein stimulated airway branching in lung explant culture (25). Oxygen toxicity rat models that exhibited features of BPD had dramatically reduced Lgl1 levels (24). Absence of the Lgl1 (Crispld2) gene product is embryonic lethal (21). Heterozygous Lgl1 KO mice had a number of features characteristic of BPD (21). To test for an in vivo role of Lgl1, we therefore attempted to develop an Lgl1 KD mouse using a lentiviral vector-based system combined with RNA interference to regulate Lgl1 expression. Repeated attempts to produce these mice were not successful in depleting LGL1 protein. Moreover, embryonic stem cells in which Lgl1 was maximally reduced appeared to have impaired growth. We therefore elected to explore the function of LGL1 in vitro. Among the advantages of this approach is that we were able to investigate over- and underexpression systems in human fetal lung cells. This is of particular relevance given that LGL1 is expressed almost exclusively in mesenchymal tissue. Evidence from other studies suggests that unique properties distinguish human from mouse lung fibroblasts. For example, Wright et al. (36) described differences in cyclooxygenase-driven prostanoid effects on cytokine production in human lung fibroblasts vs. mouse lung (36). Our approach also allowed us to explore the...
function of \textit{LGL1} in the presence of fibroblasts alone and/or in the presence of fibroblasts and epithelial cells.

Only one human cell line (MRC5, derived from a 14-wk-old male embryo) was available that we could reproducibly grow in culture following gene manipulation. Moreover, to our knowledge, no human cell lines are available from later-stage embryos. The future availability of such additional cell lines amenable to gene manipulation in culture will allow for an improved understanding of genotypic-phenotypic effects on \textit{LGL1} function in vitro.

Our studies define multiple CRISPLD2 functions that are likely to be temporally and spatially regulated in developing lung mesenchyme. To our knowledge, this is the first study to address the mechanism of \textit{LGL1} action in any in vitro system. Further definition of the temporal and spatial functions of \textit{Lgll} in human lung development, investigations that are beyond the scope of the current study, will involve studies in human fetal lung explant culture. We suppressed \textit{LGL1} expression in MRC5\textsuperscript{LGL1KD} cells. Suppression of \textit{LGL1} impaired cell growth and induced apoptosis, leading to a significant reduction in cell numbers. After 5-day DOX exposure, cell number was reduced by more than 50%. Cell-cycle analysis revealed a significant reduction in cell population in DNA synthesis phase. In addition to the positive Annexin V assay for apoptosis, we also detected an increase in multiple proapoptotic genes and caspase 3/7 activity. These findings consistently suggested a role for \textit{LGL1} in active lung growth. Clearly, the impact of \textit{Lgll} on the processes of cell proliferation and apoptosis in vivo will be modulated by coordinate expression of multiple temporally and spatially regulated genes in the developing lung mesenchyme and epithelium. For example, we show here that \textit{LGL1} suppresses apoptosis, a function important in lung airway branching. By contrast, an increase in apoptosis is critical to mesenchymal epithelial thinning in late gestation and to alveogenesis.

Mesenchymal-epithelial interactions drive lung development (6). Using a Transwell migration assay, we showed that conditional medium that contained \textit{LGL1} can regulate migration of both lung fibroblasts and epithelial cells. Migration of MRC5\textsuperscript{LGL1KD} (fibroblast) cells was suppressed compared with that of MRC5\textsuperscript{control} cells. By contrast, medium enriched in \textit{LGL1} positively regulated cell migration in primary rat lung epithelial (E20) cells and human airway epithelial 1HAEo-cells. In previous studies, we showed that \textit{Lgl1} was localized in fibroblasts adjacent to the epithelium. Maximal fetal GC-responsive \textit{Lgl1} expression occurred at gestational day 20–21 in rat (17). At this time, mesenchymal \textit{Lgl1} was restricted to the smooth muscle/α-actin-positive myofibroblasts adjacent to the epithelium that regulate the formation of new alveolar units (25). Beginning in late canalicular lung, secreted \textit{Lgll} was associated with distal lung epithelial cells (26). Our current findings show that medium enriched in secreted \textit{LGL1} has a direct impact on epithelial cell migration, confirming its potential as a regulator of mesenchymal-epithelial interactions in the developing lung. Mesenchymal-epl-
thelial signaling is essential to both lung branching and alveogenesis. Further investigation will be required to search for candidate LGL1 receptors and downstream signaling pathways.

The process of alveogenesis is coordinated by multiple interactions through paracrine mechanisms between the fibroblastic, epithelial, and microvascular lung components, and with the ECM. To provide gas exchange efficiently in the postnatal organism, the lung undergoes dramatic tissue growth and remodeling. The formation of new alveolar septation is a necessary step to increase blood-gas interface. Many key control molecules have been identified in this process, including various transcription factors and growth factors (14). At the molecular level, suppression of LGL1 in MRC5LGL1KD cells was associated with the anomalous expression of multiple ECM genes that modulate lung development and repair, including MMP1, COL15a1, and elastin (all downregulated). Col15a1 is developmentally regulated and expressed in lung (23). MMP1 (interstitial collagenase) is important in airway inflammation and the development of emphysema. Although an understanding of molecular interactions is lacking, evidence of association suggests that LGL1 may modulate alveogenesis through interactions with elastin. Elastin has been shown to

Fig. 4. LGL1 modulates migration of lung fibroblasts and epithelial cells. The effect of LGL1 on cell migration was evaluated by Transwell migration assay. A: representative images of stained cells (migrated cells) from MRC5LGL1KD and MRC5control cultures. Migrated cells were quantified in 3 independent experiments. Suppression of LGL1 in MRC5 significantly reduced cell migration (n = 3, *P < 0.01). B: representative images of migrated 1HAEo- cells incubated in conditioned medium in the presence or absence of LGL1. NC, conditioned medium collected from HEK293control; LGL1, conditioned medium from HEK293LGL1ORF. 1HAEo- cell migration was significantly increased in the presence of LGL1 enriched medium (n = 3, *P < 0.05). C: rat lung epithelial (E20) cells in culture were incubated in the presence or absence of LGL1-enriched medium, and migrated cells were quantified. There was a significant increase of migrated cells in rat lung epithelial cells treated with LGL1 medium (n = 3, *P < 0.05). D: expression of extracellular matrix genes and regulators, including MMP1, COL15a1, and elastin mRNA levels were compared in MRC5LGL1KD and MRC5control cells (n = 4, *P < 0.05, **P < 0.01). E: MRC5control and MRC5LGL1KD were immunostained with tropoelastin antibody. Expression of elastin in MRC5LGL1KD cells was decreased, compared with that in MRC5control cells. Representative images are shown. MRC5control (a) and MRC5LGL1KD (b) stained with antitropoelastin. MRC5control (c) and MRC5LGL1KD (d) stained in the absence of antitropeitastin as negative control. F: Secretion of pro-matrix metalloproteinase 1 (MMP-1) from MRC5control and MRC5LGL1KD cell supernatants were tested by ELISA. pro-MMP-1 in MRC5LGL1KD cells was expressed as a fold change over that of MRC5control cells. Values are represented by vertical bars. Mean values were significantly decreased in MRC5LGL1KD relative to MRC5control cells (n = 4, *P < 0.05).
modulate the budding of secondary septa, which is central to the process of secondary septation, by which primitive respiratory saccules are subdivided into definitive alveoli. Elastin and LGL1 colocalize at the tips of secondary septa in developing rodent lung (21). Distal airway development is impaired in mice lacking elastin (35), which exhibit deficient distal airway branching reminiscent of the effects of inhibiting LGL1 expression in fetal rat lung explants (25).

The CRISP family of more than 100 proteins is characterized by a secretory signal and two LCCL domains (9, 27, 32). Multiple functions have been attributed to the LCCL module, including roles in cell differentiation, migration (18, 33), host defense, and innate immunity (7, 10, 32). The combined findings of 1) an O2-induced BPD-like phenotype associated with dramatically reduced LGL1 levels that normalize with recovery (24), 2) BPD-like features in heterozygous LGL1 KO

Fig. 4—Continued
mice (21), and 3) in the present study, impaired fibroblast repair after wounding, which is corrected by exogenously supplied LGL1, all lend consistent support for a role for LGL1 in lung development.

Recent studies in humans and rodents attribute anti-inflammatory properties to Crispld2/Lgl1. Upregulation of the serum-soluble form of LGL1 protected mice against LPS-induced lethality (34). In a study on potential drug therapies for chronic obstructive pulmonary disease in human airway epithelial (BEAS2B) cells, combination treatment of glucocorticoid plus A2B was shown to enhance expression of anti-inflammatory genes; among genes induced by this treatment was Crispld2 (11). In future work, we will need to directly investigate the impact of inflammatory mediators on MRC5 control, MRC5KD, and LGL1-enriched MRC5/MRC5KD cells.

This study has several limitations. Because of the absence of available LGL1 protein, exogenous supplementary LGL1 was provided via enriched cellular medium. Therefore, our evaluation of the dosage response of LGL1 function in target cells was limited. Difficulties in culturing human fetal lung epithelial cells in vitro precluded analyses of the effects of LGL1 on migration of isolated human fetal lung epithelial culture.

Fig. 5. LGL1 modulates wound healing in MRC5 cells. A: wound/scratch assay was performed in MRC5LGL1KD cultures. Representative images showed that, at 40 h, the healing (measured by distance between the dashed lines) was retarded in DOX-induced MRC5LGL1KD cells compared with uninduced cells. Addition of LGL1-conditioned medium collected from HEK293LGL1ORF cells to DOX-induced MRC5LGL1KD cells attenuated healing retardation. B: distances between edges of wound quantified at 0 and 40 h after injury were presented as ratio of the distance at 0 h. There was a significant healing defect in DOX-induced MRC5LGL1KD cells, compared with uninduced cells at 40 h after injury (n = 4 for each time point, *P < 0.05). Addition of LGL1-conditioned medium to DOX-induced MRC5LGL1KD cells attenuated this difference.
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signaling pathways through which LGL1 modulates the various functions described above remain unknown. Our examination of the relevance of the magnitude of these in vitro effects must be tempered by our inability to assess the potential contribution of individual variation and sexual dimorphism. However, the present studies demonstrate, to our knowledge for the first time, that LGL1 regulates multiple specific functions within the developing human lung mesenchymal cell. Importantly, we also provide new evidence that LGL1 directs migration of lung epithelial cells (both human and rat) in the absence of any other cell type.

In summary, we demonstrated a role for LGL1 in regulating proliferation, apoptosis, and migration of human fetal lung fibroblasts. We also showed that LGL1 enhances wound repair.

To our knowledge, these are the first investigations to report on these functions of LGL1. Given the paucity of available and effective prevention and treatment for the profound burden of neonatal lung injury, testing the effects of LGL1 on postnatal lung development is of enduring interest.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: H.Z. performed experiments; H.Z. and F.K. analyzed data; H.Z., N.B.S., and F.K. conceived and designed research; figures; H.Z. and F.K. drafted manuscript; H.Z., N.B.S., and F.K. approved data; H.Z., N.B.S., and F.K. interpreted results of experiments; H.Z. prepared final version of manuscript; N.B.S. and F.K. conception and design of research; N.B.S. and F.K. edited and revised manuscript.

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