Modulation of *Lgl1* by Steroid, Retinoic Acid, and Vitamin D Models Complex Transcriptional Regulation During Alveolarization

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ABSTRACT: Alveolarization depends on circulating glucocorticoid (GC), retinoid (RA), and vitamin D (VitD). Bronchopulmonary dysplasia, a leading cause of neonatal morbidity, is associated with arrested alveolarization. In hyperoxia-exposed rats displaying features of bronchopulmonary dysplasia, reduced levels of late gestation lung 1 (*Lgl1*) normalize during recovery. We show that GC (100 nM) stimulates (7- to 115-fold) and VitD (100 μM) suppresses (twofold) *Lgl1* expression. RA (all-trans/9-cis, 10 μM) effects are biphasic. From postnatal days 7–10, RA was stimulatory (twofold) at 24 h, after which effects were inhibitory (3- to 15-fold). *Lgl1* promoter-luciferase reporter assays confirmed that these agents operated at the transcriptional level. Interestingly, the individual inhibitory effects of VitD and RA on GC induction of *Lgl1* were abrogated when both agents were present, suggesting that steric hindrance may influence promoter accessibility. Analysis of the proximity (<50 base pairs) of binding sites for overlapping VitD and RA receptors to that of the GC receptor identified 81% of promoters in 66 genes (including *Lgl1*) important in human lung development compared with 48% in a random set of 1000 genes. Complex integration of the effects of GC, RA, and VitD on gene expression in the postnatal lung is likely to contribute to the timely advance of alveolarization without attendant inflammation. (*Pediatr Res* 67: 375–381, 2010)

Bronchopulmonary dysplasia (BPD), a leading cause of morbidity in preterm infants (1), is characterized by arrested alveolarization. Part of this process, secondary septation, involves subdivision of terminal air sacs into alveoli. Multiple transcription factors regulate alveolarization, including hormone receptors that activate or repress gene expression (2,3). Glucocorticoids (GCs) and retinoids (RAs) act through the GC and RA receptors (GR, also called Nr3c1, RAR/RXR, respectively). GR signaling inhibits secondary septation by establishing a single-layered capillary network (4). GCs have been used to treat or prevent BPD (5). However, the beneficial effects of GC are now considered to be outweighed by increased risk of impaired lung and brain growth (6).

RA has been implicated in alveolar development and regeneration (7). Postnatal (PN) RA treatment counteracts GC-induced inhibition of alveolarization (8). By contrast, RA potentiates GC stimulation of expression of mesenchymal mitogens of alveolar type II cells. Vitamin D (VitD) is an important regulator of lung growth *in utero* (9).

In a search for downstream targets of GC that regulate lung maturation, we cloned late gestation lung 1 (*Lgl1*; also called Crespld2), a mesenchyme-specific gene (10,11) maximally expressed in late gestation and early PN life (10,12). Inhibition of *Lgl1* impaired airway branching (13). In *Lgl1* null mice, absence of *Lgl1* is lethal before lung formation (14). *Lgl1*$^{\pm}$ heterozygotes display a complex respiratory phenotype including delayed histologic maturation, features of inflammation in the newborn period, and altered lung mechanics at maturity. In oxygen toxicity models of BPD, reduced levels of *Lgl1* were restored during recovery (12). Therefore, we hypothesized that *Lgl1* modulates alveolarization, subject to regulation by GC, RA, and VitD.

We report on the coordinate transcriptional regulation of *Lgl1* by GC, RA, and VitD. Multiple genes important in human lung development are enriched for binding sites for GC, RA, and VitD that are in close proximity. Complex integration of the effects of GC, RA, and VitD on gene expression in the PN lung is likely to contribute to the timely advance of alveolarization without attendant inflammation.

MATERIALS AND METHODS

Fetal rat lung primary cell culture. All procedures involving animals were conducted according to criteria of the Animal Care Committees of the Canadian Council and the Montreal Children's Hospital Research Institute, McGill University Health Centre. Timed pregnant Sprague-Dawley rats (day 0, mating; term, day 22) and postnatal (PN, day 1, and birth) were from Charles-River. Primary fibroblasts originating adjacent to the epithelium were isolated as described (10).

Cell treatment protocols. Lung primary cells reached 75–80% confluence in minimal essential medium with Earle's balanced salts (MEM-EBSS) containing 10% fetal bovine serum (FBS), penicillin (100 units/mL), and

streptomycin (100 μg/mL). The culture conditions were changed for 24 h to MEM-EBSS media containing 5% charcoal-stripped FBS, penicillin (100 units/mL), and streptomycin (100 μg/mL) before being treated with hydro-cortisone (cortisol, 10 pM–100 nM), all-trans retinoic acid (ATRA, 1 μM–100 μM), 9-cis-retinoic acid (9-cis-RA, 10 μM), 1α, 25-dihydroxyvitamin D$_3$ (VitD, 0.1–1000 μM) for 12–72 h in serum-free media. Control plates contained equal volumes of ethanol or DMSO in serum-free media ($n \geq 4$).

RNA isolation. Cells were lysed using 1 mL Trizol. Total isolated RNA was resuspended in RNASecure and quantified using an ND-1000 spectro-photometer.

Quantitative real-time PCR. Real-time/quantitative real-time PCR (qRT-PCR) was performed in triplicate as described (12).

Transfection and dual reporter gene assays. A 901bp sequence of the 5′ flanking region of rat *Lgl1* [analyzed using MatInspector® promoter inspector software (15)] was inserted between the *Xho*I and *Hind*III sites upstream of the luciferase gene in the pGL3 basic plasmid to generate p*Lgl1*-luc. Embryonic day (E) 20 and PN7 rat lung fibroblasts were seeded (2.5 × 10^5 cells/well) in 24-well plates. The next day, cells were cotransfected in serum and antibiotic-free DMEM using lipofectamine 2000 reagent, with 0.8 μg p*Lgl1*-luc or with the empty pGL3Basic plasmid (control), and 4 ng of pRSV40-luc (Renilla). After 6 h, media was changed to antibiotic-free DMEM containing 5% charcoal-stripped FBS, and cells were grown for 24 h. Transfected cells were treated with 1 μM Dex, 100 nM cortisol, 10 μM ATRA, 10 μM 9-cis-RA, 100 μM VitD in DMEM containing 5% charcoal-stripped FBS ($n = 3$). After 24–72 h, cells were washed with PBS and lysed in 100 μL passive lysis buffer. Firefly- and renilla-luciferase activities were measured using the Dual Luciferase Reporter assay system kit and lysates measured using a Monolight 3010 luminometer. Relative promoter activities are reported as ratio of firefly- to renilla-luciferase activity to normalize for transfection efficiency. Assays were performed in triplicate.

Generation of deletion constructs. Deletion constructs were generated using a reverse primer on the pGL3 basic plasmid and two forward primers GRE+ (-572/-552) and GRE− (-512/-492). Resulting PCR fragments were cloned in pGL3 basic plasmid using *Xho*I and *Hind*III sites. Plasmids obtained (*GRE+* and *GRE−*) were transfected in NIH3T3 cells (in triplicate) and luciferase activity assayed as above.

Analysis of proximity of VitD receptor (VDR)/RXR or VDR/RAR consensus binding sites to GR. Analysis was carried out for 66 genes (Table 1) with a previously reported role in lung development (16). One thousand randomly chosen Refseq genes were used as controls. The regulatory framework specified overlap of bindings sites of VDR and either RXR or RAR and that the GR binding site lies within 50bp of the overlapping sites. Nineteen motifs were surveyed in http://www.biobaseinternational.com/pages/index.php?id=transfac and http://thebrain.bwh.harvard.edu/uniprobe/about.php. Clover software (17) was used to search for consensus binding sites of VDR, RXR, RAR, and GR. The Regulatory Framework Enrichment (RFE) Score was computed as the proportion of selected genes that contained the regulatory framework. The Motif score threshold determines how well a motif-sequence match suggests a true binding site. RFE of 6 is the default.

In vivo GC-treatment of rats. Sprague-Dawley rat pups were injected intraperitoneally with 100 μL of either saline or 3 mg/kg budesonide (Bud, Astra Zeneca, Lund, Sweden) on PN4, PN5, and PN6. After 72 h, (at PN7) the lungs were collected and flash frozen. RNA was isolated with Trizol, and *Lgl1* mRNA was measured by qRT-PCR. $n = 6$ for each group.

Statistical analysis. All data are presented as mean ± SEM. Statistical significance was determined by two-way analysis of variance. Pair-wise group comparisons were then assessed using Student-Neuman-Keuls test. Significance was defined as $p < 0.05$.

RESULTS

GC stimulation of **Lgl1** *in rat lung fibroblasts is temporally regulated.* During maximal septation in rat, circulating levels of plasma corticosteroid are low. Given that lung *Lgl1* levels are high during this period (12), we explored the temporal pattern of GC regulation of *Lgl1* mRNA. Primary cultures of rat lung fibroblasts from E18 to PN14 were exposed to cortisol (100 nM) for 24 h and *Lgl1* mRNA levels were determined by qRT-PCR. Cortisol stimulated *Lgl1* mRNA levels at all time points (Fig. 1*A*) with maximal stimulation of 100-fold at PN4.

Retinoids regulate **Lgl1** *expression in PN lung.* Metabolically active forms of RA in lung fibroblasts increase after

Table 1. *Genes involved in human lung development*

Genes enrichment set		
1	Bmp5	Bone morphogenetic protein 5
2	Bmp7	Bone morphogenetic protein 7
3	Crispld2	Cysteine-rich secretory protein LCCL domain containing 2 (*Lgl1*)
4	Egf	Epidermal growth factor
5	Eln	Elastin
6	Fgf10	Fibroblast growth factor 10
7	Fgf3	Fibroblast growth factor 3
8	Fgf4	Fibroblast growth factor 4
9	Fgf7	Fibroblast growth factor 7
10	Fgfr1	Fibroblast growth factor receptor 1
11	Fgfr2	Fibroblast growth factor receptor 2
12	Fgfr3	Fibroblast growth factor receptor 3
13	Fgfr4	Fibroblast growth factor receptor 4
14	Fgfrl1	Fibroblast growth factor receptor-like 1 (fgfr-5)
15	Flt1	FMS-like tyrosine kinase 1
16	Foxa2	Forkhead box A2 (HNF-3β)
17	Foxf1a	Forkhead box F1a (HFN-8)
18	Foxj2	Forkhead box J2 (HFH-4)
19	Gata5	GATA binding protein 5
20	Gars	Glycyl-tRNA synthetase (SMAD1-MAD homolog 1)
21	Gli1	GLI-Kruppel family member GLI1
22	Kdr	Kinase insert domain protein receptor
23	Mmp13	Matrix metallopeptidase 13 (Mmp1)
24	Mmp2	Matrix metallopeptidase 2
25	Mmp9	Matrix metallopeptidase 9
26	Nr3c1	Nuclear receptor subfamily 3, group C, member 1 (GR)
27	Pdgfa	Platelet derived growth factor, alpha
28	Pdgfb	Platelet derived growth factor, B polypeptide
29	Rara	Retinoic acid receptor, alpha
30	Rarb	Retinoic acid receptor, beta
31	Rarg	Retinoic acid receptor, gamma
32	Ret	Ret proto-oncogene (PTC)
33	Rxra	Retinoid X receptor alpha
34	Rxrb	Retinoid X receptor beta
35	Rxrg	Retinoid X receptor gamma
36	Sftpb	Surfactant associated protein B
37	Sftpc	Surfactant associated protein C
38	Smad1	MAD homolog 1 (GARS glycyl-tRNA synthetase)
39	Smad2	MAD homolog 2 (forkhead box H1)
40	Smad4	MAD homolog 4
41	Tgfa	Transforming growth factor, alpha
42	Tgfb1	Transforming growth factor, beta 1
43	Tgfb2	Transforming growth factor, beta 2
44	Tgfb3	Transforming growth factor, beta 3
45	Tgfbr1	Transforming growth factor, beta receptor I
46	Timp1	Tissue inhibitor of metalloproteinase 1
47	Timp3	Tissue inhibitor of metalloproteinase 3
48	Tnc	Tenascin C
49	Vdr	Vitamin D receptor
50	Vegfa	Vascular endothelial growth factor A
51	Wnt2	Wingless-related MMTV integration site 2
52	Wnt5a	Wingless-related MMTV integration site 5A
53	Wnt7b	Wingless-related MMTV integration site 7B
Genes not in enrichment set		
54	Bmp4	Bone morphogenetic protein 4
55	Egfr	Epidermal growth factor receptor
56	Gata6	GATA binding protein 6
57	Mmp12	Matrix metallopeptidase 12
58	Pdgfra	Platelet derived growth factor receptor, alpha polypeptide

(Continued)

59	Pdgfrb	Platelet derived growth factor receptor, beta polypeptide
60	Shh	Sonic hedgehog
61	Sftpa1	Surfactant associated protein A1
62	Smad3	MAD homolog 3 (Drosophila)
63	Smad7	MAD homolog 7
64	Sftpd	Surfactant associated protein D
65	Tgfbr2	Transforming growth factor, beta receptor II
66	Nkx2–1	NK2 homeobox 1

birth (7) with a parallel increase in RA receptors from PN8-PN12 (18). To determine whether RAs regulate *Lgl1* expression during this period by RARs (activated by ATRA and 9-cis-RA) or RXRs (activated by 9-cis-RA), we initially analyzed the effects of ATRA and 9-cis-RA on *Lgl1* mRNA levels in PN7 and PN10 rat lung fibroblasts. At both time points, 9-cis-RA or ATRA had a biphasic effect on *Lgl1* mRNA: weakly stimulatory after 24 h but strongly inhibitory after 48 h.

Primary rat lung fibroblasts from E18-PN14 were exposed to 10 μM ATRA for 24 or 48 h. At 24 h, ATRA stimulated *Lgl1* mRNA twofold on PN7 and PN10 (Fig. 1*B*). By contrast, after 48 h exposure, ATRA suppressed *Lgl1* levels from E18 to PN10. Maximal inhibition by ATRA after 48 h at PN10 was 15-fold.

The effects on **Lgl1** ***mRNA expression of GC and ATRA are antagonistic.*** Given GC stimulation and ATRA suppression of *Lgl1* mRNA levels, we asked whether ATRA inhibits cortisol-induced upregulation of *Lgl1* mRNA. Primary rat

lung fibroblasts isolated on PN4, when GC effects were maximal, were exposed to GC, ATRA, or both for 12–72 h (Fig. 1*C*). GC stimulated and ATRA suppressed *Lgl1* mRNA expression. Although ATRA alone had no effect on *Lgl1* mRNA levels after 12–24 h exposures, ATRA suppressed cortisol-induced *Lgl1* levels as early as 12 h. ATRA suppression of cortisol-stimulated *Lgl1* mRNA levels was stronger after 48 h but did not completely abrogate GC stimulation. Maximal suppression of GC stimulation was from 5.5- to 2-fold.

VitD suppression of **Lgl1** ***in rat lung fibroblasts.*** VitD mediates maturation of late gestation alveolar type II cells (9). To explore the effects of VitD on *Lgl1* mRNA expression, rat lung fibroblasts isolated from E20-PN14 were exposed to VitD (10 μM) for 24 h, and *Lgl1* mRNA levels were determined by qRT-PCR (Fig. 1*D*). VitD inhibited *Lgl1* mRNA levels from PN4 to PN14 (Fig. 1*D*). Maximal inhibition at PN7 was twofold.

Combined treatment with RA and VitD does not suppress GC induction of **Lgl1** ***mRNA.*** In the PN lung, synergistic action between VitD and RA stimulates growth of immature fibroblasts (19). As both of these agents are present in the lung during alveolarization, we determined the effects on *Lgl1* mRNA of combined treatment with VitD and 9-cis-RA in the presence and absence of GC. Rat lung fibroblasts isolated at PN4 were exposed to VitD, 9-cis-RA, or VitD plus 9-cis-RA for 24 h. Although RA alone did not impact *Lgl1* mRNA expression at 24 h, RA significantly suppressed GC induction of *Lgl1* mRNA at this time (Fig. 1*C* and data not shown). Combined treatment with VitD plus 9-cis-RA led to a modest
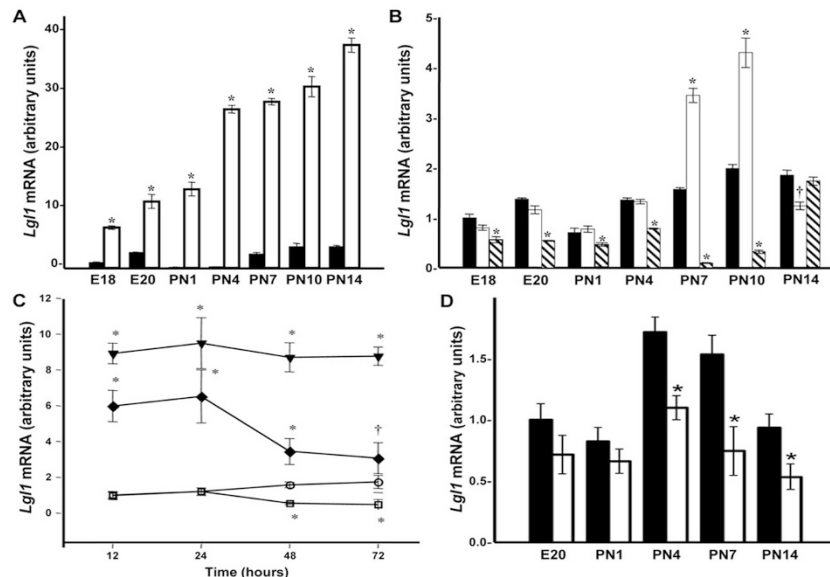

Figure 1. *A*, Temporal regulation of *Lgl1* by cortisol in rat lung fibroblasts. Rat lung fibroblasts isolated from E18 to PN14 were treated with 100 nM cortisol for 24 h as described in Materials and Methods section. *Lgl1* mRNA levels were quantified by qRT-PCR ($n = 6$). *$p \leq 0.01$ Control (■) *vs* GC-treated (□). *B*, Temporal regulation of *Lgl1* by ATRA in rat lung fibroblasts. Rat lung fibroblasts isolated from E18 to PN14 were treated with 10 μM ATRA for 24 and 48 h as described in Materials and Methods section. *Lgl1* mRNA levels were quantified by qRT-PCR ($n = 4$). †$p \leq 0.05$ and *$p \leq 0.01$ control (■) *vs* ATRA-treated 24 h (□) and (▨) ATRA-treated 48 h. *C*, Effects of coordinate treatment with cortisol and ATRA on *Lgl1* mRNA in PN4 rat lung fibroblasts. Rat lung fibroblasts isolated at PN4 were treated with 100 nM cortisol, 10 μM ATRA or both for 24 to 72 h as described in Materials and Methods section. *Lgl1* mRNA levels were quantified by qRT-PCR ($n = 4$). †$p \leq 0.05$ and *$p \leq 0.01$ control (○) *vs* treated 100 nM cortisol (▼), 10 μM ATRA (□), and 100 nM cortisol + 10 μM ATRA (◆). *D*, Suppression of *Lgl1* by VitD in rat lung fibroblasts at PN4, 7, and 14. Rat lung fibroblasts isolated from E20 to PN14 were cultivated for 24 h in media containing charcoal-stripped fetal bovine serum (FBS) and treated with 10 μM VitD for 24 h. *Lgl1* mRNA levels were quantified by qRT-PCR ($n = 6$). *$p \leq 0.01$ control (■) *vs* VitD treated (□).
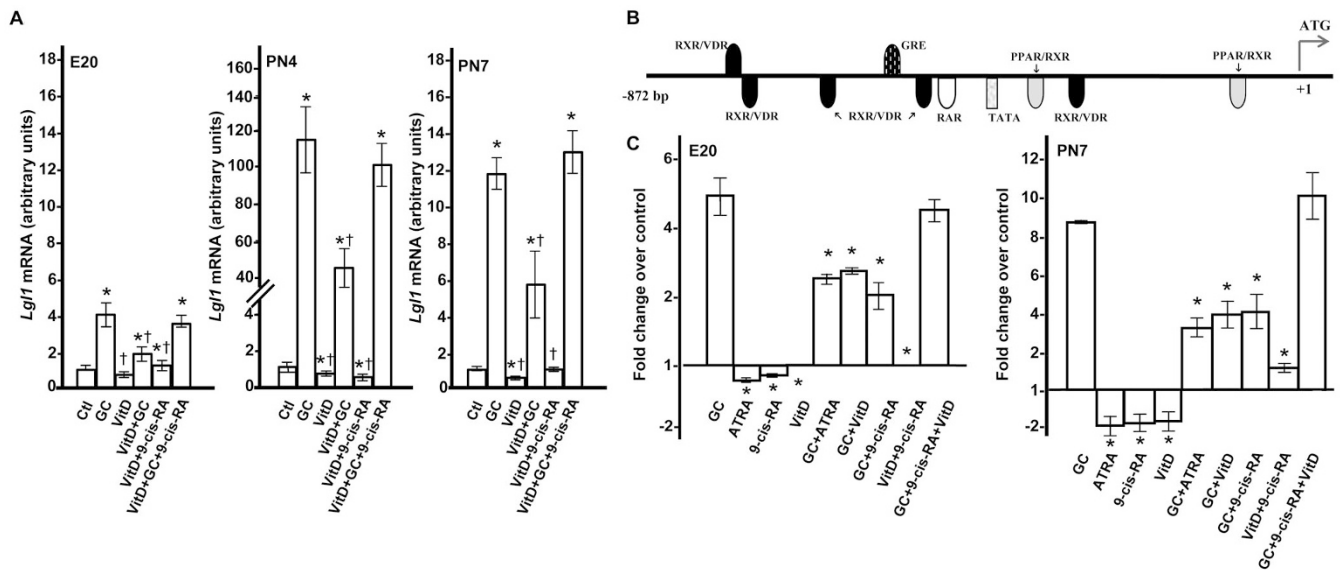
Figure 2. *A*, Effects of coordinate treatments with VitD, cortisol, and 9-cis-RA on *Lgl1* expression in rat lung fibroblasts. Rat lung fibroblasts isolated at E20, PN4, and PN7 were treated with 100 nM GC, 10 μM VitD, or combinations of 100 nM GC, 10 μM VitD, and 10 μM 9-cis-RA for 24 h as described in Materials and Methods section. *Lgl1* mRNA levels were quantified by qRT-PCR ($n = 6$). *$p \le 0.001$, control *vs* treated cells. †$p \le 0.02$, combined treatments *vs* treatment with GC alone combination. *B,* Analysis of the 901bp 5′-flanking region of *Lgl1*. Representation of the *Lgl1* 5′-flanking promoter region showing binding sites of interest. *C,* Summary of luciferase assay results. Graph depicting the fold change in *Lgl1* promoter activity at 48 h in treated compared with control (untreated) cells at E20 andPN7. *$p \le 0.003$, combined treatments *vs* treatment with GC alone.
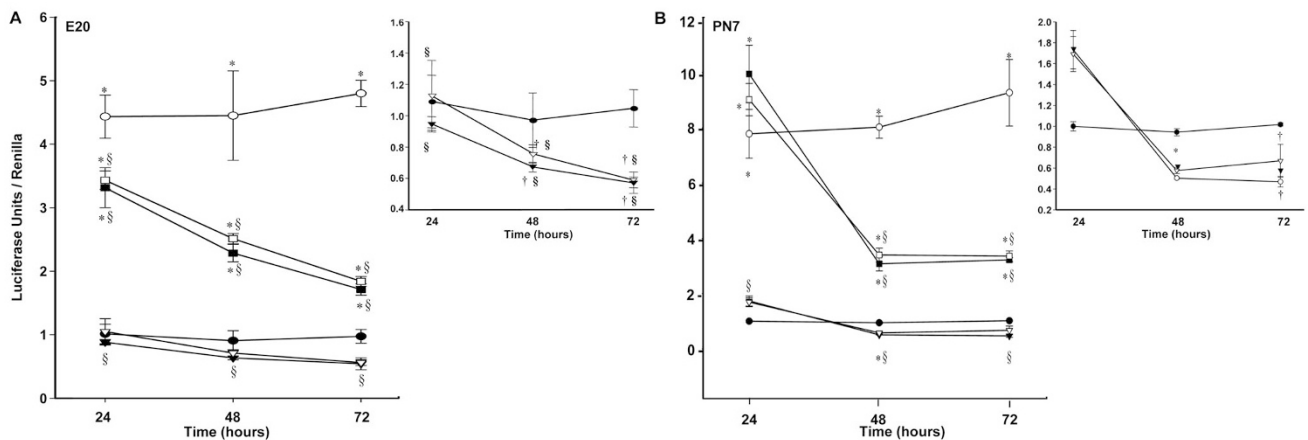

Figure 3. *A*, The effects of GC, ATRA, and 9-cis-RA on *Lgl1* promoter activity in E20 rat lung fibroblasts. Rat lung fibroblasts isolated at E20 were cotransfected with p*Lgl1*-luc and pRSV40-luc (Renilla) were treated with 100 nM cortisol, 10 μM ATRA, 10 μM 9-cis-RA, or combinations of GC plus RA or GC for 24–72 h as described in Materials and Methods section. Firefly- and renilla-luciferase activities were measured. Relative promoter activities are reported as a ratio of firefly- to renilla-luciferase activity to normalize for transfection efficiency ($n = 3$). †$p \le 0.05$ and *$p \le 0.01$ control *vs* treated. §p. ≤0.02, combined treatments *vs* treatment with GC alone. *B*, The effects of GC, ATRA, and 9-cis-RA on *Lgl1* promoter activity in PN7 rat lung fibroblasts. Rat lung fibroblasts isolated at PN7 were cotransfected, treated, and assessed as in Fig. 3A ($n = 3$). †$p \le 0.05$ and *$p \le 0.01$, control *vs* treated. §$p \le 6 \times 10^{-4}$, combined treatments *vs* treatment with GC alone. Control (●), 100 nM cortisol (○), 10 μM 9-cis-RA (▽), 10 μM ATRA (▼), 100 nM cortisol + 10 μM 9-cis-RA (□), and 100 nM cortisol + 10 μM ATRA (■).

inhibition of *Lgl1* mRNA at PN4 and PN7, which was not additive (Fig. 2*A*). VitD, similar to RA, suppressed GC induction of *Lgl1* mRNA by ~50% (Fig. 2*A*). Most interestingly, combined treatment with VitD and 9-cis-RA had no effect on GC induction of *Lgl1* mRNA at all time points (Fig. 2*A*).

GC, ATRA, 9-cis-RA, and VitD regulate Lgl1 *promoter activity.* We next explored whether GC, RA, and VitD regulate *Lgl1* expression by effects on gene transcription. Consensus recognition sequences for putative binding sites for GR, RXR, RAR, and VDR are illustrated in Figure 2*B*. Five VDR/RXR heterodimer binding sites surround the single con-

sensus binding site for GR with the most proximal located 38bp downstream. Because a weak stimulatory effect of RA on *Lgl1* expression occurred at PN7 and PN10, we carried out these experiments at E20 and PN7.

Fibroblasts isolated at E20 or PN7 were cotransfected with the luciferase expression plasmids p*Lgl1*-Luc and pRSV40-luc (Renilla). Transfected cells were exposed to GC, ATRA, 9-cis-RA, or VitD individually or in combination. Luciferase reporter activities were normalized to Renilla in two sets of experiments. In the first set, we confirmed the effects of GC and RA (ATRA and 9-cis-RA) individually or in combination.
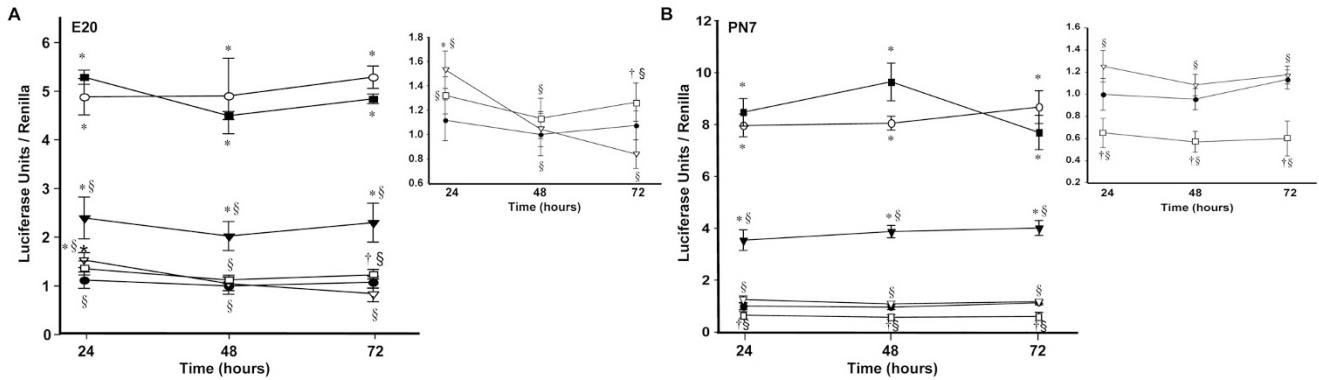
Figure 4. *A*, The effects of GC, 9-cis-RA, and VitD on *Lgl1* promoter activity in E20 rat lung fibroblasts. Rat lung fibroblasts isolated at E20 were cotransfected as in Figure 3*A* and treated with 100 nM cortisol, 10 μM 9-cis-RA, 10 μM VitD, or combinations thereof for 24 to 72 h and assessed as in Figure 3A (*n* = 3). †$p \leq 0.05$ and *$p \leq 0.01$, control *vs* treated. §$p \leq 0.009$, combined treatments *vs* treatment with GC alone. *B*, The effects of GC, 9-cis-RA, and VitD on *Lgl1* promoter activity in PN7 rat lung fibroblasts. Rat lung fibroblasts isolated at PN7 were cotransfected, treated, and assessed as in Fig. 3A (*n* = 3). †$p \leq 0.05$ and *$p \leq 0.01$ control *vs* treated. §$p \leq 6 \times 10^{-4}$, combined treatments *vs* treatment with GC alone. Control (●), 100 nM cortisol (○), 10 μM VitD (□), 100 nM cortisol + 10 μM VitD (▼), 10 μM 9-cis-RA + 10 μM VitD (▽), and 100 nM cortisol + 10 μM 9-cis-RA + 10 μM VitD (■).

In the second set, we evaluated the effects of combined treatments with VitD and 9-cis-RA in the presence or absence of GC.

The *Lgl1*-reporter plasmid demonstrated significant basal activity (Fig. 3*A*). GC, ATRA, and 9-cis-RA effects on *Lgl1*-promoter-driven luciferase reporter activities were entirely consistent with the observed effects of these agents on *Lgl1* mRNA expression levels. In E20 cells, cortisol activated the *Lgl1* promoter at all time points. No significant effect on promoter activity was observed after 24 h exposure to ATRA or 9-cis-RA. Exposure to cortisol plus ATRA or 9-cis-RA suppressed GC-induced promoter activity, which was time dependent.

At PN7 (Fig. 3*B*), GC effects on luciferase promoter activity were more pronounced. At 24 h, exposure to ATRA or 9-cis-RA for 24 h was associated with weak activation of *Lgl1* expression; combined treatment with GC plus RA-augmented luciferase transcriptional activity above that seen with GC alone, consistent with our findings that 24 h exposure to ATRA at PN7 or PN10, weakly stimulated *Lgl1* mRNA expression.

We next assessed the effects of VitD with or without RA on GC induction of *Lgl1* promoter activity (Fig. 4*A* and *B*). VitD effects on *Lgl1* promoter driven luciferase reporter activity were consistent with those on *Lgl1* mRNA expression levels. At E20, VitD alone had no significant effect and VitD plus 9-cis-RA weakly suppressed promoter activity at 72 h only (Fig. 4*A*, insert). By contrast, VitD suppressed GC induction by ~50% at all time points. Similar to the case of *Lgl1* mRNA, treatment with both VitD and 9-cis-RA failed to suppress GC induction of promoter activity. At PN7, VitD alone modestly inhibited *Lgl1* promoter-driven luciferase activity (Fig. 4*B*, insert). VitD plus 9-cis-RA failed to suppress GC induction of the *Lgl1* promoter. The results of *Lgl1* promoter-driven luciferase activity are summarized in Figure 2*C*.

***Deletion of the promoter binding site for GR suppresses GC regulation of* Lgl1.** As an independent confirmation of direct transcriptional regulation of *Lgl1* by GC, we assessed the promoter activity of two constructs in which the binding
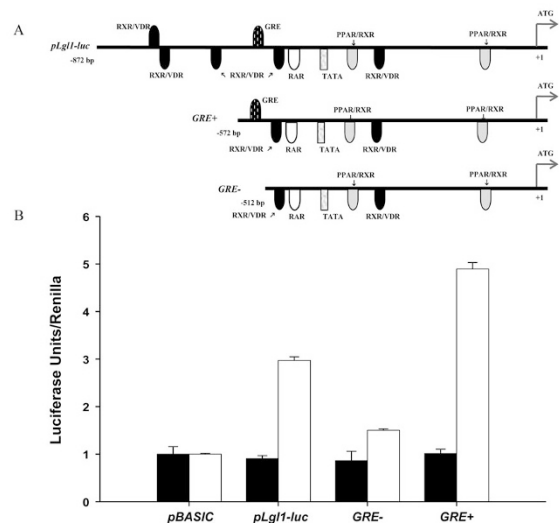

Figure 5. Deletion of GRE in the promoter of *Lgl1* abolishes the response to GC in NIH 3T3 cells. *A*, Schematic representation of luciferase reporter constructs. *B*, Effect of GC on *Lgl1* promoter activity in NIH3T3 cells. NIH3T3 cells were cotransfected with pRSV40-luc (Renilla) and one of the constructs depicted in *A* as described in Materials and Methods section. Cells were treated with serum-free media with (□) or without (■) 100 nM GC, for an additional 48 h. Firefly-luciferase activity, normalized to Renilla, was measured.

site for GR was either eliminated (GRE−) or retained (*GRE*+) (Fig. 5*A*). *Lgl1* promoter-driven luciferase activity was measured after transfection of constructs into NIH 3T3 cells in the absence or presence of GC (Fig. 5*A*). In these cells, the *Lgl1*-reporter plasmid shows a threefold increase in luciferase activity over renilla in the presence of GC (Fig. 5*B*). When transfected with the *GRE*− vector, luciferase activity was suppressed 1.5-fold. Residual *Lgl1* activity above that of *pLgl1-Luc* may reflect concomitant loss of inhibitory transcription factor effects such as the three upstream VDR/RXR sites. This interpretation is also supported by the finding that *GRE*+ shows a greater stimulation than *pLgl1-Luc*.

Enrichment of GC responsive elements adjacent to overlapping VitD and RA binding sites in the promoters of lung
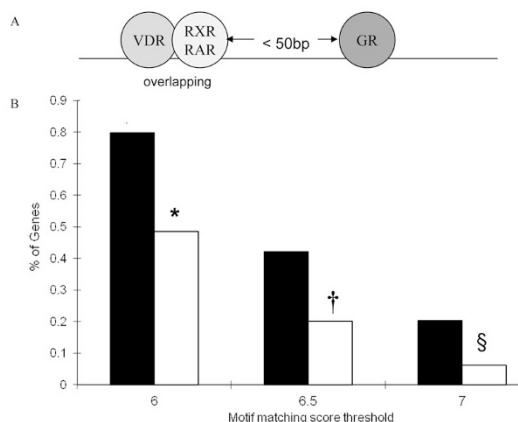
Figure 6. The promoters of genes important in lung development are enriched for VDR/RXR or VDR/RAR binding sites in close proximity to binding sites for GR. *A,* Depiction of the regulatory framework defining the gene enrichment set—VDR/RXR (or VDR/RAR) within 50bp of GR binding site. *B,* The percent of genes in the Gene Enrichment Set using motif matching score thresholds of 6, 6.5, and 7. Selected (■) and Random (□).
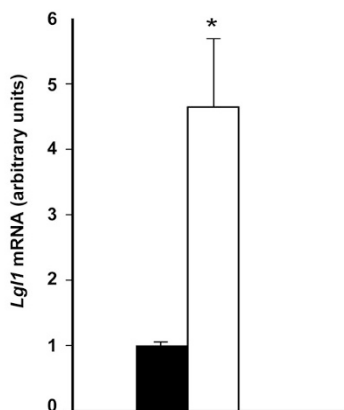

Figure 7. GC stimulation of *Lgl1* mRNA in postnatal rat lung in vivo. Rat lungs isolated at PN7 after daily treatment with 3 mg/kg budesonide from PN4-PN6 (*n* = 6). *Lgl1* mRNA levels were quantified by qRT-PCR. *p* ≤ 0.01, control (■) and treated (□).

development genes. The individual effects of RA and VitD on GC stimulation of *Lgl1* are abrogated when both were present, suggested that steric hindrance of transcription factor binding may occur when overlapping VDR/RXR sites closely abut GR binding sites. Because GC, RA, and VitD regulate multiple aspects of respiratory development, we interrogated the 5′ proximal promoters of 66 genes with known roles in respiratory development (Table 1) for GREs within a fixed distance of overlapping VDR/RXR (or VDR/RAR) compared with a set of 1000 random Refseq genes. The regulatory framework included genes for which the binding site of VDR and RXR (or RAR) are overlapping, and within 50 bp of a GRE (Fig. 6A). The RFE Score describes the proportion of the 66 selected genes that satisfy the regulatory framework (Fig. 6B). The "motif score threshold" is the likelihood that a motif represents a true binding site. Using a threshold of 6.0, 81% (Table 1, Fig. 6) of the Development Gene Set met the enrichment criteria compared with only 48% in the Random Set. When the threshold is raised

to 7, 20% of the Development Set met the enrichment criteria compared with 6% in the Random Set.

In vivo *GC stimulation of* Lgl1 *expression in PN rat.* To determine the stimulatory effects of GC on *Lgl1* expression in developing lung *in vivo*, rat pups were injected daily with 3 mg/kg budesonide from PN4-PN6. *Lgl1* mRNA expression in whole lung RNA of GC-treated pups at PN7 was 4.5-fold (Fig. 7).

DISCUSSION

The alveolar stage of lung development represents a particularly vulnerable period in lung maturation. At birth, failure to successfully adapt to air leads to respiratory distress and may be associated with long-term sequelae (BPD). A growing body of evidence points to roles of GC, RA, and VitD in regulating both lung maturation and inflammation in the newborn period. Our studies focused on the regulation of a single gene, *Lgl1*, while at the same time providing novel information about the complex interactions of GC, RA, and VitD in the regulation of PN lung development.

Information regarding the direct molecular targets of GC and RA in lung development is limited. We were the first to report on the coincident regulation of midkine by GC and RA, providing a potential mechanism for the integration of GC and RA effects on fetal lung development (20). We now report on the coordinate transcriptional regulation of *Lgl1* by GC, RA, and VitD in the PN lung. We show that the interaction of GC and RA on *Lgl1* expression can be additive or antagonistic and that this interaction is dependent on both duration of exposure and developmental stage. Each of VitD and RA is inhibitory when present alone, but together they have no effect on the induction of *Lgl1* by GC, a finding with pharmacologic implications.

GC is the most potent known modulator of *Lgl1*, stimulating expression over 100-fold at PN4. The sensitivity of *Lgl1* to GC may account in part for the high levels of *Lgl1* maintained in rat lung (12) from PN7 to PN14 when circulating levels of GC are low (21). The biphasic effects of 9-cis-RA and ATRA between PN7 and PN10 are also of interest. During this period of increasing levels of active forms of RA, these agents can have opposing effects, stimulating *Lgl1* expression after a short exposure (24 h) and suppressing *Lgl1* levels after longer exposure.

PN GC treatment inhibits alveolarization (22). In animal models, some of the detrimental effects of GC on lung development may be ameliorated by simultaneous RA treatment (23,24). However, RA does not normalize Dex-induced changes in lung function (25). The potential role of VitD as a modulator of *Lgl1* in PN lung is also of interest. To our knowledge, this is the first report of VitD modulating mesenchymal gene expression in developing lung. We speculate that *Lgl1* may be a mesenchymal mediator of VitD effects on epithelial maturation.

GC, RA, and VitD regulation of pulmonary development likely operates through both direct and indirect mechanisms. We believe that this is the first example of coordinate temporal regulation of target gene promoter activity by GC, RA, and

VitD during lung development. Interactions between RA and VitD increase combinatorial possibilities for gene regulation of *Lgl1* by VDR, RXR, and RAR (26). In the presence of increased concentrations of these hormones, steric hindrance by VDR/RXR heterodimers may compromise the effects of either agent alone.

The enrichment of consensus binding sites for GR, VDR, RXR, and RAR in the promoter region of genes essential to normal human lung development suggests that these agents are likely to impact multiple critical developmental processes in the lung. The proximity of GR and VDR/RXR or VDR/RAR sites offers a context for complex fine tuning of such regulatory mechanisms.

Deficiency of *Lgl1* in the newborn period is associated with features of neonatal lung injury in rat (12). *Lgl1* is an essential gene, required before lung formation (14). Haplo-insufficiency for *Lgl1* in knockout mice is associated with a complex respiratory phenotype including delayed histologic maturation, features of inflammation in the newborn period, and altered lung mechanics at maturity. Hormones that regulate lung development also function as mediators of inflammation. Imbalance of these factors in the newborn period is likely to alter respiratory health. The effects of GC and RA in this period are well established. It is also now becoming clear that VitD is an important modulator of immune/inflammatory response (27,28). VDR expression in the lung microenvironment is required for maximal induction of lung inflammation (27).

Specific interventions that augment pulmonary maturation in a temporally appropriate manner have the potential to ameliorate hyperoxic lung injury. Abnormal lung development is also a susceptibility factor for respiratory diseases that present later in life, including chronic obstructive pulmonary disease (COPD) and asthma (29). As such, our findings may also have implications for long-term respiratory health.

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