Identification of glucocorticoid-regulated genes that control cell proliferation during murine respiratory development

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Glucocorticoids play a vital role in fetal respiratory development and act via the intracellular glucocorticoid receptor (GR) to regulate transcription of key target genes. GR-null mice die at birth due to respiratory dysfunction associated with hypercellularity and atelectasis. To identify events associated with this lung phenotype we examined perinatal cellular proliferation rates and apoptotic indices. We demonstrate that compared to wild-type controls, day 18.5 postcoitum (p.c.) GR-null mouse lungs display significantly increased cell proliferation rates (1.8-fold $P < 0.05$) and no change in apoptosis. To examine underlying molecular mechanisms, we compared whole genome expression profiles by microarray analysis at 18.5 days p.c. Pathways relating to cell proliferation, division and cell cycle were significantly down-regulated while pathways relating to carbohydrate metabolism, kinase activities and immune responses were significantly up-regulated. Differential levels of gene expression were verified by quantitative-RT-PCR and/or Northern analysis. Key regulators of proliferation differentially expressed in the lung of 18.5 p.c. GR-null lungs included *p21*^{CIP1} (decreased 2.9-fold, $P < 0.05$), a negative regulator of the cell cycle, and *Mdk* (increased 6.0-fold, $P < 0.05$), a lung growth factor. The more under-expressed genes in 18.5 p.c. GR-null lungs included *Chi3l3* (11-fold, $P < 0.05$), a macrophage inflammatory response gene and *Ela1* (9.4-fold, $P < 0.05$), an extracellular matrix remodeling enzyme. Our results demonstrate that GR affects the transcriptional status of a number of regulatory processes during late fetal lung development. Amongst these processes is cell proliferation whereby GR induces expression of cell cycle repressors while suppressing induction of a well characterized cell cycle stimulator.

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Development of the mammalian fetal lung is a complex process requiring cell proliferation and differentiation leading to the formation of a complex morphological structure that is populated with specific respiratory cell lineages. These processes are facilitated through interactions between mesenchymal cells, epithelial cells and the extracellular matrix (ECM) and are regulated, particularly during the latter stages of development, by mechanical stimuli. The influence of circulating systemic hormones such as retinoids, thyroid hormone and glucocorticoids are also critical, and together these signalling pathways help to induce the migration, proliferation and differentiation of pulmonary cells as well as to determine the architecture of the respiratory tree (Minoo & King, 1994; Mendelson, 2000). The actions of glucocorticoids on lung development have been extensively studied, particularly their role in enhancing surfactant production and promoting distal alveolar development (Grummer & Zachman, 1998; Nakamura *et al.* 2000; Flecknoe *et al.* 2004). Synthetic glucocorticoids, such as betamethasone and dexamethasone are used antenatally to reduce the incidence of preterm infants suffering respiratory distress (Liggins & Howie, 1972; Lyons & Garite, 2002; Jobe & Soll, 2004), yet their benefits are countered by reported side-effects in non-human primates. These include reduced body growth, disrupted immune cell proliferation and perturbed central nervous system development, particularly following the use of multiple doses (Coe & Lubach, 2005).

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Glucocorticoids exert the majority of their effects through binding to the intracellular glucocorticoid receptor (GR), which acts as a ligand-dependent transcription factor. The GR belongs to the steroid receptor family, a subgroup of the nuclear receptor superfamily (Robinson-Rechavi *et al.* 2003). Once bound by ligand, the GR dimerizes and translocates to the nucleus where it can directly or indirectly regulate expression of specific target genes (McKenna & O'Malley, 2002; Robinson-Rechavi *et al.* 2003). Treatment of fetal rat lung explants with dexamethasone retards growth, distorts branching, dilates proximal tubules, and reduces proliferation of epithelial cells within the distal tubules (Oshika *et al.* 1998). Furthermore, a number of biochemical and morphological features associated with accelerated maturation are observed following dexamethasone treatment. These features include flattened or cuboidal epithelial cells lining the distal tubules, rudimentary septa and large airspaces, compressed and attenuated mesenchymal tissue between adjacent epithelial tubules and increased transcription of genes associated with epithelial growth and differentiation including surfactant proteins A, B and C (*Sftpa*, *Sftpb* and *Sftpc*), secretory globulin 1a1 (*Scgb1a1*, *CC10*) and fibroblast growth factor 7 (*Fgf7*).

We and others have previously generated GR-null mice via gene targeting (Cole *et al.* 1995; Brewer *et al.* 2002). On a complete 129sv or C57BL/6 genetic background, all GR-null mice die at birth, due to respiratory dysfunction with severe atelectasis. We further demonstrated that the lungs of GR-null fetal mice have higher proportions of type-II and undifferentiated alveolar epithelial cells (AECs) as well as peri-saccular hypercellularity leading to increased DNA content (Cole *et al.* 2004). These findings suggest that GR activation is not essential for differentiation into the type-II AEC phenotype, but plays an important role in regulating lung cell proliferation and lung structural development and in enhancing the differentiation of primordial AECs.

Although glucocorticoid signalling can induce both cell cycle arrest and apoptosis in different tissues (Sanchez *et al.* 1993; Baghdassarian *et al.* 1998; Zilberman *et al.* 2004), it is unclear how these mechanisms are involved in the glucocorticoid-induced maturation of the developing lung. To further investigate the role of glucocorticoid signalling in fetal lung development, we have now analysed GR-null mice to explore a possible role for glucocorticoid signalling in the regulation of cell proliferation or controlled cell death. We find that the lungs of day 18.5 p.c. GR-null fetal mice display significantly higher rates of cellular proliferation relative to wild-type littermates. To identify genes potentially responsible for the hyper-proliferation, as well as other glucocorticoid-induced gene networks, we have used mouse whole genome microarrays on fetal lung tissue. We show that processes regulating cell proliferation, cell division and the cell cycle are among the most significantly altered ontologies in the lung of day 18.5 p.c. GR-null fetal mice. Quantitative Real Time PCR (qRT-PCR) of selected genes supported this conclusion and demonstrated that mRNA levels for $p21^{CIP1}$, a well characterized cyclin-dependent kinase inhibitor (Corroyer *et al.* 1997), and Fgf7 (Ulich *et al.* 1994), a known stimulator of pulmonary surfactant production during late fetal lung development, were both significantly reduced. On the other hand, mRNA levels for midkine (a retinoic acid induced lung growth factor; Kaplan *et al.* 2003) were significantly increased. We conclude that glucocorticoid signalling regulates hypercellularity of the fetal lung by affecting genes involved specifically in proliferation and not in apoptosis.

Methods

Mice

GR-null mice (on an isogenic 129sv genetic background) were generated by gene targeting as previously described (Cole *et al.* 1995). All animal experimentation was approved by the School of Biomedical Sciences Animal Ethics Committee (project no. BAM/B/2004/36) at Monash University. Pregnant heterozygous female mice were killed using carbon dioxide asphyxiation at day 18.5 p.c. (0.5 days before birth) and fetal mice were collected by caesarian-section. A small piece of tail from fetal mice was collected and used for genotyping at the GR locus by PCR as previously described (Cole *et al.* 1999).

Immunohistochemistry and cell morphometric analysis

Lung tissue was fixed overnight in 4% paraformaldehyde, embedded in paraffin and 5 μm sections were prepared for immunohistochemical analyses. Fetal lung sections were incubated at 60°C (2 h) and then deparaffinized using three washes of xylene. Sections were hydrated using several washes of 100%, 95% and 70% ethanol and finally phosphate buffered saline (PBS). Antigen retrieval was enhanced by microwaving slides (on high) in 0.01 M sodium citrate for 20 min. Sections were then treated with 3% hydrogen peroxide in methanol for 30 min to quench endogenous peroxidases and blocked with 20% normal goat serum in PBS for 30 min. Sections were then incubated with primary antibody: Ki67, a marker for cell proliferation (1 : 100, rabbit anti-mouse, Labvision, Fremont, CA, USA) or cleaved-caspase 3, a marker of cell apoptosis (1 : 250, goat anti-mouse, Cell Signalling, Beverly, MA, USA). Following a 90 min incubation at room temperature, slides were washed in PBS (with 0.1% Tween-20) and incubated for 1 h at room temperature with either a biotinylated goat anti-rabbit secondary antibody (1 : 700; Vector Laboratories, Burlingame, CA,

USA) or a biotinylated donkey anti-goat secondary antibody (1 : 1000, Sigma, St Louis, MO, USA) depending upon the primary antibody used. The sections were again washed in PBS–0.1% Tween 20 and the biotinylated secondary antibody detected using a Vectastain ABC detection kit (Vector laboratories, Burlingame, CA, USA) as per the manufacturer's instructions. Sections were then counterstained with haematoxylin, and then washed, dehydrated and mounted with DPX (Dibutyl polystyrene xylene) mounting medium (Merck Pty Ltd, Kilsyth, Australia). Sections were viewed using a light microscope (×100) and images from proximal and distal regions of the fetal lung from each animal were captured. For cell counting analyses, at least 1000 cells were counted for each animal using multiple sections and different fields of view. Numbers of immuno-positive cells (brown from the horseradish peroxidase/diaminobenzidine (HRP–DAB) reaction) were compared to the number of immuno-negative cells (purple from haematoxylin histological stain) and a labelling index was calculated.

TUNEL analysis

Terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labelling (TUNEL) was performed using an ApopTag Plus Peroxidase In Situ Detection Kit (Chemicon International, Temecula, CA, USA) as per the manufacturer's instructions. Briefly, hydrated fetal lung sections (obtained as per previous immunohistochemistry methods) were treated with proteinase K (20 g ml^{-1}) for 15 min at room temperature, washed in dH_2O, then treated with 3% hydrogen peroxide in PBS for 5 min at room temperature to quench endogenous peroxidases, then washed again in dH_2O. Sections were then treated with terminal deoxynucleotidyl transferase (TdT) at 37°C for 1 h, followed by 10 min at room temperature in stop/wash buffer. Sections were washed in PBS and then treated with antidigoxignenin conjugate for 30 min at room temperature. After washing again in PBS, sections were treated with peroxidase substrate to visualize staining, washed in dH_2O, and counterstained with Nuclear Fast Red. Slides were dehydrated and mounted (as per above).

Isolation of total lung RNA

Total RNA was prepared from isolated fetal mouse lung by homogenization in TRIzol reagent (Gibco/BRL, Rockville, MD, USA), a guanidinium isothiocyanate/phenol-based solution. Following, chloroform extraction, RNA was precipitated from the aqueous phase with isopropanol, washed in 70% ethanol and redissolved in nuclease-free sterile water. RNA was further purified using an RNeasy cleanup kit (Qiagen, Valencia, CA, USA).

Northern blot analysis

Briefly, 8 μg of lung total RNA was separated by electrophoresis on 1.2% agarose/2.2 M formaldehyde gels and blotted onto Genescreen Plus (Perkin Elmer, Boston, MA, USA) nylon membranes for Northern blot analyses. Filters were hybridized with ^{32}P-labelled RNA probes derived from pGEM-T (Promega, Madison WI, USA) plasmids containing a cDNA fragment for Elastase 1 (549 bp). The Ela1 plasmid was linearized to generate a ^{32}P-labelled antisense riboprobe using a riboprobe kit (Promega). All filters were hybridized at 68°C in Ultrahyb buffer (Ambion, Austin, TX, USA) and washed at 75°C in 0.1% SSC, 0.1% SDS three times for 20 min each. Filters were rehybridized with a ^{32}P-labelled 323 bp cDNA fragment of mouse 18S rRNA to control for loading.

Synthesis of cDNA and quantitative real-time PCR

To determine relative levels of specific mRNAs between wild-type and GR-null fetal mouse lung, quantitative real time PCR (qRT-PCR) was performed using cDNA prepared from fetal wild-type and GR-null lung total RNA. To obtain cDNA from fetal lungs, total lung RNA (isolated as previously described) was reverse transcribed into cDNA using random hexamers and M-MLV reverse transcriptase, RNase H Minus, Point Mutant (Promega, Madison WI, USA). cDNA concentration was measured by UV spectrophotometry. With the exception of primer pairs for H2-Ab1 and H2-Eb1 (Superarray Bioscience, Frederick, MD, USA), oligonucleotide primer pairs (forward and reverse) were designed specifically for qRT-PCR analyses using the web based Primer3 software (Rozen & Skaletsky, 2000). Where possible, at least one oligonucleotide primer from any given primer pair was designed to overlap an exon–exon boundary to ensure specific amplification of a given mRNA transcript. qRT-PCR reactions were then optimized for each primer pair with varying concentrations of primer and cDNA. Three wild-type and three GR null fetal lung cDNA samples were used for each gene of interest, and each assay performed in triplicate to account for operator error. Each qRT-PCR assay was repeated at least twice and also included amplification of the 18S ribosomal RNA as a normalizing control. Cycling was performed using Platinum® SYBR® Green qPCR SuperMix (Invitrogen, Carlsbad, CA, USA) on a Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Detection of fluorescence was measured following elongation during cycling. Upon completion of cycling, a melt curve of fluorescence *versus* temperature was generated to ensure a single amplification product for each primer pair. qRT-PCR data were analysed using RotorGene 6.0 software (Corbett Research, Sydney, Australia) and differential expression determined using the comparative $\Delta\Delta C_T$ method (Pfaffl, 2001).

Gene microarray analysis

Microarray analysis was performed using the 36K Mouse Genome Codelink Bioarray System according to manufacturer's instructions (GE Healthcare, Chandler, AZ, USA). Initially, total RNA was isolated from the day 18.5 p.c. GR-null and WT lung as described above. Subsequently, 2 μg of this RNA, together with spiked bacterial RNA, was used to synthesize cDNA. The resultant double-stranded cDNA was purified using a QLAquick purification kit (Qiagen). cRNA was generated via overnight *in vitro* transcription with biotin-11-uridine (Perkin Elmer, Foster City, CA, USA) and purified using an RNeasy column, then quantified by UV spectrophotometry. Ten micrograms of cRNA was then fragmented before hybridizing overnight onto microarray slides. After hybridization and washing, the slides were incubated with Cy^{TM}5-Streptavidin to activate and stain cRNA. Slides were washed and dried, then scanned using a GenePix scanner (Axon Instruments, Union City, CA, USA) and expression analysis was performed using the GE Healthcare Codelink Expression Analysis software. Preliminary data obtained were uploaded and analysed using GeneSifter (VizX Laboratories LLC, Seattle, WA, USA; http://www.genesifter.net). Poor quality (very low-intensity, irregular, contaminated) signals were removed by filtering as they were indistinguishable from background non-specific binding (Treister *et al.* 2005). The Filtered data were normalized to a median value and differential expressed genes were assessed by Student's *t* test (two-tailed, unpaired) and Benjamini–Hochberg correction (to statistically eliminate any false positives). Only genes that passed the intensity filter, *P*-values < 0.05, Benjamini–Hochberg correction and fold change greater than 1.5 were used to generate gene ontology reports (Ashburner *et al.* 2000) and *z*-score reports for further analysis. The data from these arrays are accessible from the European Bioinformatics Institute (EBI) database (http://www.ebi.ac.uk/arrayexpress/).

Statistical analysis

For Northern blot analyses, RNA levels were quantified by densitometry on a Storm Phosphorimager (Molecular Dynamics, Sunnyvale, CA, USA) using ImageQuant software, and standardized to the expression of 18S rRNA with statistical significance set at $P < 0.05$. The sample variance of the cell proliferation index count, microarray expression profiles, and quantitative real time PCR relative expression profiles within wild-type and knockout was analysed using SigmaStat 3.1 software (Systat Software Inc., San Jose, CA, USA). All data were tested and verified as being normally distributed although data for slitrk6 were first log transformed before analysis for normal distribution. Statistical significance was determined using Student's *t* test with statistical significance set at $P < 0.05$.

Results

The lungs of GR-null fetal mice display hyperproliferation, but not apoptosis

To determine whether the hypercellular morphology of the lung is caused by increased proliferation or decreased apoptosis, fetal lung sections were immunostained with the cell proliferation marker Ki67, the apoptotic marker, cleaved-caspase 3, as well as a TUNEL stain which is an additional marker of apoptosis. Ki67 staining was much more abundant in the distal lung, particularly in the interstitial mesenchyme, of GR-null fetal mice compared with WT controls, although staining in proximal airway compartments and endothelial cells appeared unchanged (Fig. 1*A*). The proportion of total lung cells labelled in S-phase with Ki67 (an indicator of relative proliferation rates) was markedly increased from $31.7 \pm 8.3\%$ of all cells in WT to $55.3 \pm 4.3\%$ in GR-null mice ($P < 0.05$; Fig. 1*B*). Cleaved-caspase 3 and TUNEL staining revealed no detectable differences in the level of cell apoptosis between lung sections from WT and GR-null fetal mice; the very low number of immuno-positive cells per whole lung section prevented a statistical analysis (Fig. 1*C* and *D*, respectively). These results indicate that GR activation mediates a repression of cellular proliferative mechanisms in the fetal lung, particularly in the peri-saccular mesenchyme of the distal lung, but appears to have little effect on levels of cell apoptosis.

Microarray analysis of the GR-null fetal lung

To identify genes that may contribute to the abnormal respiratory phenotype of GR-null fetal mice, we used Codelink microarray slides containing 36 000 separate features and representing nearly all genes expressed from the mouse genome. A Genesifter microarray analysis of these data identified 1496 genes that were differentially expressed in the lungs of GR-null compared to WT fetal mice, using a cut-off criterion of 1.5-fold differential expression and with statistical significance set at $P < 0.05$. The data from these arrays are accessible from the European Bioinformatics Institute (EBI) database (http://www.ebi.ac.uk/arrayexpress/). A shortened list showing potential positive and negative GR-regulated genes is shown in Table 1. To confirm the validity of the microarray data, we analysed several of the most strongly differentially expressed genes using quantitative real time PCR (qRT-PCR) in both GR-null and WT fetal mice

(Fig. 2). All data were tested for normal distribution and found to be normally distributed (see Methods). Consistent with our microarray data, we showed a reduction in mRNA levels for *chitinase 3-like-3* (*Chi3l3*, 6.1-fold, $P = 0.002$), *melanin-concentrating hormone receptor1* (*Mchr1*, 10.4-fold, $P = 0.0001$) and *glycine N-methyltransferase* (*Gnmt*, 14-fold, $P = 0.007$), in the lungs of GR-null relative to WT fetal mice. We also showed an increase in mRNA levels for *SLIT and NTRK-like family, member 6* (*Slitrk6*, 10.2-fold, $P = 0.009$), *histocompatibility 2, class II antigen A, beta 1* (*H2-Ab1*, 5-fold, $P = 0.01$) and *histocompatibility 2, class II*

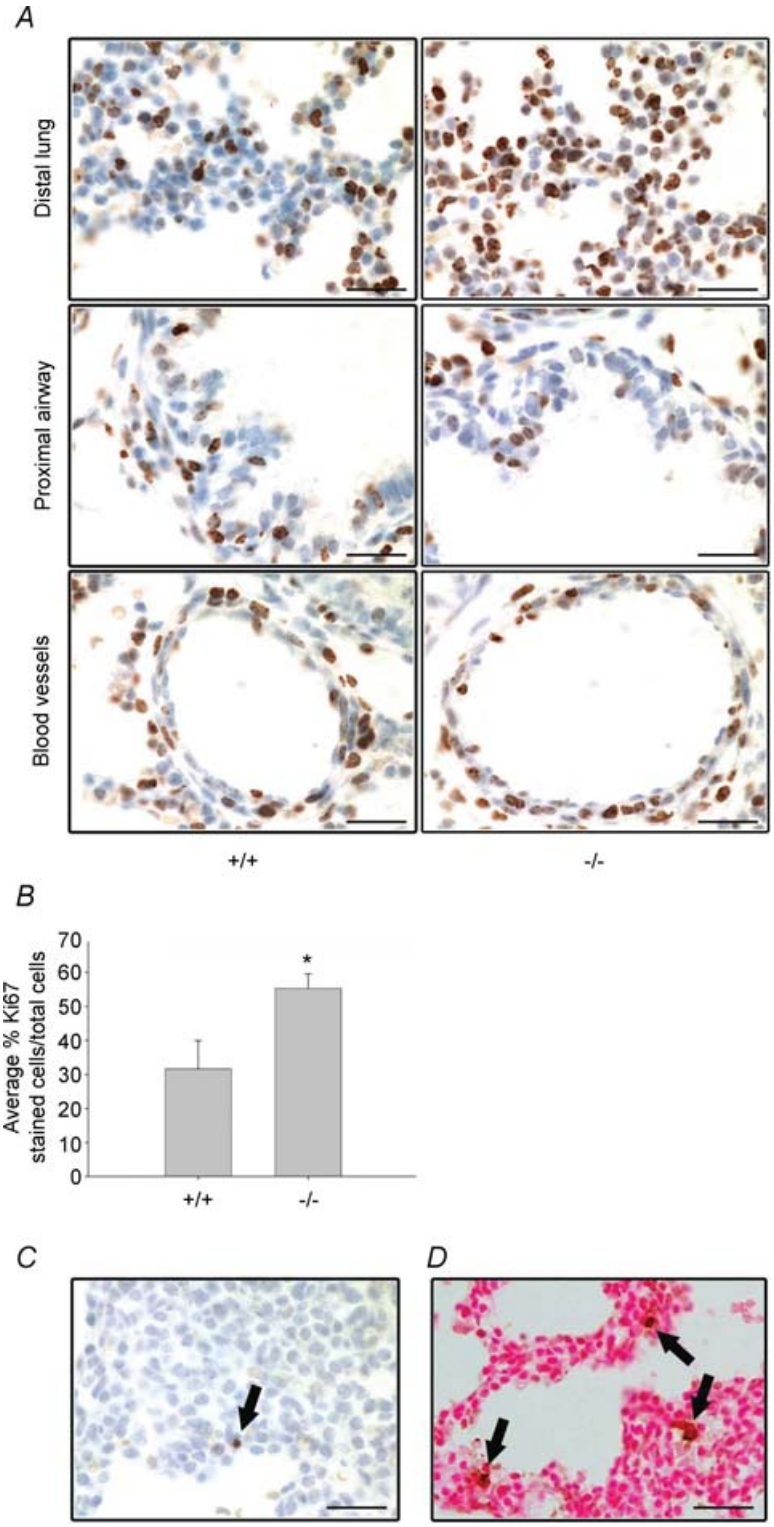

Figure 1. The GR-null fetal lung displays increased cell proliferation in the distal mesenchyme but a normal level of apoptosis
Immunohistochemical analysis of day 18.5 p.c. GR-null and WT lung sections (5 μm) by light microscopy. *A*, Ki67 staining (brown) of proliferating nuclei in WT and GR-null lung in distal lung, proximal airway and blood vessel compartments. Scale bar = 25 μm *B*, quantification of Ki67 staining in whole lung: nuclei were assigned as 'Ki67 positive' or 'Ki67 negative' according to the observed staining pattern. Average proportions of Ki67 stained nuclei were then measured for GR-null and WT from several fields of view ($n = 3$) and means tested for statistical difference using Student's *t* test. Asterisks indicate statistical significance ($P < 0.05$). *C*, cleaved-caspase 3 staining (brown) of apoptotic cells. Arrow indicates a single apoptotic cell. All sections were counterstained with haematoxylin (blue). Scale bar = 25 μm *D*, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end-labelling (TUNEL) staining of apoptotic cells. Arrows indicate a single apoptotic cell. All sections were counterstained with Nuclear Fast Red. Scale bar = 25 μm.

Table 1. Annotated target genes from Genesifter DNA Microarray analysis: Differentially expressed genes in fetal lung RNA (day 18.5 pc) from GR-null mice compared to WT fetal mice ($n = 3$)

Accession No.	Gene name	Fold change*	*P* value†	Function
A. Genes with mRNA levels significantly lower in GR-null relative to WT (GR up-regulated)				
NM_009892	Chitinase 3-like 3 (Chi3l3)	11	0.026	Inflammatory response
AW047155	G protein coupled receptor 24 (Gpr24)	9.81	0.011	Rhodopsin-like receptor activity
NM_033612	Elastase 1, pancreatic (Ela1)	9.42	0.022	Serine-type endopeptidase activity
AI893740	Glycine N-methyltransferase (Gnmt)	9.3	0.019	Glycine N-methyltransferase activity
NM_009978	Cystatin-related epididymal spermatogenic protein (Cst8)	7.86	0.023	Cysteine protease inhibitor activity
BC061226	Similar to class-α glutathione S-transferase	6.08	0.005	Unknown
NM_007894	Eosinophil-associated ribonuclease (Ear1)	5.79	0.030	Endonuclease activity
NM_144547	Anti-Mullerian hormone type 2 receptor (Amhr2)	5.15	0.005	Protein amino acid phosphorylation
NM_007607	Carbonic anhydrase 4 (Car4)	5.1	0.028	Extracellular pH buffer
NM_010405	Hemoglobin X, α-like embryonic chain in Hba complex (Hba-x)	4.96	0.023	Oxygen transporter activity
NM_011134	Paraoxonase 1 (Pon1)	4.89	0.027	Antioxidant enzyme
NM_153801	Steroid 5 α-reductase 2-like 2 (Srd5a2l2)	4.79	0.007	Regulator of androgen activity
NM_134102	Phospholipase A1 member A (Pla1a)	4.67	0.030	Lipid metabolism
NM_018857	Mesothelin (Msln)	4.66	0.027	Unknown
NM_016693	Mitogen-activated protein kinase kinase kinase 6 (Map3k6)	4.66	0.005	Protein amino acid phosphorylation
B. Genes with mRNA levels significantly higher in GR-null relative to WT (GR down-regulated)				
NM_175499	SLIT and NTRK-like family, member 6 (Slitrk6)	8.27	0.037	Neuron development
NM_207105	Histocompatibility 2, class II antigen A, beta 1 (H2-Ab1)	5.1	0.031	Antigen processing
NM_010382	Histocompatibility 2, class II antigen E beta (H2-Eb1)	4.84	0.031	Antigen processing
NM_010378	Histocompatibility 2, class II antigen A, α (H2-Aa)	3.36	0.040	Antigen processing
NM_021886	Centromere autoantigen H (Cenph)	3.17	0.037	Chromosome segregation
NM_019696	Carboxypeptidase X 1 (M14 family) (Cpxm1)	3.16	0.023	Cellular macromolecule metabolism
NM_178788	DCMP deaminase (Dctd)	2.8	0.023	Nucleotide biosynthesis
NM_183014	Zinc finger protein 184 (Kruppel-like) (Zfp184)	2.65	0.031	Regulation of transcription
NM_172453	DNA helicase-like protein (Pif1)	2.65	0.041	Helicase activity
NM_009765	Breast cancer 2 (Brca2)	2.62	0.039	DNA repair
AK084628	Fibrillin 2 (Fbn2)	2.57	0.039	Extracellular matrix microfibrils
NM_008318	Integrin binding sialoprotein (Ibsp)	2.59	0.044	Bone remodeling
NM_010387	Histocompatibility 2, class II, locus Mb1 (H2-DMb1)	2.52	0.035	Antigen processing
NM_009472	Unc-5 homolog C (C. elegans) (Unc5c)	2.52	0.029	Brain development
NM_011234	RAD51 homolog (S. cerevisiae) (Rad51)	2.47	0.031	DNA repair

*Measured as a ratio of normalized signal spot intensities of WT/KO (GR-up-regulated) or KO/WT (GR-down-regulated). †Determined by a Student's *t* test between the mean of WT and GR-null lung normalized signal spot intensities.

antigen E, beta (*H2-Eb1*, 6-fold, $P = 0.004$) relative to WT controls. To further validate our microarray data, mRNA levels of *Elastase 1* (Ela1), one of the most differentially expressed genes (Table 1), were investigated using both qRT-PCR and Northern blot analysis. Consistent with our microarray data, mRNA levels of *Ela1* were significantly reduced in the fetal lung of GR-null mice using both qRT-PCR (25-fold, $P = 0.005$) and Northern blot analysis (Fig. 2).

Microarray analysis identified altered gene ontologies in the GR-null fetal lung

The use of Genesifter microarray analysis software also allowed for the investigation of significantly affected gene ontologies. These ontologies define gene groups based on biological function and are categorized according to the tenets of the Gene Ontology Consortium (Ashburner *et al.* 2000), which classify differentially expressed genes according to three organizing principles: 'cellular component', 'biological process' and 'molecular function'. Within these ontologies are various subontologies which provide gene lists based on increasing levels of functional specificity. For each ontology, a '*z*-score' for wild-type (WT/KO) and knockout (KO/WT) is listed. Positive *z*-score values indicate a greater number of genes present in that ontology than would be expected by chance, whereas negative values indicate a lesser number of genes present in that ontology than expected by chance. We present ontologies over-represented in wild-type (relative to KO) and knockout (relative to WT) fetal mice, which have *z*-score values greater than 2.0 (Tables 2 and 3).

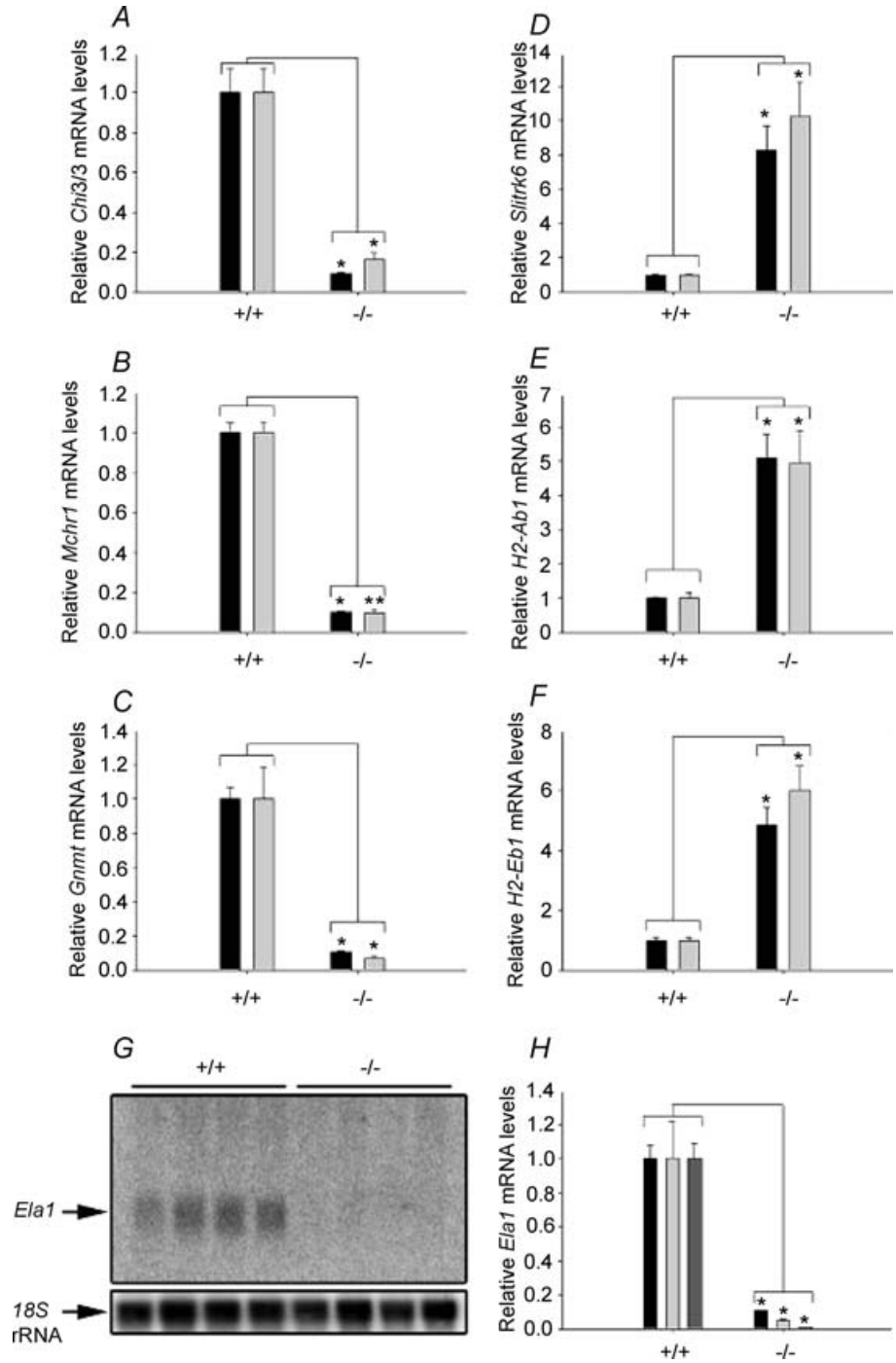

Figure 2. Comparative mRNA levels of the most significant microarray gene targets in the lungs of fetal GR-null mice

Microarray, quantitative real-time PCR and Northern blot analysis of the most differential microarray gene targets in the GR-null fetal lung. Relative mRNA levels were determined by qRT-PCR in the GR-null fetal lung relative to WT controls ($n = 3$) for *Chi3l3* (*A*), *Mchr1* (*B*), *Gnmt* (*C*), *Slitrk6* (*D*), *H2-Ab1* (*E*) and *H2-Eb1* (*F*). *G*, Northern blot analysis of *Ela1* mRNA levels in the GR-null fetal lung relative to WT controls ($n = 4$). Northern blot quantification, microarray ($n = 3$) and quantitative real-time PCR ($n = 3$) analysis of mRNA levels for *Ela1* in the GR-null fetal lung relative to WT. A single asterisk indicates statistical significance of $P < 0.05$ and double asterisks indicates statistical significance of $P < 0.001$. Statistical significance for microarray analysis and qRT-PCR analysis is calculated separately. Black bars, microarray analysis; light grey bars, qRT-PCR analysis; dark grey bars, Northern blot analysis.

Table 2. Ontologies most down-regulated in the lungs of d18.5 GR-null mice

Ontology	List	Genes ↑+/+	Genes ↑−/−	Array	+/+ z-score	−/− z-score
Biological process						
DNA metabolism	37	29	8	442	5.07	−2.64
DNA repair	15	13	2	141	4.78	−1.7
Response to endogenous stimulus	18	14	4	173	4.38	−1.3
DNA replication	12	10	2	108	4.2	−1.26
Response to DNA damage stimulus	17	13	4	168	4.03	−1.24
Antigen processing	6	4	2	30	3.58	0.64
Cell division	17	12	5	180	3.28	−1.02
Cell cycle	51	26	25	529	3.18	0.49
M phase of meiotic cell cycle	9	5	4	52	3.06	1.21
Meiosis	9	5	4	52	3.06	1.21
Meiotic cell cycle	9	5	4	52	3.06	1.21
Integrin-mediated signalling pathway	13	6	7	71	2.98	2.31
M phase	17	10	7	156	2.85	0.11
Regulation of cell proliferation	28	12	16	212	2.66	2.35
Immune cell activation	12	8	4	122	2.62	−0.56
Cell activation	12	8	4	123	2.59	−0.58
Cell adhesion	42	21	21	455	2.54	0.33
Nuclear import	6	4	2	46	2.5	0.01
Protein import into nucleus	6	4	2	46	2.5	0.01
T cell activation	6	5	1	68	2.36	−1.16
Protein import	7	4	3	51	2.25	0.56
Chromosome organization and biogenesis	14	11	3	215	2.18	−2.12
Chromatin modification	9	7	2	118	2.16	−1.4
Negative regulation of cell proliferation	11	5	6	74	2.14	1.62
Molecular function						
Chromatin binding	12	6	6	70	3.04	1.73
RNA binding	30	20	10	443	2.4	−2.21
Protein kinase binding	7	3	4	31	2.4	2.33
Kinase binding	7	3	4	32	2.33	2.26
Microtubule motor activity	7	4	3	51	2.27	0.53
Helicase activity	7	7	0	115	2.25	−2.3
Protein dimerization activity	14	7	7	119	2.15	0.82
Protein homodimerization activity	8	4	4	54	2.14	1.1
Transcriptional repressor activity	13	7	6	125	2.01	0.24
Cellular component						
Chromosome, pericentric region	6	6	0	40	4.78	−1.33
Chromosome	17	14	3	219	3.38	−2.13
Nucleus	204	111	93	3144	3.28	− 4.18
Intracellular membrane-bound organelle	330	152	178	4741	2.69	−2.16
Membrane-bound organelle	330	152	178	4746	2.68	−2.18
Intracellular organelle	370	168	202	5331	2.65	−2.22
Chromosomal part	12	10	2	180	2.36	−2.1
Nuclear chromosome	6	4	2	51	2.26	−0.11
Intracellular part	413	180	233	5912	2.21	−1.63
Condensed chromosome	6	3	3	34	2.19	1.33

This list contains all ontologies over-represented in wild-type lungs relative to GR-null lungs at day 18.5 p.c. Criteria for inclusion in the table were: an ontology containing = 5 genes with a positive *z*-score of = 2.0 in the '+/+z-score' column. For the 'Biological process' and 'Cellular component' sets, the top 25 and 10 ontologies are shown, respectively.

As shown by *z*-score analysis glucocorticoid signalling via GR exerts effects on a wide range of biological processes, molecular functions and cellular components in the fetal lung. Since this investigation primarily focuses on glucocorticoid-mediated effects on whole lung morphogenesis and identifying genes involved in the regulation of cell proliferation, we concentrated our analysis on biological function ontologies. Within this

Table 3. Ontologies most up-regulated in the lungs of d18.5 GR-null mice

Ontology	List	Genes ↑+/+	Genes ↑−/−	Array	+/+ z-score	−/− z-score
Biological process						
Regulation of kinase activity	13	2	11	74	−0.01	4.49
Cellular polysaccharide metabolism	7	0	7	36	−1	4.48
Polysaccharide metabolism	7	0	7	36	−1	4.48
Regulation of transferase activity	13	2	11	75	−0.03	4.44
Cellular carbohydrate metabolism	24	2	22	214	−1.62	4.35
Regulation of MAPK activity	8	1	7	41	−0.11	4.03
Energy derivation by oxidation of organic compounds	15	2	13	107	−0.54	4.02
Positive regulation of protein kinase activity	7	0	7	42	−1.09	3.95
Carbohydrate metabolism	30	3	27	307	−1.9	3.93
IMMUNE response	51	15	36	461	0.72	3.79
Cofactor metabolism	20	4	16	153	−0.08	3.77
Group transfer coenzyme metabolism	7	0	7	45	−1.12	3.72
Positive regulation of transferase activity	7	0	7	46	−1.14	3.65
Defense response	58	17	41	559	0.48	3.62
Response to biotic stimulus	59	17	42	580	0.32	3.58
Glucose metabolism	9	0	9	72	−1.42	3.44
Monosaccharide metabolism	11	0	11	98	−1.66	3.39
Intracellular signalling cascade	63	16	47	712	−0.8	3.12
Biosynthesis	82	26	56	883	0.43	3.11
Immune cell migration	5	1	4	23	0.48	3.09
Molecular function						
Lyase activity	22	7	15	133	1.83	3.93
Haematopoietin/interferon-class (D200-domain) cytokine receptor activity	9	1	8	54	−0.39	3.77
Chemokine activity	6	0	6	36	−1	3.62
Hydro-lyase activity	8	1	7	47	−0.24	3.54
Chemokine receptor binding	6	0	6	37	−1.01	3.54
Carbon-oxygen lyase activity	10	3	7	56	1.23	2.99
G-protein-coupled receptor binding	6	0	6	48	−1.16	2.77
Kinase activity	68	20	48	783	−0.26	2.5
Iron ion binding	22	4	18	235	−0.95	2.5
Protein kinase binding	7	3	4	31	2.4	2.33
Cellular component						
Integral to organelle membrane	7	1	6	35	0.05	3.8
Intrinsic to organelle membrane	7	1	6	38	−0.03	3.54
Membrane	365	118	247	5110	−2.41	2.83
Golgi membrane	10	3	7	62	1.03	2.76
Basolateral plasma membrane	6	0	6	50	−1.18	2.73
Extracellular space	146	46	100	1855	−0.69	2.69
Golgi apparatus	24	7	17	221	0.41	2.57
Extracellular region	158	51	107	2034	−0.65	2.52
Microsome	11	1	10	113	−1.2	2.45
Extracellular region part	152	50	102	1950	− 0.46	2.39

This list contains all ontologies over-represented in GR-null lungs relative to wild-type lungs at day 18.5 p.c. Criteria for inclusion in the table were: an ontology containing = 5 genes with a positive *z*-score of = 2.0 in the '−/− z-score' column. For the 'Biological process', 'Molecular function' and 'Cellular component' sets, the top 20, 10 and 10 ontologies are shown, respectively.

grouping the most significantly up-regulated genes in GR-null fetal lungs are involved in regulating kinase activity, carbohydrate metabolism and immune response, indicating that glucocorticoid signalling normally represses these activities in the fetal lung of WT mice (Table 3). In contrast, glucocorticoid signalling via GR was shown to induce biological functions predominantly relating to DNA metabolism, replication and repair (Table 2). Interestingly, processes such as regulation of cell proliferation, cell division and cell cycle were down-regulated and not up-regulated in the lungs of GR-null fetal mice (Table 2). We suggest therefore

that glucocorticoid signalling via GR is likely to be important for inducing expression of genes responsible for inhibition of fetal lung cell proliferation rather than repressing gene expression of mechanisms which increase cell proliferation; this is analysed in greater detail below. Furthermore, as expected, we did not find any significantly affected ontology relating to cellular apoptosis.

Gene expression analysis of ontologies related to regulation of cell proliferation

To investigate glucocorticoid-mediated regulation of genes involved with the regulation of cell proliferation in more detail, we analysed differential gene expression of several targets which are involved in the 'regulation of cell proliferation' ontology and important for cell cycle control (Table 4). We investigated *cyclin-dependent kinase inhibitor 1A* ($p21^{CIP1}$, *cdkn1a*) because its gene product is a well characterized cyclin-dependent kinase inhibitor (CKI) which arrests the cell cycle in the G1 phase of mitosis (Golias *et al.* 2004). Using qRT-PCR analysis, we found a 2.9-fold reduction in mRNA levels for $p21^{CIP1}$ ($P = 0.02$) in the lungs of GR-null fetal mice relative to WT (Fig. 3). Another CKI from our microarray list, *cyclin-dependent kinase inhibitor 1C* ($p57^{KIP2}$, *Cdkn1c*) was also investigated. Although not significant (1.2-fold, $P = 0.2$), a trend for reduction in mRNA levels was detected in the lungs of GR-null fetal mice (Fig. 3). A 6-fold increase in *midkine* mRNA levels ($P = 0.007$), a retinoic-acid induced growth factor (Kaplan *et al.* 2003), was also observed in the lungs of GR-null fetal mice relative to WT controls (Fig. 3). *Fgf7*, which promotes surfactant production during late gestation (Chelly *et al.* 2001) displayed a 1.8-fold reduction in mRNA levels ($P = 0.04$) (Fig. 3).

Discussion

Glucocorticoids are important for the coordinate regulation of cell differentiation and proliferation in the developing mammalian lung. A key morphological feature during the final stages of lung maturation is the thinning of the mesenchymal septum to reduce the air–blood barrier and facilitate the process of alveolarization. Previous work from our laboratory and others have demonstrated that the fetal lungs of GR-null mice are hypercellular, displaying a thickened septum, and the distal airways are structurally immature at birth (Cole *et al.* 1995; Brewer *et al.* 2002). Furthermore, the lungs of GR-null mice exhibit marked alterations in the proportions of AECs, with a reduction in the numbers of type I AECs and an increase in the number of type II AECs (Cole *et al.* 2004). Given the marked hypercellular phenotype, we have investigated whether this is associated with changes in cellular proliferation in the lung just prior to birth in GR-null mice. Our findings indicate that the hypercellularity of the lung in GR-null fetal mice is likely to result from increased cellular proliferation, not from reduced levels of apoptosis. In addition, given that the GR mediates its direct effects primarily as a ligand-bound transcription factor, we have identified potential gene candidates which mediate the glucocorticoid regulation of cell proliferation in the fetal lung.

Using Ki67 immunostaining, we found that cellular proliferation was increased 1.7-fold in GR-null fetal lungs at day 18.5 p.c. relative to WT controls. This predominantly occurs in the distal lung mesenchyme, with no detectable difference in proximal airway compartments or large blood vessels. These results are similar to a previous finding that corticotrophin-releasing hormone (CRH) knockout mice, where endogenous glucocorticoids are markedly reduced, display significantly increased cellular proliferation at day 18.5 p.c. (Muglia *et al.* 1999). Furthermore, dexamethasone treated fetal rat lungs show a decrease in proliferating cell nuclear antigen (PCNA) protein levels at 20 days gestation (Arai *et al.* 2005). It is likely therefore that glucocorticoid signalling functions to restrain proliferative mechanisms in the distal parenchyma of the fetal lung, probably to facilitate thinning of the interalveolar septum and surrounding mesenchyme. Interestingly, studies have shown that day 17.5 p.c. and 18.5 p.c. fetal mouse explants exposed to dexamethasone have increased cell proliferation at low (0.1 nM) doses whereas at high doses (10–100 nM) cell proliferation is reduced (Shan *et al.* 2004). On the other hand, evidence supports the concept that glucocorticoids induce cellular proliferation earlier in lung development. For example, fetal sheep given single and multiple doses of dexamethasone between 76 and 105 days GA (term = 150 days), exhibited increased cell proliferation in airway epithelium and distal lung parenchyma (Pua *et al.* 2005). Interestingly, a recent study involving cell specific GR expression in lung epithelium in mice on a GR-null background has shown reduced lung cellularity compared to lungs from GR-null fetal mice causing only a mild reduction in the septum between adjacent airways (Gagnon *et al.* 2006). This indicates that glucocorticoid signalling between the epithelium and mesenchyme is likely to be necessary for reduced cell proliferation.

Using cleaved-caspase 3 and TUNEL immunostaining, we have detected very low levels of apoptosis in the lung of day 18.5 p.c. fetal mice, which did not differ between GR-null and WT control fetal mice. This was also observed through gestational days 14.5–18.5 p.c. in the lungs of CRH-KO fetal mice (Muglia *et al.* 1999). These data are in contrast to earlier results which demonstrated considerably higher levels of apoptosis in the fetal lung of rabbits and rats late in gestation (Kresch *et al.* 1998; De Paepe *et al.* 1999). Furthermore, it has been suggested that apoptotic activity in the fibroblasts of the fetal rat lung distal parenchyma at day 17.5 p.c. facilitates thinning

Table 4. Differential gene expression in ontologies relating to regulation of cell proliferation in the lungs of d18.5 GR-null mice

Genes with decreased expression in GR-null (relative to GR+/+)	Genes with increased expression in GR-null (relative to GR+/+)
Regulation of cell proliferation	
Baculoviral IAP repeat-containing 6	Androgen-induced proliferation inhibitor
Calcitonin receptor-like	CD276 antigen
Calcium homeostasis endoplasmic reticulum protein	Cell division cycle associated 7
Caveolin 2	Fanconi anaemia, complementation group A
Caveolin, caveolae protein 1	Glypican 3
Cell division cycle 2-like 5	Integrin β1 (fibronectin receptor β)
Cyclin-dependent kinase inhibitor 1A (P21)	Jumonji, AT rich interactive domain 2
Fibroblast growth factor 7	Mdm2, transformed 3T3 cell double minute p53 binding protein
Homeodomain interacting protein kinase 1	Ornithine decarboxylase, structural 1
Leukaemia inhibitory factor receptor	Parathyroid hormone receptor 1
Prokineticin 1	Pre B-cell leukaemia transcription factor 1
Schlafen 1	Protein-kinase, interferon-inducible double stranded RNA dependent inhibitor
Sphingosine kinase 1	
Tumor necrosis factor	
Vascular endothelial growth factor A	
Zinc finger and BTB domain containing 16	
Cell division	
Cell division cycle 2-like 5	Cell division cycle associated 1
Protein phosphatase 1, catalytic subunit, α isoform	Centrosomal protein 55
SLIT-ROBO Rho GTPase activating protein 2	Cyclin B1
Stromal antigen 1	Cyclin T1
Transforming, acidic coiled-coil containing protein 1	Minichromosome maintenance deficient 5, cell division cycle 46
Zinc finger and BTB domain containing 16	Nanog homeobox
protein regulator of cytokinesis 1	
Rac GTPase-activating protein 1	
Septin 9	
SLIT-ROBO Rho GTPase activating protein 2	
Cell cycle	
Amyloid β (A4) precursor protein	Aurora kinase B
Calcium/calmodulin-dependent protein kinase II α	CDK2 (cyclin-dependent kinase 2)-associated protein 1
Cell division cycle 2-like 5	Centromere protein H
Cyclin-dependent kinase inhibitor 1 A (P21)	Centrosomal protein 55
Cyclin-dependent kinase inhibitor 1C (P57)	Chromatin assembly factor 1, subunit A (p150)
Dual specificity phosphatase 1	Chromatin assembly factor 1, subunit B (p60)
Fibroblast growth factor 7	Cyclin B1
Gardner-Rasheed feline sarcoma viral (Fgr) oncogene homologue	Cyclin T1
Growth arrest and DNA-damage-inducible 45 γ	Cytoplasmic polyadenylation element binding protein 1
Harvey rat sarcoma oncogene, subgroup R	discs, large homologue 7 (*Drosophila*)
Kinesin family member 11	Extra spindle poles-like 1 (*S. cerevisiae*)
MutL homologue 3 (*E coli*)	Fanconi anaemia, complementation group A
Neuroblastoma, suppression of tumorigenicity 1	Glucocorticoid receptor DNA binding factor 1
P53-associated parkin-like cytoplasmic protein	Integrin β 1 (fibronectin receptor β)
Protein phosphatase 1, catalytic subunit, α isoform	Mdm2, transformed 3T3 cell double minute p53 binding protein
Replication protein A1	Minichromosome maintenance deficient 2 mitotin (*S. cerevisiae*)
Retinoblastoma-like 2	Minichromosome maintenance deficient 6 (MIS5 homologue, *S. pombe*)
S100 calcium binding protein A6 (calcyclin)	protein regulator of cytokinesis 1
Schlafen 1	Rac GTPase-activating protein 1
Sestrin 1	RAD51 homologue (*S. cerevisiae)*
Stromal antigen 1	Regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing
Testis specific gene A2	Septin 9
Transforming, acidic coiled-coil containing protein 1	Topoisomerase (DNA) II β binding protein
V-crk sarcoma virus CT10 oncogene homologue (avian)	TPX2, microtubule-associated protein homologue (*Xenopus laevis*)
Vascular endothelial growth factor A	Transformation/transcription domain-associated protein

This list contains all genes described as showing differential mRNA levels in the lungs of GR-null fetal mice using Genesifter analysis, and which meet the criterion for the 'regulation of cell proliferation', 'Cell division' and 'Cell cycle' ontologies. It should be noted that genes can meet the criterion for multiple ontologies.

of the interalveolar walls (Bruce *et al.* 1999). While it is well known that apoptosis is difficult to detect *in vivo*, it is hard to reconcile the different findings between these studies. Indeed, all these studies have used the same apoptotic-detection techniques, such as activated caspase 3 immuno-labelling, TUNEL labelling and electron microscopy, but have found very different results. Perhaps apoptosis does play a role in the thinning of the alveolar septum, but only at specific developmental time points. Indeed, most of the studies mentioned above which describe apoptotic effects focus on the postnatal rodent lung where final alveolarization and microvascularization take place. Therefore, further studies are needed to definitively describe the possible role of cell apoptosis in fetal lung remodelling over a wide range of developmental stages.

Whole genome microarray expression analysis was performed to investigate differential gene expression in the lungs of day 18.5 p.c. GR-null mice. Our data have been analysed by Genesifter bioinformatic software and have been validated by comparing the expression levels of the most differentially expressed genes by qRT-PCR. Most of these genes demonstrated an expression pattern consistent with our gene microarray analysis and therefore this indicates that the microarray data represent an accurate overview of gene expression profiles in the day 18.5 p.c. fetal lung lacking glucocorticoid signalling via GR. Several of the genes which had the strongest differential expression are known glucocorticoid targets, albeit in other tissues. For instance, *Gnmt* is induced by glucocorticoids in hepatocytes (Stipanuk, 2004) and *H2-Ab1* is suppressed at the transcriptional level by corticosteroids in B cell and macrophages (Celada *et al.* 1993). Gnmt catalyses the transmethylation of methionine to form sarcosine, which is an important detoxifying process in the liver. Conversely, H2-Ab1 and also H2-Eb1, H2-Aa and H2-DMb1 (shown in Table 1) have a vital role in specific recognition by the immune system, presenting peptide antigens to T cells (Ting & Trowsdale, 2002).

Of the other glucocorticoid-regulated gene targets, Chi3l3, otherwise known as Ym1, is a heparin binding lectin which localizes to alveolar macrophages in the late gestational fetal lung (Hung *et al.* 2002). However, others genes such as *Ela1*, *Mchr1* and *Slitrk6* are novel glucocorticoid targets whose functions and/or mRNA expression have not been previously described in the fetal lung. Ela 1 or pancreatic elastase is a member of the pancreatic family of serine proteases and can hydrolyse elastin but also a variety of collagens (Swift *et al.* 1984). Mchr1 binds the orexigenic melanin concentrating

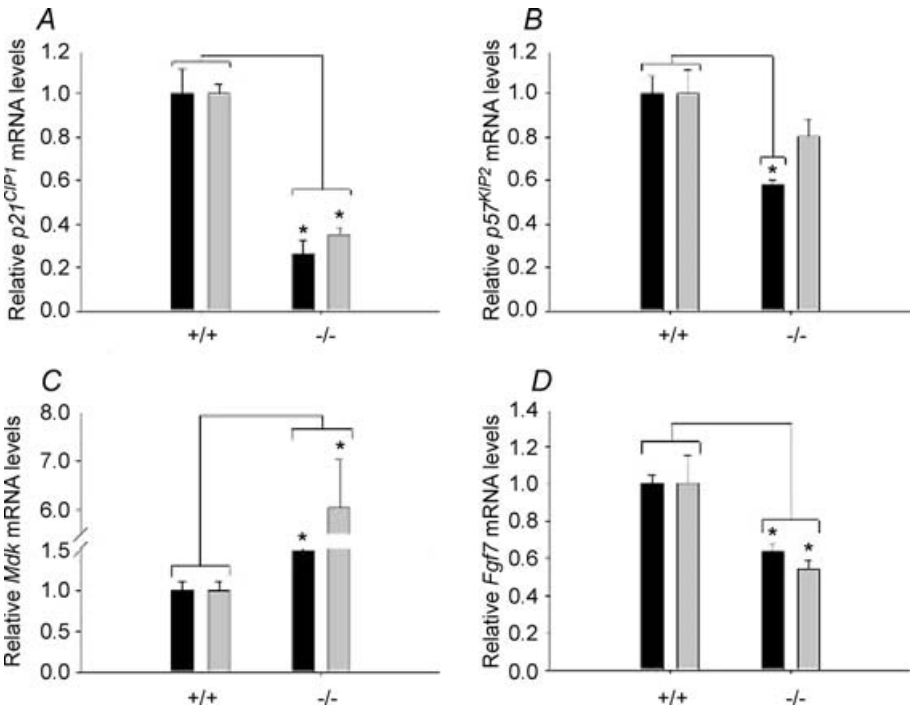

Figure 3. Comparative mRNA levels of gene targets relating to regulation of cell proliferation in the lungs of fetal GR-null mice and WT controls
Microarray and quantitative real-time PCR analysis of genes related to regulation of cell proliferation in the GR-null lung. Relative mRNA levels were determined by qRT-PCR in the GR-null fetal lung relative to WT controls ($n = 3$) for $p21^{CIP1}$ (*A*), $p57^{KIP2}$ (*B*), *midkine* (*mdk*) (*C*), *Fgf7* (*D*). Single asterisk indicates statistical significance of $P < 0.05$. Statistical significance for microarray analysis and qRT-PCR analysis is calculated separately. Black bars, microarray analysis; light grey bars, qRT-PCR analysis.

hormone (MCH) to regulate energy balance and body weight and is predominantly expressed throughout the brain, particularly in the hypothalamus (Saito *et al.* 2001). Finally, slitrk6 is a member of the Slitrk family of neuronal transmembrane proteins, which are regulators of neurite outgrowth (Aruga, 2003). Further investigation on the regulation of these genes in the fetal lung will be necessary to elucidate their specific roles in respiratory development.

Ontology analysis of our microarray data has also provided several interesting avenues of future research. Glucocorticoid signalling via GR was shown to induce expression of genes which meet the criterion for ontologies predominantly relating to DNA metabolism, replication and repair. Conversely glucocorticoid signalling via GR was shown to repress expression of genes which meet the criterion for ontologies relating to kinase activity, carbohydrate metabolism and immune response. We do not discuss these processes in depth as this study focuses on glucocorticoid-induced effects on cell proliferation and apoptosis. Further investigation will be needed to understand the glucocorticoid regulation of these processes in the fetal lung. Our finding that glucocorticoid signalling via GR induces gene expression of processes relating to regulation of cell proliferation, cell cycle and cell division in the fetal lung is consistent with the finding of altered proliferation rates in the lung of GR null fetal mice. Furthermore, our finding that ontologies relating to apoptosis were not significantly affected in GR-null fetal mice is consistent with our immunostaining results of no change in either cleaved-caspase 3 or TUNEL staining. We suggest therefore that glucocorticoid signalling via GR has no effect on gene expression relating to apoptosis, but induces expression of genes responsible for the inhibition of cell proliferation in the fetal lung.

Glucocorticoid signalling has previously been shown to induce cell cycle arrest in a variety of tissues (Sanchez *et al.* 1993; Baghdassarian *et al.* 1998) and several studies have investigated glucocorticoid-mediated regulation of the important proteins controlling cell cycle progression, including cyclins, cyclin-dependent kinases (CDKs) and CKIs (Corroyer *et al.* 1997; Greenberg *et al.* 2002). Thus, we selected several genes in our microarray list from ontology groups relating to regulation of cell proliferation, cell cycle and cell division for validation by qRT-PCR. We have found a significant reduction in mRNA levels of the *CKI* family member *p21^{CIP}*, in the lungs of GR-null fetal mice. Furthermore, qRT-PCR indicated a trend reduction in p57^{KIP2} expression in the GR-null fetal lung, a finding supported by the significant reduction observed following microarray analysis. Glucocorticoids have previously been shown to regulate protein levels of both CKIs in isolated and cultured lung epithelium. Dexamethasone treatment induced p21^{CIP1} protein levels in early postnatal lung and lung cancer cell lines (Corroyer *et al.* 2002; Greenberg *et al.* 2002) and a glucocorticoid responsive region has been characterized in the promoter region of the *p21^{CIP1}* gene (Cha *et al.* 1998). Similarly, dexamethasone increased *p57^{KIP2}* mRNA levels in human bronchial epithelial cells (Puddicombe *et al.* 2003) and a functional GRE has been described in the promoter region of the human *p57^{KIP2}* gene (Alheim *et al.* 2003), Interestingly, another report has shown an increase of protein, but not increased mRNA levels of *p21^{CIP1}* in cultured neonatal type II AECs following dexamethasone treatment (Corroyer *et al.* 1997). The authors proposed that this was due to glucocorticoid-mediated post-transcriptional effects which increased p21^{CIP1} protein levels only. In addition to epithelial cells, our *in vivo* study indicates that glucocorticoid signalling via GR probably inhibits proliferation primarily in fetal lung mesenchymal cells, and indicates that mesenchymal cells such as fibroblasts are amajor target of p21^{CIP1}-induced cell cycle arrest. In a mesenchymal specific fashion, glucocorticoid-regulated *p21^{CIP1}* and *p57^{KIP2}* expression may thus represent a key regulator of peri-saccular septation, one of the more important processes of lung structural development.

We also show differential mRNA levels of various growth factors in the lungs of GR-null fetal mice, particularly increased mRNA levels of the retinoic-acid responsive growth factor *midkine*, which can promote proliferation in a variety of cell types, including pulmonary cells (Salamaa *et al.* 2005). Our results are consistent with the finding in another GR-null mouse line, using a limited gene microarray analysis, immunohistochemistry and *in situ* hybridization, that *midkine* gene and protein expression are relatively increased in GR-null fetal lungs just before birth (Kaplan *et al.* 2003). Furthermore, another study has shown that midkine protein levels are reduced in lungs of GR-null mice expressing a functional rat GR under the control of a human epithelial SP-C promoter (Gagnon *et al.* 2006). Therefore, glucocorticoid-mediated regulation of *midkine* may also contribute toward cell proliferation mechanisms in the fetal lung.

The data described here also reveal significantly reduced *Fgf7* mRNA levels in 18.5 p.c. GR-null mouse lungs compared to wild-type controls. These data support other work describing an increase in *Fgf7* mRNA levels in fetal lung mesenchyme following dexamethasone treatment (Chelly *et al.* 2001). *Fgf7*, expressed in the mesenchyme, has a variety of functions during late gestation, including the stimulation of surfactant production in the adjacent epithilium (Ulich *et al.* 1994; Chelly *et al.* 2001). Previous work from our laboratory has shown that glucocorticoid signalling via GR is not necessary for type II AEC differentiation in the fetal lung (Cole *et al.* 2004). Furthermore, electron microscopy analysis of the lungs of GR-null fetal mice displayed no visible reduction in lamellar bodies or lack of airway surfactant (Cole *et al.* 2004). This may indicate that although excess FGF7 can induce Sftpc, glucocorticoid signalling through *Fgf7* is not

a prerequisite for appropriate type II AEC development, and thus implicates other key regulators in this process.

In summary, we have provided insights into glucocorticoid-induced control over cellular proliferation mechanisms in the developing fetal lung. Analysis of the lung of GR-null fetal mice indicates that glucocorticoid signalling via GR restrains cellular proliferation in late gestation, particularly in the distal lung mesenchyme, and this process is associated with thinning of the alveolar septum. Gene microarray analysis of the lungs of GR-null fetal mice has provided a number of gene targets which may contribute to these regulatory effects. Glucocorticoid-mediated induction of important CKIs such as $p21^{CIP1}$ may play a dominant role in restraining hyperproliferation by down-regulation of the cell-cycle. Furthermore glucocorticoid-mediated down-regulation of particular growth factors such as midkine may also contribute toward the inhibition of cell proliferation in the developing lung. However, further investigation will be necessary to determine whether the changes in gene expression we describe stem from direct GR-mediated transcription or secondary effects. This will show with greater specificity the role glucocorticoids play in regulating gene transcription in the mammalian fetal lung.

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