Gene Induction during Differentiation of Human Pulmonary Type II Cells *In Vitro*

Kelly C. Wade, Susan H. Guttentag, Linda W. Gonzales, Kathryn L. Maschhoff, John Gonzales, Venkatadri Kolla, Sunil Singhal, and Philip L. Ballard

Department of Pediatrics, Division of Neonatology, the Children's Hospital of Philadelphia; and Section of Thoracic Surgery, Department of Surgery, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania

Mature alveolar type II cells that produce pulmonary surfactant are essential for adaptation to extrauterine life. We profiled gene expression in human fetal lung epithelial cells cultured in serum-free medium containing dexamethasone and cyclic AMP, a treatment that induces differentiation of type II cells. Microarray analysis identified 388 genes that were induced > 1.5 -fold by 72 h of hormone treatment. Induced genes represented all categories of molecular function and subcellular location, with increased frequency in the categories of ionic channel, cell adhesion, surface film, lysosome, extracellular matrix, and basement membrane. In time-course experiments, self-organizing map analysis identified a cluster of 17 genes that were slowly but highly induced (5- to ~ 190 -fold) and represented four functional categories: surfactant-related (*SFTPC*, *SFTPA*, *PGC*, *SFTPB*, *LAMP3*, *LPL*), regulatory (*WIF2*, *IGF2*, *IL1RL1*, *NR4A2*, *HIF3A*), metabolic (*MAOA*, *ADH1B*, *SEPP1*), and transport (*SCNN1A*, *CLDN18*, *AQP4*). Induction of both mRNA and protein for these genes, which included nine newly identified regulated genes, was confirmed, and cellular localization was determined in both fetal and postnatal tissue. Induction of lysosomal-associated membrane protein 3 required both hormones, and expression was localized to limiting membranes of lamellar bodies. Hormone-induced differentiation of human type II cells is associated with genome-wide increased expression of genes with diverse functions.

Keywords: cyclic AMP; epithelial differentiation; glucocorticoid; human fetal lung; type II cell

Pulmonary type II cells have a number of known physiologic functions. During lung development, they are the progenitor cell for type I cells that constitute the gas exchange surface in alveoli. After lung injury, type II cells proliferate to repopulate the pulmonary epithelium. These cells participate in transepithelial movement of sodium and water to clear fetal lung fluid at birth and to maintain alveolar fluid homeostasis with air breathing. Type II cells produce a variety of cytokines, chemokines, and other proteins involved in the inflammatory response, as well as proteins that have either immunomodulatory or antioxidant roles. The most specialized function of type II cells is the production and secretion of surfactant, the complex mixture of lipid and surfactant proteins (SPs) that maintains alveolar stability and is required for successful adaptation to extrauterine life (1–3).

Differentiation of type II cells from undifferentiated precursor epithelial cells is marked by the disappearance of glycogen, which serves as a substrate for surfactant phospholipid synthesis, the formation of lamellar bodies, which are the intracellular storage sites for secreted surfactant, and expansion of the apical cell surface in the form of microvilli (4–7). A number of classes of proteins are developmentally increased during type II cell differentiation, including SPs, lipogenic enzymes, selected water and ion transporter/channels, metabolic enzymes, and structural proteins (8).

Studies of alveolar type II cell differentiation in cultured fetal lung explants and *in vivo* indicate that both glucocorticoids and cyclic AMP (cAMP) are important agents in the maturational process (5–9). Hormone treatment of explants or epithelial cells from second trimester human fetal lung results in differentiation of type II cells that contain lamellar bodies and secrete surface-active surfactant. A limited number of glucocorticoid-regulated genes have been identified (7, 8). In general, hormonally induced genes are also developmentally regulated, consistent with the view that glucocorticoid treatment causes precocious maturation and mimics the role of endogenous corticosteroids in lung development. In a survey of hormonal responses in fetal lung epithelial cells, most ($\sim 90\%$) induced genes responded to both glucocorticoid and cAMP in either an additive or synergistic manner (7). Although many of the recognized inductive responses to glucocorticoid/cAMP exposure occur in lung epithelial cells, mesenchymal fibroblasts are also target cells for these hormones, and mesenchymal–epithelial interactions are important in lung development (10). Other reported regulators of lung development include thyroid hormones, epidermal growth factor, gastrin-releasing peptide, IL-1, and parathyroid hormone-related peptide; however, there is limited information regarding target genes for these agents (11).

Primary culture of fetal lung epithelial cells is a useful model system for examining changes in gene expression during type II cell differentiation. In this experimental approach, an enriched population of epithelial cells, isolated from second trimester human fetal lung tissue, is cultured in serum-free medium containing dexamethasone and 8-bromoadenosine 3',5'-cAMP (8-Br-cAMP) (7). Within 4 d, many of the hormone-treated cells develop intracellular lamellar bodies and secrete surface-active surfactant in response to secretagogues; cells cultured without hormones do not undergo differentiation. We previously used DNA microarray analysis to generate an initial, limited survey of regulated genes after 3 d of hormone-induced type II cell differentiation *in vitro* (7). In the present study, we have expanded the survey and analysis of induced genes and profiled gene expression at time points during the process of type II cell differentiation. We have analyzed the chromosomal distribution of induced genes and determined categories of the encoded proteins with regard to their molecular function and subcellular location. A cluster of 17 known genes that were highly induced contained 9 newly identified, hormonally responsive genes. Preliminary results have been published as an abstract (12).

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Correspondence and requests for reprints should be addressed to Philip L. Ballard, M.D., Ph.D., Children's Hospital of Philadelphia, 416 Abramson Research Center, 3516 Civic Center Boulevard, Philadelphia, PA. E-mail: ballardp@email.chop.edu

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MATERIALS AND METHODS

Cell Culture

We isolated enriched populations of epithelial cells from second trimester human fetal lung tissue under institutional review board-approved protocols, as previously described (6). After overnight culture (Day 1), we cultured the cells for an additional 3 d in serum-free Waymouth's medium alone (control), or with dexamethasone (10 nM)/8-Br-cAMP (0.1 mM)/isobutylmethylxanthine (0.1 mM) (DCI), or with dexamethasone or 8-Br-cAMP/isobutylmethylxanthine separately. These concentrations maximally induce surfactant components in human lung explant cultures (9). For time-course experiments, cells were collected at 4, 8, 24, 48, and 72 h after addition of DCI or diluent (control). Epithelial cell purity by this procedure was $86 \pm 2\%$ ($n = 6$), with fibroblasts as the primary contaminating cell type (6, 7).

DNA Microarray Analysis

Total RNA was extracted from control and DCI-treated cells and converted to biotin-labeled cRNA using Affymetrix reagents (Affymetrix, Santa Clara, CA) and protocol. Biotin-labeled cRNA was hybridized to U133A Affymetrix microarray chips that contain 16–20 unique 25 mer oligonucleotide probes for $\sim 14,500$ human genes plus corresponding 12–16 probes with a single nucleotide change (mismatch control). U133A hybridization, washing, staining, and scanning were performed by the Stokes Research Institute Nucleic Acid Core Facility using procedures described in the Affymetrix GeneChip Expression Analysis technical manual. A total of 18 chips were analyzed with cells cultured from 13 individual lungs. Gender was known for seven lungs (four female and three male). For studies of DCI effects at 72 h, a total of 10 chips (5 control and 5 DCI-treated) were used. In the first two experiments (4 chips) RNA was prepared from cells isolated from individual specimens of lung tissue (13- and 14-wk gestation) that were cultured in the presence or absence of DCI. In an additional three experiments (6 chips), equal amounts of RNA from 72-h control and DCI-treated cells (derived from 3–4 different lungs for each pool, 16- to 20-wk gestation) were pooled for analysis. To study the time course of gene induction, cells from 4 individual lung specimens (all ~ 18 -wk gestation) were cultured in the presence of DCI or Waymouth's media for 4, 8, 24, 48, and 72 h, and equal amounts of RNA from each experiment were pooled for analysis with 2 chips (DCI and control) used at each time point; data from the 72-h point in this experiment represent 1 of the 3 RNA pools noted previously here. RNA from all 13 cell preparations was analyzed by dot-blot hybridization, as previously described (7), to confirm induction of SP-B mRNA after 72 h DCI treatment. The strategy of pooling of RNA samples from separate experiments for microarray analysis has been found not to affect the inference for most genes (13).

Affymetrix Microarray Suite 5.0 was used to quantitate and analyze mRNA content for expressed genes. Default values provided by Affymetrix were applied to all analysis parameters. Probes for 70 control genes on each chip were used to normalize fluorescence intensity between chips, and arrays were scaled to an average intensity of 1,500 fluorescence units and analyzed independently. For very-low-abundance mRNAs in control cells (< 20 fluorescence units), a value of 20 units was assigned. The Microarray Suite software uses Wilcoxon's signed rank tests to evaluate whether a transcript is detectable on the array (present, marginal, absent), and the probability of a significant change between arrays (*i.e.*, DCI-treated versus control), assigning *P* values and a change call (increase, decrease, no change) for each probe. For the current analysis, induced genes were defined as those that were detected as present and increased (DCI-treated for 72 h versus control) in all 5 experiments, with $P < 0.003$ for at least four experiments and a mean fold induction > 1.5 -fold. We also determined *q* values for the entire data set using QVALUE software (available online at <http://genome.org/qvalue>). This analysis indicated that induced genes met a false discovery rate of < 0.025 (14). When more than one probe set was present for the same gene, data were combined to provide a mean value. Fold-stimulation results are expressed as mean $\pm$ SE.

Database Analyses

We used databases and software in the public domain to compare various properties of induced genes of fetal epithelial cells with human

genomic data. Distribution of induced genes among chromosomes (as % of chromosomal genes represented on the chip) and close clustering of induced genes was determined using data extracted from the NCBI website (available online at www.ncbi.nlm.nih.gov/mapview/maps.cgi?ORG=humandMAPS=ideogr.cntg.ugHs.genesandCHR=1).

The molecular function and subcellular location of induced genes was determined from the Swiss-Prot database (available online at <http://us.expasy.org/sprot/sp-docu.html>), and the relative distribution of genes between categories was compared with data for all human entries in this database (10,125 at the time of analysis). Subcellular location was primarily a single designation in this database, whereas multiple terms were generally assigned for gene molecular function.

Clustering Analysis for Self-Organizing Maps

GeneCluster 1.0 from the Whitehead Institute/Massachusetts Institute of Technology Center for Genome Research (available online at: <http://www.genome.wi.mit.edu/cancer/software.html>) was used to cluster differentially expressed genes in the time course by self-organizing maps (SOM). Cluster analysis was restricted to the 5,570 probes on U133A that met the following two criteria by Microarray Suite 5.0 analysis: (1) the probe was designated as "present" on at least one chip in the time course studies; and (2) in comparative analysis between control and DCI-treated cells, the probe was designated either "increased" or "decreased" for at least one time point. For our SOM analysis, we used a 6×4 matrix and the default settings of the software to create 24 patterns of gene expression.

Real-Time RT-PCR

RNA was prepared using RNA STAT (Tel-Test, Inc., Friendswood, TX) per the manufacturer's instructions. Samples were then treated with RQ1 RNase-free DNase (Promega, Madison, WI) and ethanol precipitated after phenol-chloroform extraction. Integrity, purity, and concentration were confirmed using an Agilent 2,100 bioanalyzer (Agilent Technologies, Palo Alto, CA) in the Nucleic Acid Core Facility at Children's Hospital of Philadelphia. cDNA was synthesized from 2 μ g RNA samples using the SuperScript First-Strand RT-PCR kit (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Real-time PCR reactions using a singleplex format were performed with an ABI Prism 7,000 (Applied Biosystems, Foster City, CA) in the Real-Time PCR Core Facility of the Children's Hospital of Philadelphia. We used the standard PCR protocol recommended by the manufacturer of Assay-on-Demand kits (Applied Biosystems, Foster City, CA). The specific primer and probe sequences are available from the authors on request. The assays were determined to be in the linear amplification range in each experiment using cDNA standards derived from RNA of cells treated for 5 d with DCI.

Antibodies

Antibodies were obtained from the following sources and used for immunofluorescence staining (dilutions shown) and Western analysis: lipoprotein lipase (LPL; 1:100, monoclonal clone 5D2; gift of J. Brunzell, University of Washington, Seattle); alcohol dehydrogenase (ADH; 1:250, monoclonal; Research Diagnostics, Inc., Flanders, NJ); Wingless-Int (Wnt) inhibitory factor-1 (WIF-1; 1:100, goat polyclonal; R&D Systems, Minneapolis, MN); epithelial sodium channel α (ENaC α ; 1:200, rabbit polyclonal; Alpha Diagnostic International, San Antonio, TX); IL-1 receptor-like (IL1RL1, IL-1R4, ST2; 1:200, goat polyclonal; R&D Systems); monoamine oxidase (MAO; 1:500, chicken polyclonal; US Biological, Swampscott, MA); Aquaporin 4 (AQP4; 1:1000, rabbit polyclonal; US Biological); hypoxia-inducible factor 3 α (HIF3 α ; 1:100; Novus Biologicals Inc., Littleton, CO); lysosomal-associated membrane protein 3 (LAMP3, dendritic cell (DC)-LAMP, CD208; 1:250, monoclonal; Immunotech, Marseille, France); insulin-like growth factor (IGF)-II (1:100, goat polyclonal; R&D Systems); nuclear-related receptor 1 (Nurr-1, NR4A2; 1:1000, rabbit polyclonal; Santa Cruz Biotechnology, Santa Cruz, CA). Antibodies that failed to give positive immunostaining results with cultured lung cells were as follows: IGF-II (rabbit polyclonal; Chemicon International, Inc, Temecula, CA); WIF-1 (polyclonal; Santa Cruz Biotechnology); selenoprotein P (monoclonal; BD Transduction Laboratories, San Diego, CA).

Western Analysis

We performed immunoblotting using previously described procedures (7) and NuPAGE Bis-Tris gels with MES (2-[morpholino] ethane sulfonic acid) SDS Running Buffer, per the manufacturer's protocol (Invitrogen, Carlsbad, CA). Proteins were transferred to Duralose membrane (Stratagene, La Jolla, CA) and probed with primary antibodies and appropriate horseradish peroxidase-tagged secondary antibody. Signal was detected using the enhanced chemiluminescence kit (SuperSignal West PICO kit; Pierce, Rockford, IL) and blots exposed to Kodak Biomax MS film (Eastman Kodak Co., Rochester, NY). Films were scanned with an Agfa Argus II scanner and FotoLook SA scanning software on a Macintosh G4 computer (Apple Computer, Inc., Cupertino, CA). Semiquantitative densitometric analysis was done using MacBAS version 4.2, after background subtraction.

Immunofluorescence

Lung tissue (fetal uncultured or after explant culture, or normal human infant 1–3 mo of age) was fixed in cold 1% paraformaldehyde, washed in 1 mM NH₄Cl in PBS followed by cold PBS, then cryoprotected by 5% sucrose in PBS, and embedded in Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC); ~5 µm frozen sections were prepared for immunostaining. Isolated epithelial cells were cultured on glass coverslips for cell culture (Fisher Scientific, Pittsburgh, PA), then fixed with cold methanol (–20°C) and permeabilized with 0.3% Triton X-100 before immunostaining, as previously described (7). Secondary antibodies for immunofluorescence detection were tagged with Alexa 488 or Cy3 and used at 1:200 or 1:300. After immunostaining, some sections were exposed to 4', 6-diamidino-2-phenylindole (0.1 µg/ml, Molecular Probes, Eugene, OR) for 10 min to stain nuclei.

In Situ Hybridization

Nonisotopic *in situ* hybridization on frozen sections was performed with fetal lung tissue (uncultured or after explant culture) that was fixed in cold 1% paraformaldehyde and embedded in tissue freezing medium. Sections (10 µm) were fixed in 4% paraformaldehyde, treated with proteinase K, and then refixed in paraformaldehyde, acetylated, and dehydrated. Antisense probes (2.5 µg), prepared using the Dig RNA Labeling kit (Roche, Indianapolis, IN) were diluted in 1 ml hybridization solution, placed on each slide, and incubated overnight at 70°C. After hybridization, slides were washed briefly in 5× SSC and high-stringency wash solution and then treated with RNase A. Slides were blocked with 1% Blocking Reagent (Roche) and incubated overnight at 4°C with alkaline phosphatase-coupled sheep anti-digoxigenin Fab fragments (Roche) diluted 1:5,000 in 1% Blocking Reagent in 1× PBS-0.1% Tween. Alkaline phosphatase activity was detected by incubation in 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium mixture for 24–72 h. The slides were counterstained with Nuclear Fast Red (Sigma, St. Louis, MO), allowed to air-dry, and mounted with VectaMount (Vector Laboratories, Burlingame, CA). Slides were examined with an Olympus 1X70 microscope (Olympus, Melville, NY) and Metamorph imaging system (Universal Imaging, West Chester, PA).

RESULTS

Number of Induced Genes

To assess the overall profile of gene expression in differentiated fetal lung epithelial cells, we identified those transcripts that were scored as “present” in cells treated with DCI for 72 h using the U133A Affymetrix microarray chip, which contains ~22,500 probes for ~14,500 human genes (available online at www.affymetrix.com). There were 8,453 mRNAs that were identified as present in each of five experiments. Because of redundancy in probe sets, the 8,453 mRNAs likely represent ~5,450 separate gene transcripts. Assuming that the gene probes on this chip are representative of all the named human genes, we estimate that 38% of the human genome is expressed at detectable levels in fetal lung epithelial cells after 72 h of DCI treatment.

Analysis of microarray data for 5 experiments, representing 13 different fetal lungs between 13- and 20-wk gestation, revealed that 388 mRNAs were reproducibly induced > 1.5-fold by 72 h

of DCI treatment compared with control cells. This represents ~2.7% of the genes that are probed by the chip and ~7.1% of the genes that are expressed at a detectable level in these cells. Of the 388 induced genes, 350 encode for proteins of currently known or putative function, and the remaining 38 genes are currently described as expressed sequence tags (ESTs) or encoding unknown proteins. The list contains 78 genes that we previously identified using a first-generation microarray chip (Affymetrix HuFL) containing probes for ~5,500 human genes (7). The full list of induced genes is provided online at <http://stokes.chop.edu/web/neoresearch>, and original data for gene expression profiling after 72-h hormone exposure are deposited at the Gene Expression Omnibus (accession number GSE3306) at the National Center for Biotechnology Information (available online at www.ncbi.nih.gov). Downregulated genes, which are not a focus of this report, are included at the Gene Expression Omnibus website.

Chromosomal Locus of Induced Genes

The chromosomal locus was known for 377 genes induced by 72-h DCI exposure. Induced genes were localized to each of the chromosomes, except for chromosome Y, at a mean frequency of 1.1% (range 0.4–2.1%). The U133A chip has 42 probes for Y chromosome genes, representing 19% of genes currently assigned to the Y chromosome. It is possible that the failure to detect reproducibly induced genes of the Y chromosome reflects a relative scarcity of male lungs in the specimens studied.

Induced Genes Related to Phospholipid Biosynthesis

Hormone treatment of fetal lung cells increases the synthetic rate and content of phosphatidylcholine (PC) and alters the molecular composition of both PC and phosphatidylinositol (7). Accordingly, we examined induced genes of known protein function for those involved in phospholipid biosynthesis. A total of 13 genes were identified that participated in uptake of lipids (4 genes), biosynthesis (7 genes), or remodeling (2 genes) of phospholipids (Figure 1). Highly induced genes included lipoprotein lipase (*LPL*, 13-fold), fatty acid synthase (*FAS*, 6-fold), glycerol kinase (*GK*, 5-fold), and phosphatidylglycerophosphate

Figure 1. Induced genes in phospholipid biosynthesis. A simplified pathway of lipid uptake and biosynthesis of phosphatidylcholine and phosphatidylglycerol (PG) with induced genes is shown. Mean induction in five microarray experiments was ~2-fold except for genes where the fold induction is noted. ADFP, adipose differentiation-related protein; EC, extracellular; FA, fatty acid; MGLL, monoglyceride lipoprotein lipase; PAP, phosphatidic acid phosphatase; PG-P, phosphatidylglycerol phosphate.

synthase (*PGSI*, 3-fold); the other genes were all induced 1.6- to 2.5-fold. In addition, there were induced genes encoding a putative membrane phospholipid transporter (*ABCA3*), a putative acyltransferase (*FLJ12443*), and five induced genes related to cholesterol uptake or synthesis (*LDLR*, *LSS*, *DHCR7*, *DHCR24*, *ACATI*).

Molecular Function of Induced Genes

There is limited information about biological functions of type II cells other than production of pulmonary surfactant. To assess whether changes in gene expression during type II cell differentiation were restricted to selected types of biological activities, we examined the major categories of molecular function among induced genes. At the time of data summary (August 2005), the molecular function for encoded proteins of 299 induced genes was available in Swiss-Prot and TrEMBL databases (<http://ca.expasy.org/sprot/>). To examine the distribution of induced genes among 24 major functional categories, we calculated the percentage of induced genes in each category and compared those values with the categorical distribution of all classified genes in the databases (a total of 10,125 entries). These results are shown in Table 1. On regression analysis, there was a close correlation ($r = 0.92$) between the percentage of genes in each category for fetal lung epithelial cells versus the available data for the human genome. The percentage in different categories ranged from 1.0% (surface film) to 28.1% (signal), and represented a range of 2–84 induced genes per category. Of note, three functional categories were enriched in type II cells, with a > 8-fold higher percentage of induced genes compared with the genome: ionic channel (3.3 versus 0.2%); cell adhesion (3.3 versus 0.4%); and surface film (1.0 versus 0.03%). The 10 induced ionic channel genes are *CDH4*, *CDH5*, *FBLN5*, *ICAN4*, *LAMA2*, *LAMA3*,

PCDH9, *PECAM1*, *SPON1*, and *ALCAM*. The 10 induced cell adhesion genes, as identified by this microarray analysis, include *CACNA2D2*, *CLIC3*, *CLIC5*, *FXYD3*, *KCNJ15*, *KCNJ2*, *KCNJ8*, *PLLP*, *SCNN1A*, and *SCNN1B*. The three induced surface film genes (*SFTA*, *SFTB*, *SFTC*) represent all of the genes in this category listed in the protein databases.

Subcellular Location of Induced Genes

The subcellular location of proteins often reflects their general area of biological function (e.g., signaling and transport functions for membrane proteins). To further assess the spectrum of functional changes with type II cell differentiation, we categorized induced genes by location of encoded proteins. At the time of data summary, the subcellular location of 183 (47%) of the induced genes was listed in the Swiss-Prot and TrEMBL databases. Distribution of induced genes among 14 major subcellular categories was compared with that for all 8,534 proteins in the databases with subcellular location assignments. Regression analysis indicated a close correlation ($r = 0.98$, excluding “plasma”) between the percentage of genes in each category for fetal lung epithelial cells and the available data for the human genome. Approximately 80% of the induced genes were in either transmembrane/membrane or nuclear protein categories of subcellular location, consistent with the high frequency of these assignments in the protein databases. There was a relatively low percentage (3.8%) of induced genes in the plasma category, which represents secreted proteins, primarily of hepatic origin. The extracellular matrix (10 genes), lysosome (5 genes), and basement membrane (2 genes) categories were represented at an ~ 3-fold higher percentage for induced genes compared with the genome.

Time Course of Gene Expression in Response to DCI

To evaluate kinetics of gene induction, we performed an initial microarray study for control and DCI-treated cells at 4, 8, 24, 48, and 72 h. We then used GeneCluster 1.0 software to perform SOM analysis, which categorizes genes by their temporal pattern of hormone response (induced, repressed, or no change) and has been used to identify groups of regulated genes that share regulatory elements or functional relationships (15, 16). Figure 2 shows the time course for four clusters of genes with mean induction values > 1.5-fold for at least one time point. Cluster 1 contained 18 highly induced genes, including SP-A, SP-B, and SP-C, and was selected for further study and confirmation of hormonal responsiveness. Real-time RT-PCR analysis ($n = 3$) confirmed the time-course data obtained by microarray (Figure 2). The

TABLE 1. INDUCED GENES CATEGORIZED BY MOLECULAR FUNCTION

Category	Type II cells	Genome
Signal	28.4	21.1
Phosphorylation	12.4	10.6
Hydrolase	11.7	8.7
DNA binding	5.0	9.1
Transferase	10.7	8.2
Oxidoreductase	6.7	3.8
ATP-binding	8.0	7.3
Transcription regulate	6.7	10.4
Receptor	8.0	4.7
Metal-binding	9.4	6.2
Transport	9.4	5.9
Acetylation	3.0	2.1
Calmodulin-binding	0.7	1.0
Extracellular matrix	3.0	1.4
Ion transport	3.3	1.9
Ionic channel	3.3	0.2
Calcium-binding	2.0	3.1
Cell adhesion	3.3	0.4
Kinase	4.3	1.1
Developmental	2.0	2.8
Serine protease	2.3	1.1
Isomerase	1.3	0.7
Lyase	1.3	0.9
Surface film	1.0	0.03

For type II cells, functional category assignment was made for 299 induced genes that had data available in the Swiss-Prot and TrEMBL databases, and percent for each category was calculated (2–84 genes/category). Data for the genome represent the same calculation for all human proteins in the databases (10,125 entries). The percentages of induced type II cell genes in each functional category is similar to those for the human genome, except for the categories of ionic channel, cell adhesion, and surface film (shown in bold).

Figure 2. Time course of induced gene clusters. Cells were exposed to DCI or diluent for 4, 8, 24, 48, or 72 h. Time-course data by microarray, examined by SOM analysis, revealed 4 clusters of induced genes with mean induction > 1.5-fold for at least 1 time point (dashed lines).

mRNA content for the 17 genes of Cluster 1 was also determined by real-time RT-PCR analysis (solid line) and mean $\pm$ SE values ($n = 3$) are shown. The time-course patterns by microarray and RT-PCR were comparable for both mean values and for individual genes (not shown), with all genes demonstrating continued induction between 48 and 72 h.

expression pattern of this gene cluster was distinct from other induced clusters with regard to the level of induction and the continuing increase in expression between 48 and 72 h.

Excluding one gene of unknown function (DKFZP586A0522), Cluster 1 contained genes in 4 general categories of protein function: surfactant-related, regulatory, metabolic, and transport, and included 9 genes not previously known to be hormonally responsive in fetal lung (*LAMP3*, *LPL*, *WIF1*, *IL1RL1*, *NR4A2*, *HIF3A*, *MAOA*, *ADH1B*, and *SEPP1*). Table 2 lists mRNA abundance and induction data for these 17 genes from microarray analysis of 72 h DCI-treated cells. In separate experiments under the same treatment conditions, induction at 72 h was determined for all the genes of Cluster 1 using real-time RT-PCR. Induction of SP-A, SP-B, SP-C, and PGC mRNA has been previously demonstrated in this system by Northern, dot-blot analysis, and real-time RT-PCR (7, 17). There were some differences in fold induction between microarray and real-time RT-PCR, which are likely attributable to inaccuracies in calculation of fold change in instances where the control level of gene expression was very low or undetectable (*SFTPA*, *SFTPC*, *PGC*, *IL1RL1*). Because optimal induction of type II cell differentiation occurs in the presence of both glucocorticoid and cAMP, we also examined the response to individual hormones (dexamethasone or cAMP/isobutylmethylxanthine) by microarray and/or RT-PCR. All types of hormonal responsiveness were represented in the gene cluster: induction by only dexamethasone (two genes) or only cAMP (four genes), additive or synergistic effects between dexamethasone and cAMP (eight genes), and a requirement for both hormones with no response from one hormone alone (permissive effect, three genes).

Detection of DCI-Induced Proteins

We used Western analysis and immunofluorescence staining to investigate the cellular localization and content of 11 newly identified proteins encoded by genes of Cluster 1. Induction and epithelial localization of the SPs, pepsinogen C and claudin 18,

have been previously described (6, 7, 17, 18). The antibody against selenoprotein P did not detect immunoreactive protein in control or DCI-treated cells by either Western analysis or immunostaining.

Western analysis confirmed that DCI treatment increased protein expression for 5 of the 11 newly identified genes of Cluster 1 (Figure 3). The increases were in the 2- to 4-fold range for LPL, ENaC, WIF-1, and ADH, and higher (22-fold) for IL1RL1 (ST2), which was faintly detected in control tissue and strongly present after DCI treatment. IL1RL1 (ST2) protein was only detected in DCI-treated lung explants (row 2) and not in epithelial monolayer cultures, perhaps reflecting protein abundance and stability to enzymatic digestion of lung tissue. WIF-1 from DCI-treated cells migrated slightly slower than for control, perhaps reflecting a hormone effect on posttranslational modification as well as gene expression. One or more available antibodies for MAO, HIF3 α , AQP4, LAMP3 (DC-LAMP), IGF-II, and NR4A2 were tested, but failed to detect specific bands at the expected locations on protein blots.

Frozen sections of lung explants, either uncultured (preculture) or cultured 4 d in control or DCI-containing media, were immunostained with available antibodies (Figure 4). Explants retain intercellular morphologic relationships comparable to *in vivo* tissue, allowing discrimination of staining to epithelial and/or mesenchymal cell compartments. Antibodies for eight of the proteins detected primarily epithelial cell immunostaining, whereas immunoreactivity with the ADH antibody was localized primarily to mesenchymal cells. HIF3 α staining was mesenchymal in control tissue and present in both mesenchyme and epithelium in DCI-treated tissue. For each of the proteins, minimal staining was detected in uncultured tissue, and DCI treatment increased staining intensity compared with control tissue. Different patterns of epithelial cell staining were evident: cytoplasmic (LAMP3, HIF3 α , MAO), basal (LPL), basolateral (ENaC, AQP4), and apical (IL1RL1, NR4A2). Two antibodies against IGF-2 provided no fluorescent signal in explants.

TABLE 2. mRNA CONTENT FOR GENES OF CLUSTER 1

Functional Category	Gene Symbol	Common Name	Microarray Relative Abundance Induction (Mean f.u.)	Microarray Fold Induction (Mean $\pm$ SE)	RT-PCR Fold Induction (Mean $\pm$ SE)	Hormonal Response
Surfactant-related	<i>SFTPC</i>	SP-C	5137	123.8 $\pm$ 25.2	42.5 $\pm$ 18.9	Synergy*
	<i>SFTPA</i>	SP-A	4507	145.6 $\pm$ 58.0	18.9 $\pm$ 7.2	DEX-permissive*
	<i>PGC</i>	Pepsinogen C	1385	49.7 $\pm$ 15.2	192.4 $\pm$ 4.1	Synergy*
	<i>SFTPB</i>	SP-B	8190	17.1 $\pm$ 4.2	37.2 $\pm$ 15.5	Synergy
	<i>LAMP3</i>	Lysosomal protein	1396	13.8 $\pm$ 4.3	13.4 $\pm$ 6.2	Synergy
Regulatory	<i>LPL</i>	Lipoprotein lipase	337	10.7 $\pm$ 1.0	23.3 $\pm$ 9.1	Synergy*
	<i>WIF1</i>	Wnt inhibitory factor 1	421	12.9 $\pm$ 3.6	16.3 $\pm$ 4.5	cAMP only
	<i>IGF2</i>	Insulin-like growth factor 2	879	10.7 $\pm$ 3.1	26.3 $\pm$ 20.1	DEX only
	<i>IL1RL1</i>	IL-1 receptor-like 1	105	7.2 $\pm$ 0.7	19.0 $\pm$ 3.6	Synergy
	<i>NR4A2</i>	Nuclear receptor 4A2	147	6.1 $\pm$ 1.2	4.8 $\pm$ 0.6	cAMP only
Metabolic	<i>HIF3A</i>	Hypoxia induced factor 3 α	57	7.7 $\pm$ 1.4	30.7 $\pm$ 36.1	cAMP only
	<i>MAOA</i>	Monoamine oxidase A	454	8.6 $\pm$ 4.0	11.8 $\pm$ 3.5	cAMP-permissive*
	<i>ADH1B</i>	Alcohol dehydrogenase 1 β	165	8.2 $\pm$ 1.5	26.3 $\pm$ 10.1	DEX only*
	<i>SEPP1</i>	Selenoprotein P	267	3.2 $\pm$ 0.4	6.0 $\pm$ 0.9	Synergy
	<i>SCNN1A</i>	Epithelial sodium channel- α	966	17.0 $\pm$ 2.5	23.6 $\pm$ 8.7	Synergy*
Transport	<i>CLDN18</i>	Claudin 18	750	9.8 $\pm$ 3.5	15.5 $\pm$ 8.2	DEX-permissive
	<i>AQP4</i>	Aquaporin 4	321	6.9 $\pm$ 0.8	5.9 $\pm$ 3.8	Additive*

Definition of abbreviations: cAMP, cyclic AMP; DEX, dexamethasone; f.u., fluorescence units; SP, surfactant protein; Wnt, Wingless-Int.

* Data from Ref. 4.

Genes of Cluster 1 as identified by self-organizing map analysis are shown by functional categories. Fold induction data are mean $\pm$ SE from 5 (microarray) and 3 (RT-PCR) experiments with exposure of cells to dexamethasone/8-Br-cAMP/isobutylmethylxanthine (DCI) for 72 h. Hormonal response data were obtained using RNA from cells exposed to DEX alone, cAMP/isobutylmethylxanthine (cAMP) alone, DCI, or untreated with analysis by RT-PCR or microarray (7). A DEX-permissive effect is defined as no response to DEX alone but increased responsiveness to cAMP in the presence of DEX compared to cAMP alone. A cAMP-permissive effect reflects the opposite response to the two hormones.

Figure 3. Western blot analysis of LPL, IL1RL1, ENaC α , WIF-1, and ADH. Undifferentiated epithelial cells were isolated from individual fetal lung specimens and cultured in control media or DCI to promote differentiation. Equal amounts of protein from total cell extracts of control and DCI-treated cells were run on SDS-PAGE gels, blotted to Duralose, and incubated with primary antibodies. For IL1RL1 (row 2), hormone treatment was performed with fetal lung explants due to lack of immunoreactivity with sonicates of monolayer cells. Representative blots from two experiments are shown. Bands of the expected molecular size were detected for LPL (58 kD), IL1RL1 (72 kD), ENaC α (74 kD), WIF-1 (42 kD), and ADH-1 β (39 kD). In 3–5 experiments, mean $\pm$ SE values for fold-induction by densitometric scanning were: LPL, 2.3 ± 0.5 ; IL1RL1, 21.6 ± 2.4 ; ENaC, 3.3 ± 0.4 ; WIF-1, 4.3 ± 1.1 ; and ADH, 1β 2.3 ± 0.2 .

Immunostaining was also performed with sections of normal postnatal lung (Figure 5). The pattern of staining was generally comparable to that observed in hormone-treated fetal tissue. LPL and AQP4 staining localized to the surface of epithelial cells, with regions of increased intensity. The distribution of DC-LAMP staining was consistent with type II cell-specific expression. NR4A2, ENaC and weak HIF3 α staining appeared to be cytoplasmic in epithelial cells, whereas ADH staining occurred primarily in mesenchymal cells. Staining of postnatal tissue was not detected for MAO and IL1RL1 under the conditions used, perhaps representing age-dependent expression.

We immunostained fetal lung cells in monolayer culture for better visualization of intracellular staining (Figure 6). Increased staining in epithelial cells with DCI treatment was confirmed for AQP4 (plasma membrane), LAMP3 (punctate cytoplasmic), and HIF3 α and NR4A2 (cytoplasmic). LPL (Figures 6a and 6b) appeared as punctate cytoplasmic immunostaining in epithelial cells, whereas localization to the basal membrane was observed in explants. Confirming the observation in explants, ADH immunoreactivity was most intense in fibroblasts that contaminate the culture (Figures 6i and 6j), although some staining was evident in epithelial cells. IGF-II immunoreactivity (Figures 6k and 6l), which was not detected in explants, was present as faint cytoplasmic staining in DCI-treated but not in control epithelial cells.

In Situ Hybridization

To establish cellular localization of gene expression, *in situ* hybridization was performed on sections of uncultured, control, and DCI-treated explants for proteins without a suitable anti-

Figure 4. Immunostaining of highly induced proteins in fetal lung explants. Human fetal lung (16- to 19-wk gestation) was cultured as explants for 5 d in control or DCI media, fixed, and frozen sections of each group (uncultured, control, DCI-treated) were placed on the same slide for processing with each antibody as described in MATERIALS AND METHODS. Uncultured tissue is in the left panel of each row (a, d, g, j, m, p, s, v, y), control tissue at 5-d culture is in the middle panel (b, e, h, k, n, q, t, w, z), and DCI-treated tissue is shown in the right panel (c, f, i, l, o, r, u, x, aa). Results were similar with three separate lung specimens, and representative staining is shown. For each of the proteins, uncultured tissue showed low to absent immunostaining. Fluorescence signal was also low for control tissue, except for somewhat increased signal with AQP4, IL1RL1, HIF3 α , MAO, and ADH; HIF3 α staining in control explants was restricted to the mesenchyme. For all antibodies, staining was most intense for tissue cultured in DCI and was localized to epithelial cells, except for ADH (primarily mesenchymal) and HIF3 α (epithelial and mesenchymal). Different patterns of epithelial cell staining are evident: cytoplasmic (LAMP3, HIF3 α , MAO), basal (LPL), basolateral (ENaC, AQP4), apical (IL1RL1, NR4A2). Arrows indicate epithelial cells. A, alveolar space; m = mesenchyme.

body (WIF1), immunolocalization to both mesenchymal and epithelial cells (MAO), and faint fluorescence signal (IGF-II) (Figure 7). WIF-1 and MAO mRNAs were not detected in uncultured lung tissue, were present in epithelial cells of control

Figure 5. Immunofluorescence staining of postnatal lung for proteins of Cluster 1. Frozen sections of postnatal lung (1–3 mo normal infant) were immunostained as described for fetal lung explants (Figure 4). The staining patterns for most genes were similar to those observed in the explants. LPL and AQP4 staining localized to epithelial cell membranes with areas of stronger intensity; the pattern of DC-LAMP was consistent with restricted type II cell expression; NR4A2, ENAC, and weak punctate HIF3 α staining appeared to be cytoplasmic in epithelial cells; and ADH staining occurred primarily in mesenchymal cells. Staining of postnatal tissue was not detected for MAO and IL1RL1 under the conditions used. Nonspecific staining (not shown) was consistently low for postnatal tissue.

explants, and signal intensity was increased in DCI-treated tissue. IGF-2 mRNA was detected in epithelial cells of uncultured tissue, with greater expression in cultured control tissue and highest expression in DCI-treated tissue.

Characterization of DC-LAMP Induction

We further characterized expression of LAMP3 (DC-LAMP), one of the newly identified hormonally regulated genes. Induction of both DC-LAMP mRNA and protein on exposure to DCI occurred with a time course consistent with Cluster 1 genes, with an initial lag and continuing increase between 48 and 72 h, and the fold increase comparable for transcript and protein (Figures 8A and 8B). There was no significant response of DC-LAMP protein to either hormone alone (Figure 8C). In additional immunofluorescence studies, expression of DC-LAMP in DCI-treated cells colocalized with SP-B at lower magnification. With high power confocal microscopy, DC-LAMP staining was localized to the lamellar body, limiting membranes surrounding SP-B staining that localized to a discreet area within the lamellar bodies (Figure 9). These findings indicate that DC-LAMP expression in human lung is restricted to lamellar body membranes in type II cells.

DISCUSSION

Maturation of the lung *in utero*, in particular differentiation of type II cells and surfactant production, is critical for normal lung function and survival at birth; however, the scope of cellular responses regulated by this treatment is not known, and relatively few responsive genes have been identified. Our earlier study in the human fetal lung epithelial cells, a model system for type II cell differentiation, surveyed a limited number of genes and characterized induction of one gene of interest (thy-

roid transcription factor [TTF]-1) (7). In the current study, we determined the genome-wide profile of gene expression in response to hormone treatment. There are two new major findings from this study. First, we found that the induced genes represent all major functional categories; in addition to increased expression of SPs and genes related to phospholipid biosynthesis, as anticipated for surfactant-producing cells, differentiation involved increased expression of genes of all other major molecular functions. This novel finding implies that numerous new cellular functions, many of which are currently unexplored, are acquired on transition from precursor epithelial cell to mature type II cell. It is uncertain whether the scope of differentially expressed genes is unique to type II cell differentiation, as this issue has not been addressed in other differentiating systems, such as adipocytes, hematopoietic cells, and osteoblasts (15, 16, 19). Second, we identified and confirmed induction of protein and/or mRNA for 17 known genes, including 9 newly identified, regulated genes in the lung that represent novel markers of type II cell differentiation. It is likely that some of these coordinately induced genes share regulatory mechanisms or functional relationships. Overall, these results provide the first detailed description of specific gene expression during human type II cell differentiation. New regulated genes of diverse molecular functions have been identified that may be critical for numerous physiologic activities, some of which may be currently unappreciated, of the mature lung.

DNA microarray approaches have been used in other studies to assess differential gene expression in lung tissue and cells under various conditions. Recent reports describe changes in gene expression in response to transforming growth factor- β treatment of lung fibroblasts (20), effects of treatment of newborn mice with dexamethasone and retinoic acid (21), changes in gene expression during development of mouse lung (22), and effects of phosphorylation status of TTF-1 (23). In the dexamethasone study (21), a significant difference in gene expression was detected for 499 genes of $\sim 12,000$ queried—a percentage comparable to that for induced plus repressed genes in our study.

There are some limitations to both the experimental model and microarray methodology used in this study. Hormonal treatment of fetal lung cells in primary culture may not completely replicate type II cell differentiation as occurs *in vivo*. However, several lines of evidence support the appropriateness of this culture system as a model for *in vivo* lung development. Key lipogenic enzymes are induced, PC synthesis increases with a shift in composition toward that of mature surfactant, content of SPs increases many-fold, the cells develop lamellar bodies, and surface-active surfactant is secreted in response to secretagogue treatment (6, 7). Collectively, these responses represent a coordinated, precocious expression of the surfactant system. In addition, there is induction of a number of other nonsurfactant-related genes (e.g., *SCNN1A*, *ATP1B1*, *ATP1A1*, *FASN*) whose expression is known to increase during type II cell differentiation *in vivo* (8). A more definitive assessment of the cultured cell model and profile of induced genes will be provided by a future study comparing gene expression in cultured fetal versus mature type II cells obtained from postnatal lung specimens. The study is also limited by conditions inherent to microarray analysis (e.g., false discovery rate) and by the number of lungs and chips used, which suggests caution in interpreting global expression results (e.g., enrichment of functional categories).

DNA microarray technology is a powerful tool for quantitative analysis of gene expression on a nearly genome-wide basis. Nevertheless, there are a number of caveats associated with this experimental approach that may lead to false-positive or false-negative results for changes in gene expression. Genes that are targeted by more than one probe set, which was 35% of the

Figure 6. Fluorescence immunostaining of fetal lung cells in monolayer culture. Control Day 5 cells are in the left panel of each pair (a, c, e, g, i, k, m) and DCI-treated cells are in the right panel of each pair (b, d, f, h, j, l, n). Control and treated cells for each antibody were photographed under identical exposures to allow direct comparison of staining intensities, and representative images from three or more lungs are shown. LPL immunostaining was greater in DCI-treated cells (b) compared with control cells (a), and was detected as punctate cytoplasmic staining. AQP4 stained minimally in control cells (c) and intensely stained the epithelial plasma membrane (pm) in treated cells (d). LAMP3 showed minimal staining of control cells (e) and intense perinuclear, punctate staining in treated cells (f). (g and h) HIF3 α staining overlaid with 4', 6-diamidino-2-phenylindole staining (blue) of nuclei. Staining occurred in intranuclear loci, consistent with nucleoli, in both control (g) and DCI-treated cells (h), as well as diffuse cytoplasmic staining, especially in DCI-treated cells. ADH showed intense generalized cytoplasmic staining in mesenchymal cells in both control (i) and DCI-treated (j) epithelial cells, with some staining of epithelial cells. IGF2 showed a modest increase in staining of DCI-treated cells (l) with a perinuclear vesicular pattern. NR4A2 staining was weak in control cells (m), with increased intensity in the cytoplasm of DCI-treated cells (n). N, nucleus; LB, lamellar bodies; FB, fibroblast; E, epithelial cell.

induced genes in this study, are less susceptible to errors for these reasons. Microarray data require confirmation by other measurements of mRNA content. Because protein levels do not always mimic changes in RNA levels, protein confirmation and immunohistochemistry are valuable. In our study, induction of all 17 genes of known function in Cluster 1 was confirmed by real-time RT-PCR. Induction was also found by Western analysis and/or immunostaining for 16 of 17 genes examined. The failure to confirm immunoreactive selenoprotein P, which has been previously detected in lung tissue (24), could reflect inappropriate specificity of the antibody used, low abundance of the protein, or induced transcription without increased translation.

We found 13 induced enzymes or transporters related to synthesis of PC and phosphatidylglycerol, key phospholipids of surfactant, with only one gene (*LPL*) included in Cluster 1. Hormonal induction of some of the biosynthetic proteins (fatty acid synthase, choline phosphotransferase, stearyl-coenzyme A desaturase) has been previously described (8, 25). It is noteworthy that molecules involved in lipid uptake (*LPL*, monoglyceride lipoprotein lipase, LDL receptor, membrane phosphatidic acid phosphatases, and adipose differentiation-related protein) or with a putative transport function (*ABCA3*) are hormonally responsive, as determined by microarray analysis, suggesting that the increased production of surfactant phospholipids involves

Figure 7. *In situ* hybridization for *WIF1*, *MAO*, and *IGF2* expression. Human fetal lung (16- to 19-wk gestation; $n = 3$) was cultured as explants for 5 d in control or DCI media. Explant tissue was fixed, and frozen sections from each group (uncultured, control, DCI-treated) were placed on the same slide for processing with each probe. Uncultured tissue is in the left panel of each row (a, d, g), control tissue at 5 d culture is in the middle panel (b, e, h) and DCI-treated is in the right panel (c, f, i). *WIF1*, *MAOA*, and *IGF2* were all expressed to the highest degree in epithelial cells of DCI-treated explants (c, f, i). *IGF2* was the only one of these messages expressed in the cells prior to culture, whereas *MAOA* showed some expression after culture in the absence of DCI, and *WIF1* was expressed only in DCI-treated explants.

enhanced uptake of extracellular substrate as well as increased synthetic activity. Induction of *LPL* is consistent with a model in which fibroblasts store triglyceride that is then provided to epithelial cells (26).

When gene expression was analyzed over time, SOM analysis revealed a variety of kinetic patterns, likely reflecting, in part, both primary and secondary hormone responses. A cluster of 17 genes of known function, including the SP genes, was upregulated after 4- to 8-h lag period to relatively high levels of expression by 72 h. As coordinate regulation of genes can indicate regulatory or functional relationships, some of the newly identified genes of this cluster may have key roles in the type II cell. The cluster contains eight genes (*SFTPA*, *SFTPB*, *SFTPC*, *PGC*, *IGF2*, *SCNN1A*, *AQP4*, *CLDN18*) that are known to be hormonally responsive in fetal lung (6–9, 17, 18, 27, 28). Although *SFTPD* was also induced by DCI, the time course of expression and fold induction differed from the other SPs and, therefore, it clustered with another group of induced genes. *PGC* encodes pepsinogen C, also referred to as gastricsin, which is an aspartic protease and member of the pepsin family that is type II cell specific and may have a role in SP-B processing (17). The function of IGF-II in alveolar epithelial cells may relate to cell proliferation. *SCNN1A* and *AQP4* both function in alveolar fluid homeostasis (27). DCI treatment also increased expression of *SCNN1B*, but expression of the γ subunit was not detected. Of the nine AQP genes probed by the chip, expression was detected for only AQP3 and AQP4. The tight-junction protein, claudin 18, is involved in paracellular ion permeability and epithelial cell barrier function, and induction of claudin 18 is associated with changes in transepithelial resistance of type II cell monolayers (18).

In addition, Cluster 1 contains 9 newly identified, regulated genes of fetal lung epithelial cells. Seven genes (*LAMP3*, *LPL*, *MAOA*, *WIF1*, *HIF3A*, *SEPI*, and *ADH1B*) have been detected

Figure 8. Properties of hormone-induced DC-LAMP gene expression. (A) Exposure of cells to DCI increased DC-LAMP mRNA in a linear fashion between 8 and 72 h of exposure, as assessed by both cDNA microarray (RNA pooled from cells of 4 lungs) and qPCR in a separate representative experiment. (B) DC-LAMP protein increased progressively between 12 and 72 h of DCI exposure compared with control cells. Values are mean $\pm$ SEM ($n = 5$). The inset shows a representative Western blot for DCI exposure plus lanes for control cells ("C") at 12 and 72 h. (C) Exposure to DCI, but not to dexamethasone or cAMP alone, induced DC-LAMP protein. The inset shows a blot from a representative experiment. Detection of DC-LAMP staining was performed with the Supersignal West Femto-ECL detection reagent. Values are mean $\pm$ SE for three experiments. * $P < 0.05$ versus control; ** $P < 0.05$ versus 24 h DCI.

in the lung, including three (*LAMP3*, *LPL*, *MAOA*) in type II cells; *LAMP3* has been previously localized to lamellar bodies. Three of these genes (*LPL*, *MAOA*, *ADH1B*) are hormonally regulated in other tissues. In lung cells, we found that *ADH1B*

was responsive only to dexamethasone, three genes (*WIFI*, *NR4A2*, *HIF3A*) were induced by cAMP but not by dexamethasone, and the other five genes were regulated by an interaction of glucocorticoid and cAMP. The pattern of hormonal responsiveness for gene induction emphasizes the physiologic importance of both glucocorticoid and cAMP in differentiation of type II cell function as well as ultrastructure (7). Mechanistically, the variety of hormone responses rules out the possibility that the genes of Cluster 1 are all regulated by a common pathway (e.g., hormonally induced transcription factor).

LPL, which hydrolyses serum lipoprotein triglycerides, has been detected previously in lung tissue, and its activity increases after birth in temporal association with increased numbers of lipofibroblasts (26, 29). We report the new observation that *LPL* is hormonally regulated in the lung similar to previous findings in selected other tissues, such as adipocytes (30). Immunostaining indicated localization of LPL to type II cells, with basolateral expression as required for hydrolysis of triglycerides derived from serum or adjacent fibroblasts.

LAMP account for about half of the total lysosomal membrane protein. The newest member of this family, *LAMP3* (also referred to as DC-LAMP/CD208) (31), has been previously identified in lung tissue and localized to lamellar bodies of mouse type II cells (32). We found that DC-LAMP was highly induced by combined hormone treatment in differentiating human type II cells. Moreover, DC-LAMP was localized exclusively to limiting membranes of lamellar bodies in a pattern identical to that for *ABCA3* (33). *LAMP1* and *LAMP2* were also expressed in the human fetal lung cells, but were not affected by hormone treatment. A role for DC-LAMP in surfactant modification and/or in secretion has been proposed (32).

WIFI encodes an inhibitor of Wnt proteins, a large family of secreted signaling molecules that interact with the receptor Frizzled and regulate nuclear transcription through the action of β -catenin (34). Targeted deletion of β -catenin in mouse lung epithelial cells at $< E15$ results in a more proximal epithelial cell phenotype and decreased branching of secondary bronchi (35). In murine fibroblasts, *WIFI* is strongly induced during BMP-2-mediated osteoblast differentiation (19). Our data represent the first description of *WIFI* expression in human fetal lung epithelial cells and its hormonal responsiveness. This finding is consistent with a role for Wnt signaling in lung development.

HIF3A was a highly induced gene in cAMP-treated lung cells. *HIF1A* was also induced (1.7-fold) by DCI treatment; however,

Figure 9. Induction of DC-LAMP in epithelial cells by confocal immunofluorescence microscopy. Isolated cells were cultured from Days 1 to 5 in the absence (control, lower panels) or presence of DCI (upper panels) and stained for DC-LAMP (left panels; 1:100 dilution) and SP-B (center panels; 1:100 dilution), and the images were overlaid (right panels). The yellow color of the

overlaid image shows colocalization of the DC-LAMP and SP-B in vesicles (e.g., lamellar bodies) surrounding the nuclei (dark). A higher power confocal microscopy (6,300 $\times$) image of lamellar bodies (LB, far-right panel) shows localization of DC-LAMP (red) in lamellar body membranes surrounding SP-B (green) staining within lamellar bodies.

transcripts for the HIF-associated proteins pVHL (von Hippel-Lindau tumor suppressor protein) and ARNT (aryl hydrocarbon receptor nuclear translocator) were expressed at low levels in the cells and were not induced. HIF-3 α has been previously described in adult lung, where it is greatly increased by hypoxia, induces angiogenesis factors, and triggers proliferation of pulmonary vascular smooth muscle cells (36, 37). A possible role for HIF3 α in the developing lung is to facilitate induction of angiogenesis coordinately with alveolization.

Novel IL-1 receptor-like genes, including *IL1RL1* (T1/ST2), IL-1 receptor-related protein and IL-1 receptor-related protein 2, comprise a cytokine receptor gene cluster at chromosome locus 2q12 (38). We found synergistic induction of *IL1RL1* during type II cell differentiation; in addition, *IL1R1* was induced (2.2-fold), whereas *IL1R2* and *IL1RN* (receptor antagonist) transcripts were not detected. Although there has been extensive study of *IL1RL1* isoforms in hematopoietic cells, the precise role of *IL1RL1/ST2* in human lung type II epithelial cells is unknown.

The *NR4A2* gene encodes a member of the nuclear receptor superfamily known as nuclear-related receptor 1, a transcription factor that has been shown to regulate midbrain development, in particular dopaminergic neurons, and aldosterone synthesis in the adrenal cortex (39). Expression of *NR4A2* has been described in rat lung (40), and we report responsiveness to cAMP, suggesting a possible role in cell differentiation.

ADH and monamine oxidases are nicotinamide adenine dinucleotide oxidoreductases that catalyze metabolism of alcohols and biogenic amines, respectively. Class I ADHs are expressed in a variety of tissues with epithelial localization (41). By contrast, we found that immunoreactivity localized predominantly to lung fibroblasts with the one antibody available for study. ADH is glucocorticoid responsive in hepatocytes (42); however, there are no previous reports of glucocorticoid regulation of *ADH1B* in the lung. Of the different *ADH* genes surveyed, only the β isoform of Class I *ADH* was induced. Increased ADH activity could provide glycerol as substrate for phospholipid synthesis or promote production of retinoic acid from retinol (41). Retinoic acid has a number of effects in the developing lung, including coactivation of the SP-B gene, promotion of phospholipid synthesis, and enhancement of alveolization (43–45).

We found that the MAO-A, but not MAO-B (not detected), was induced in lung cells and was localized to epithelial cells by immunofluorescence. Both isozymes are expressed in a variety of tissues, including the adult lung, where MAO-A is the more abundant form and localizes to the alveolar wall and smooth muscle cells (46). Induction of both ADH and MAO-A are consistent with upregulated catecholamine metabolism at the airway surface and/or essential housekeeping oxidoreductase functions in lung cells preparatory to the availability of oxygen at birth.

The time-course study also identified a subset of induced genes that were maximally upregulated soon after hormone treatment. It is likely that transcription factors in this subset are primary hormonal targets (versus being induced secondarily by other induced factors) and potential mediators of hormonal effects on other genes. Current studies are investigating the role of two of these factors, TTF-1 and “homeodomain-only” protein (HOP), in type II cells. Our findings indicate that while TTF-1 is required for DCI-induction of many genes, an increase in TTF-1 alone induces only a small subset of genes, implicating a role for other hormone-induced regulatory factors. HOP is regulated by TTF-1 and appears to act as a negative regulator of SP gene expression (V.K., unpublished data). Future studies will focus on the role of other induced transcriptional regulators during type II cell differentiation.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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