Differentiation of human pulmonary type II cells in vitro by glucocorticoid plus cAMP

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1Division of Neonatology, Department of Pediatrics, The Children’s Hospital of Philadelphia, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104; and 2Child Health, Division of Infection, Inflammation, and Repair, Southampton General Hospital, University of Southampton School of Medicine, Southampton SO16 7PX, United Kingdom

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Gonzales, Linda W., Susan H. Guttentag, Kelly C. Wade, Anthony D. Postle, and Philip L. Ballard. Differentiation of human pulmonary type II cells in vitro by glucocorticoid plus cAMP. Am J Physiol Lung Cell Mol Physiol 283: L940–L951, 2002—Mature alveolar type II cells that produce pulmonary surfactant are essential for adaptation to extrauterine life and prevention of infant respiratory distress syndrome. We have developed a new in vitro model to further investigate regulation of type II cell differentiation. Epithelial cells isolated from human fetal lung were cultured in serum-free medium on plastic. Cells treated with dexamethasone + cAMP analog and isobutylmethylxanthine for 4 days exhibited increased phosphatidylcholine synthesis and content of disaturated phosphatidylcholine species, manifolds increases in all surfactant proteins with processing to mature forms, and abundant lamellar bodies. DNA microarray analysis identified ~3,100 expressed genes, including subsets of genes induced 2- to >100-fold (~2.5%) or repressed 2- to 18-fold (~1.2%) by hormone treatment. Of the highly regulated genes, most were coregulated in an additive or synergistic manner by dexamethasone and cAMP agents. Approximately 90% of the regulated genes identified by this initial microarray analysis have not been previously recognized as hormone responsive. One newly identified hormone-induced gene is Nkx2.1 (thyroid transcription factor-1), which has a critical role in surfactant protein gene expression. Our findings indicate that glucocorticoid + cAMP is sufficient and necessary for precocious induction of functional type II cells in this in vitro system and that these hormones act primarily in combination to regulate expression of a subset of specific genes.

surfactant development; human fetal lung; thyroid transcription factor-1; cDNA microarray

DIFFERENTIATION OF SPECIALIZED cells is a critical developmental process for organ function and adaptation to physiological events. Notable examples include maturation of intestinal epithelia before weaning, development of the mammary gland during pregnancy, and maturation of key organs in the fetus before birth (37, 43, 47). In the lung, development of effective pulmonary gas exchange structures and production of surfactant are necessary for successful adaptation to extrauterine life. These key processes in lung maturation require differentiation of epithelium into type II cells, which produce surfactant, and further differentiation into type I cells, which establishes the thin alveolar-capillary membrane for efficient gas exchange. Morphologically, type II cell differentiation is marked by the disappearance of glycogen stores, a resource for surfactant phospholipid, by formation of lamellar bodies, the intracellular storage site and secreted form of surfactant, and expansion of the apical cell surface in the form of microvilli. The major component of surfactant is disaturated phosphatidylcholine (PC), principally the dipalmitoyl species (PC16:0/16:0), which forms the surface-active film at the alveolar surface to prevent collapse of air spaces. This function requires the presence of surfactant proteins (SP), in particular SP-B and/or SP-C (32).

Developmental regulation of the surfactant system involves transcriptional and posttranslational processes, with distinct temporal patterns for various surfactant components. In human fetal lung, mRNAs for SP-B and SP-C are first detected at ~12 wk of gestation, whereas SP-A mRNA is undetectable until 20–24 wk (3). Despite the relatively early appearance of SP-B/C transcripts, mature proteins that result from processing of proproteins are not found until after 20 wk of gestation (10, 28, 56). An increase in PC16:0/16:0 content in lung tissue similarly is delayed until >20 wk gestation (3, 31). Infants born prematurely at 24–30 wk of gestation have a >50% risk of developing respiratory distress syndrome as a result of immaturity of lung structure and surfactant deficiency. Even with the beneficial effects of antenatal glucocorticoid therapy to hasten fetal lung maturation and postnatal replacement surfactant treatment, respiratory distress syndrome and associated disorders remain a major cause of morbidity and mortality in premature infants (58).

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Studies of alveolar type II cell differentiation in cultured fetal lung explants and in vivo indicate an important role for glucocorticoids and cAMP in the differentiation process (3, 4, 6, 26, 46). A limited number of glucocorticoid-regulated genes have been identified, including the SPs, lipogenic and antioxidant enzymes, insulin-like growth factor and insulin-like growth factor binding proteins, tropoelastin, and water and ion transporter/channels (4). Other reported regulators of lung maturation include thyroid hormones, epidermal growth factor, gastrin-releasing peptide, interleukin-1 (IL-1), and parathyroid hormone-related peptide (PTHrP); however, there is little information regarding signaling pathways and target genes for these agents (3, 23, 26, 48, 50, 59).

Although the fetal lung explant model has been widely used to study type II cell differentiation, this system has limitations in experimental approaches and data interpretation. For example, many reagents do not penetrate into the explant interior; studies of surfactant secretion are not technically feasible, the role of other lung cell types in type II cell responses cannot be determined, and interpretation of results from hormone treatment is complicated by the accelerated maturation of type II cells induced by explant culture in the absence of hormones (3, 5, 46). There has been no description of a fully dehormonized preparation of human lung tissue under Institutional Review Board-approved conditions, as described previously (25). Nonadherent cells were plated on 60-mm plastic culture dishes in Waymouth medium containing 10% fetal calf serum. After overnight culture as explants without hormonal treatment is complicated by the acceleration of type II cell differentiation with regard to cell ultrastructure, production of surfactant components, and identification of hormone-regulated genes by DNA microarray analysis.

**MATERIALS AND METHODS**

**Cell culture.** We isolated enriched populations of epithelial cells from second-trimester (13–20 wk gestation) human fetal lung tissue under Institutional Review Board-approved protocols. After overnight culture as explants without hormones (26), we digested the tissue with trypsin, collagenase, and DNase and removed fibroblasts by differential adherence, as described previously (25). Nonadherent cells were plated on 60-mm plastic culture dishes in Waymouth’s medium containing 10% fetal calf serum. After overnight culture (day 1), we cultured attached cells for an additional 3–9 days in 1 ml serum-free Waymouth’s medium alone (control), 10 nM dexamethasone + 0.1 mM 8-bromo-cAMP and 0.1 mM isobutylmethylxanthine together (referred to as DCI), or dexamethasone or 8-bromo-cAMP-isobutylmethylxanthine separately. These concentrations maximally induce surfactant components in human lung explant cultures (5, 26, 39, 40). In preliminary experiments, cells were plated on Madin-Darby canine kidney matrix-coated dishes (25), and 3 ml of media were added to some cells cultured on plastic or the matrix. Responses to DCI treatment were comparable with cells cultured on plastic or Madin-Darby canine kidney matrix-coated plastic; however, the response was, in general, greater with 1 ml than with 3 ml of culture medium, and thus 1 ml was used for subsequent experiments.

**Choline incorporation into PC.** We cultured cells for 4 days without (control) or with DCI, added choline-free medium for 2 h to deplete intracellular choline, and labeled the cells with [3H]choline (10 μCi/ml) overnight. Total PC was isolated by thin-layer chromatography, and incorporation into PC was determined as described elsewhere (25).

**Electrospray ionization mass spectrometry of phospholipid molecular species.** We extracted cell phospholipids with chloroform and methanol (13) and performed electrospray ionization mass spectrometry of PC and phosphatidylcholinolinositol (PI) on a triple quadrupole mass spectrometer (Quatro Ultima, Micromass, Wythenshaw, UK) equipped with an electrospray ionization interface. PC species were detected by positive ionization; PI and other acidic phospholipids were preferentially detected using negative ionization (16, 30). We acquired and processed data using MassLynx NT software and expressed PC and PI species as percentages of their respective totals in the sample. The predominant molecular species present for each ion peak was determined by analysis of fatty acyl fragments generated by collision gas-induced fragmentation under negative ionization.

**RNA preparation and hybridization.** We extracted total RNA by the acidic guanidinium thiocyanate method (18). Contents of specific mRNAs were analyzed by dot-blot hybridization and scanning densitometry using 32P-labeled human cDNA probes (SP-A, SP-B, SP-C, SP-D, and fatty acid synthase), as described elsewhere (25). Briefly, four aliquots of total RNA (0.5–4 μg) were applied to the Duralose membrane (Stratagene), hybridized with 32P-labeled cDNA probes for each mRNA, and washed under high stringency conditions. The blots were reprobed with β-actin for normalization of results; there was no apparent change in β-actin signal per microgram of RNA under all culture conditions, consistent with previous observations for fetal lung and isolated type II cells (3, 11, 25). In previous studies (25), we demonstrated quantitatively similar results by dot-blot analysis and Northern blots. To provide comparison data for hormonal responsiveness of each cell preparation, we included on all blots RNA prepared from differentiated type II cells freshly isolated from explants cultured in DCI for 4 days prepared from the same lung specimens (25). Thus responsiveness of cells in monolayer culture was expressed as a percentage of the mRNA signal (densitometric units/μg RNA) by cells differentiated in the previously characterized explant system. For Northern analysis, poly(A)+ RNA was isolated with the FastTrack 2.0 kit (Invitrogen, Carlsbad, CA), subjected to electrophoresis as described elsewhere (3, 11, 25), and probed with 32P-labeled thyroid transcription factor-1 (TTF-1) cDNA (1.2 kb) and cDNAs for SP-B and β-actin, as described above.

**DNA microarray analysis.** We prepared total RNA using the RNeasy kit (Qiagen, Valencia, CA) from day 4 control and hormone-treated cells (DCI, dexamethasone, or cAMP + isobutylmethylxanthine) isolated from four separate type II cell preparations using 14- to 17-wk fetal lung tissue. Total RNA (10–15 μg) was used to prepare cDNA and, subsequently, biotin-labeled cRNA according to the protocol provided by the manufacturer (Affymetrix, Santa Clara, CA) and as previously published (24, 41). Fragmented biotin-labeled cRNA was suspended in standard Affymetrix hybridization buffer at 0.5 μg/μl and hybridized for 16 h at 45°C with Affymetrix human array HuGeneFL (9000183) or HG-U95Av2 (900303). HuGeneFL contains 16–20 unique 25mer oligonucleotide
probes for each of ~5,600 full-length human cDNAs plus corresponding probes with a single nucleotide change (mismatch control). Array HG-U95Av2 contains oligonucleotide probes for ~12,500 genes, including most of the ~5,600 genes on the HuGeneFL array. Probe sequences for both chips are based on UniGene and GenBank databases and are generally selected within 600 bp of the polyadenylation site. Overlap of the target sequences on the two chips for individual genes was 60–100%. In the first two experiments, cRNAs were prepared from day 4 epithelial cells exposed to control media, dexamethasone, cAMP + isobutylmethylxanthine, or DCI and hybridized with HuGeneFL arrays. In the next two experiments, cRNAs were prepared from epithelial cells exposed to control media or DCI and hybridized with HG-U95Av2 arrays. Labeled arrays were washed and stained with streptavidin-phycoerythrin (Molecular Probes) using the standard Affymetrix protocol. Arrays were scanned using the Affymetrix scanner and fluorescence quantitated using Affymetrix Microarray Suite 4.0 software. Raw data from array scans were averaged across all gene probes on each array, and a scaling factor was applied to bring the average intensity for all probes on the array to a set value of 1,500 fluorescence units. Gene expression in hormone-treated cells was compared with the baseline gene expression in control cells from the same cell preparation. We report data on mRNAs detected by probes for genes common to HuGeneFL and HG-U95Av2 arrays that increased more than twofold or decreased more than twofold in each of the four experiments.

Western analysis. We performed immunoblotting for SP-A, SP-B, TTF-1, and β-actin using previously described procedures (28) and NuPAGE bis-Tris gels with MES SDS running buffer according to the manufacturer’s protocol (Invitrogen). For SP-C immunoblots, samples were run on 16.5% SDS-PAGE with a Tris-tricine buffer system. Proteins were transferred to Duralose membrane and probed with rabbit polyclonal human anti-SP-A (62), rabbit polyclonal human anti-SP-B (10), rabbit polyclonal antiserum to mature recombinant SP-C in which Phe was substituted for Cys residues 3 and 4 (Byk Gulden, Konstanz, Germany), and mouse monoclonal anti-β-actin (Abcam, Cambridge, UK) using enhanced chemiluminescence detection (Dupont-NEN, Bradford, MA). For Western analysis of TTF-1 protein, nuclei were purified and extracted (21), and aliquots of nuclear protein (10 μg/well) were separated on NuPAGE bis-Tris gels. Immunoblots of TTF-1 protein were stained with monoclonal TTF-1 antibody (1:1,000; Neomarker, LabVision, Fremont, CA) using enhanced chemiluminescence detection.

**RESULTS**

**Morphology.** Epithelial cell purity, assessed on day 4 by immunostaining for cytokeratin and nuclear staining with 4',6-diamidino-2-phenylindole, was 83 ± 2% (mean ± SE, n = 9), with a yield of 95 ± 12 × 10⁶ cells/g tissue (Fig. 1A). Total RNA yield per dish was similar for control and DCI-treated cells at day 4 of culture (107 ± 4 and 106 ± 10% of day 1 yield, respectively, n = 5), consistent with the absence of cell proliferation during the course of the experiments. Cells cultured in the presence of DCI developed progressively more and larger cytoplasmic inclusions that stained positively with Nile red, a lipophilic stain.
known to stain lamellar bodies (Fig. 1B, DCI day 5),
with ~20 and 40% of the cells strongly positive after 4
and 7 days of culture, respectively. There was little
change in Nile red staining for cells cultured under
control conditions (Fig. 1B, control day 5). By electron
microscopy, epithelial cells after 24 h of culture (Fig.
2A) displayed microvilli and occasional small dense
cytoplasmic inclusions but no lamellar bodies charac-
teristic of type II cells. After 4 days of culture without
hormones, cells had minimal or absent microvilli and
cytoplasmic lamellar inclusions (Fig. 2B). Many, but
not all, cells cultured for 4 days in the presence of DCI
exhibited apical microvilli and abundant, mature la-
mellar bodies (Fig. 2C). Cells cultured with dexameth-
asone or cAMP agents alone had fewer and smaller
lamellar bodies than DCI-treated cells (not shown).

**Phospholipid synthesis.** Cells cultured in the pres-
ence of DCI demonstrated a 66% greater rate of choline
incorporation into PC than control cells (15.6 ± 2.6 vs.
9.4 ± 1.1 nmol·mg protein⁻¹·h⁻¹, n = 6, P < 0.05),
comparable to the stimulation (+91%) observed in
DCI-treated vs. control lung explants (6.37 ± 0.66 and
3.32 ± 0.16 nmol/mg protein, respectively). Treatment
with DCI, but neither hormone alone, markedly in-
creased mRNA for fatty acid synthase, a key lipogenic
enzyme known to be hormonally regulated (Fig. 3) (61).

We examined cell phospholipid composition by tan-
dem mass spectroscopy. After culture for 4 days without
hormones, cellular PC was dominated by monoun-
saturated species, principally PC16:0/16:1, PC16:0/
18:1, and PC18:1/18:1 (Fig. 4A). The fractional
concentrations of the disaturated species PC16:0/14:0
and PC16:0/16:0 were very low (2.7 ± 0.2 and 4.8 ± 0.9
mol%, respectively), similar to that of undifferentiated
cells maintained in culture (data not shown). When
cells were cultured with DCI, the concentrations of
these two disaturated PC species increased to 5.2 ± 2.1
and 7.4 ± 1.8 mol%, respectively (P < 0.05 vs. control
cells).

**PI species** were the principal acidic phospholipids
detected in the cultured cells. The fractional concen-
trations of the monounsaturated species of PI (16:1/
18:1, 16:0/18:1, 18:1/18:1, and 18:0/18:1) were signi-
ficantly increased in cells exposed to DCI, whereas
concentrations of the polyunsaturated species of PI
(18:0/20:4 and 18:0/20:3) decreased (Fig. 4B). The pro-
portion of monounsaturated PI species increased from
26 mol% in control cells to 37.3 mol% in DCI-treated
cells (P < 0.05). There were no changes in phospha-
didyglycerol concentration or composition of epithelial
cells, similar to findings from hormone-treated vs. con-
trol lung explants (26). By comparison, concentrations
of disaturated PC species and monounsaturated PI
species remained low in parallel primary cultures of
fetal lung fibroblasts treated with DCI (results not
shown).

**Fig. 2.** Electron-microscopic images of precursor and cultured cells. Epithelial cells from a lung at 17 wk of
gestation were examined on day 1 of culture (A) or after culture for 4 days in the absence (control, B) or presence
of DCI (C). Control cells on day 4 resembled day 1 cells, with small cytoplasmic inclusions (arrows) and absence
of lamellar bodies. Many cells cultured in DCI had large lamellar bodies with characteristic electron-dense core.
Morphological responses to DCI treatment were similar for 3 additional lung specimens (15, 15, and 18 wk of
gestation). N, nucleus; m, mitochondria; MV, microvilli.

**Fig. 3.** Hormonal induction of fatty acid synthase (FAS) mRNA. Content was decreased in day 4 control cells compared with day 1
cells and increased ~6-fold by DCI but not by dexamethasone (Dex)
or cAMP-isobutylmethylxanthine (cAMP) alone. Levels were deter-
mined by dot-blot hybridization and are relative to level in differenti-
tated type II cells freshly isolated from day 4 DCI-treated explants
(100%) (25). Values are means ± SE for 3 lungs. *P < 0.05 vs. day 1;
**P < 0.05 vs. all other groups.
Induction and processing of SPs. SP-A mRNA was undetectable in day 1 cells and day 4 control cells and was markedly induced by cAMP; levels were increased a further fivefold by DCI treatment (Fig. 5A). Content of 21-kDa pro-SP-C was maintained during culture in DCI, and processing to mature 3.7-kDa SP-C did not occur until 96–120 h (Fig. 7B). By immunodot assay (not shown), DCI treatment increased SP-A and SP-B total immunoreactive content by ≥100-fold on day 4 compared with lesser responses to dexamethasone alone (SP-B ~14-fold) and cAMP alone (SP-A ~70-fold).

Regulated genes. We performed DNA microarray analysis to identify new hormonally regulated genes and to explore the scope of gene regulation during in vitro type II cell differentiation. Cultured cells expressed ~3,100 of the 5,600 genes represented on the HuGeneFL chip, with signal intensities ranging from <100 to ~70,000 fluorescence units. With DCI treatment, mRNA content in four experiments was consistently increased at least twofold for 78 genes (Fig. 8A) and decreased for 37 genes (Fig. 8B). Most induced with day 4 controls. SP-A demonstrated a pericellular pattern of staining, as expected for constitutive secretion, and punctate staining was observed for SP-B and SP-C, consistent with accumulation in multivesicular bodies and lamellar bodies (Fig. 6). By Western analysis, cells initially expressed low levels of only 42-kDa pro-SP-B; however, by 72 h with DCI, 25-kDa intermediate and mature 8-kDa SP-B forms were detected, indicating full processing (Fig. 7A). Content of 21-kDa pro-SP-C was maintained during culture in DCI, and processing to mature 3.7-kDa SP-C did not occur until 96–120 h (Fig. 7B). By immunodot assay (not shown), DCI treatment increased SP-A and SP-B total immunoreactive content by ≥100-fold on day 4 compared with lesser responses to dexamethasone alone (SP-B ~14-fold) and cAMP alone (SP-A ~70-fold).

Induction and processing of SPs. SP-A mRNA was undetectable in day 1 cells and day 4 control cells and was markedly induced by cAMP; levels were increased a further fivefold by DCI treatment (Fig. 5A). There were small increases in SP-B, SP-C, and SP-D mRNA contents with individual hormones and synergistic responses to combined treatment after 4 days (Fig. 5B), with levels of all four SP mRNAs equaling or exceeding values found in type II cells freshly isolated from day 4 hormone-treated fetal lung explants. In a separate study of the time course for SP-B mRNA, content in control cells was 9 ± 2% on day 0 of culture vs. 2 ± 0.3% on day 4 (P < 0.05, n = 3) relative to content in type II cells isolated from differentiated explants. The maximal response of SP-A and SP-B mRNAs occurred after 48–72 h of exposure to DCI and the maximal response of SP-C mRNA after 5–6 days (data not shown).

Immunostaining for SP-A, SP-B, and pro-SP-C was markedly enhanced in DCI-treated cells compared...
genes (65%) were increased 2- to 6-fold, with 28 genes increased 6- to >100-fold. All highly induced genes (>20-fold) had low expression in control cells (<1,000 fluorescence units). The majority of genes that were induced <10-fold were also expressed at relatively low abundance in control cells (<2,000 fluorescence units), although a number of genes with less than fourfold induction had higher expression levels in control cells (to ~9,000 fluorescence units). For repressed genes (Fig. 8B), abundance in control cells was not strongly associated with the level of repression.

Twenty-eight genes were highly induced (>6-fold) by DCI treatment in four experiments (Table 1). Among these are eight genes previously known to be hormonally induced in fetal lung [SP-A, SP-B, SP-C, epithelial Na⁺ channel (ENaC) α- and β-subunits, fatty acid synthase, and aquaporin] or airway epithelial cells (antileukoprotease) and two novel sequences (G protein-coupled receptor and KIAA0022 gene). The remaining induced genes of known identity belong to
Microarray data are means ± SE of 4 experiments with RNA from fetal lungs of 14–19 wk gestation. Epithelial cells were cultured for 4 days in the absence or presence of dexamethasone + 8-bromo-cAMP + isobutylmethylxanthine (DCI) for the last 72 h. Two experiments used chip HUGeneFL and 2 experiments used chip HG-U95Av2, as described in MATERIALS AND METHODS. SP, surfactant proteins. Data for SP-A are means for 4 different probes on chip HUGeneFL (no appropriate probes were present on chip HG-U95Av2), and data for SP-B are means for 2 different probes on chip HG-U95Av2 (no appropriate probes were present on chip HUGeneFL). ENaC, epithelial Na channel.

several different protein classes, with most of yet undefined relationship to mature type II cell function.

Genes highly repressed (>5-fold) by DCI treatment (Table 2) included five of uncertain function (LIM protein, S100A2, DRAL, 3′-untranslated region, and phosphoprotein) and only one previously known to be hormonally responsive (plasminogen activator). The full listing of DCI-regulated genes is available on-line (http://stokes.chop.edu/web/neoresearch).

We also examined separate effects of cAMP and dexamethasone on gene expression. For these studies, we cultured epithelial cells with individual hormones, DCI, or no treatment. Among 27 highly induced genes (Table 3), 21 responses were additive or synergistic, 1 required both hormones, and 2 responded only to dexamethasone (data not shown). These findings support a general combinatorial role for the two hormones in gene regulation during vitro type II cell differentiation.

Of the 78 induced genes identified by microarray analysis, >7 have a known or likely role in surfactant production or lipid metabolism. These include all four SPs and fatty acid synthase, confirming previous and present results (Figs. 3 and 5–7), lipoprotein lipase, and ABCA3 (a lipid transporter). At least one of the induced proteases (cathepsin H) may be involved in processing of SP-B (29) and/or SP-C (15). Of interest
Treatment with dexamethasone or cAMP-isobutylmethylxanthine alone did not significantly increase TTF-1, whereas DCI exposure maintained TTF-1 content at a level comparable to that in day 1 cells.

Immunofluorescent staining for TTF-1 demonstrated nuclear localization of the protein and increased staining intensity (2.2-fold greater, \( P < 0.05 \)) after 4 days of DCI treatment (Fig. 9D) compared with day 4 control (Fig. 9C). Granular cytoplasmic staining with regard to the inhibitory effect of transforming growth factor-\( \beta \) (TGF-\( \beta \)) on surfactant components (11), four genes involved in TGF-\( \beta \) signal transduction were induced or repressed by DCI treatment. In addition, four known transcription factors were induced by microarray analysis after DCI treatment, including TTF-1, an essential factor for expression of the SP genes (14, 45, 57).

**Induction of TTF-1.** By microarray, TTF-1 was induced 4.3 \( \pm \) 0.7-fold by DCI treatment and 1.4- and 2.2-fold by dexamethasone and cAMP-isobutylmethylxanthine individually. We carried out additional studies to confirm and characterize hormonal regulation of TTF-1 in cultured lung epithelial cells. TTF-1 mRNA was present in cells on day 1 of culture, consistent with its known expression pattern in vivo. Levels decreased by day 4 in the absence of hormones but were maintained in the presence of DCI. By Northern analysis, treatment with DCI for 4 days increased expression of TTF-1 mRNA 3.8 \( \pm \) 0.8-fold (normalized to \( \beta \)-actin) compared with day 4 control (Fig. 9A, \( n = 4 \), \( P < 0.05 \)). By comparison, SP-B mRNA was undetectable on day 1 and on day 4 in the absence of hormones but was strongly expressed after 4 days of DCI treatment.

Generally parallel results were found for TTF-1 protein content by Western analysis (Fig. 9B). Culture for 4 days in the absence of DCI markedly reduced content of nuclear TTF-1 protein compared with day 1 cells.
for SP-B protein was apparent after DCI treatment (green stain) but not in control cells.

**DISCUSSION**

Lung maturation, including differentiation of type II cells and surfactant production, is critical for normal lung function and survival at birth, particularly after preterm delivery. Clinically, women in premature labor are treated with glucocorticoid to enhance fetal lung maturation and reduce the occurrence of respiratory distress; however, the scope of cellular responses to this treatment is not known. In this report, we describe a simple, fully defined culture system for differentiation of human pulmonary type II cells in vitro in the presence of glucocorticoid + cAMP, identify new regulated genes by microarray analysis, and present new information regarding one of these genes, TTF-1.

Differentiation of type II cells from precursor lung epithelial cells has been extensively studied in organ culture of fetal lung (3–5, 10, 11, 26–28, 39, 40, 46, 56, 59–61). In explants prepared from fetal lung tissue and cultured without serum or hormones, epithelial cells spontaneously develop lamellar bodies and demonstrate increased synthesis of PC and SP-A. This spontaneous ex vivo differentiation process is accelerated in the presence of cAMP and glucocorticoid, with interactive effects on induction of SPs. Explants have been a useful model system; however, multiple cell types are present, and it has not been possible to study surfactant secretion or to carry out efficient transfection or transduction of epithelial cells.

In earlier studies, it was found that differentiated fetal type II cells, when isolated from hormone-treated explants and cultured on plastic, rapidly lost the ability to synthesize surfactant components, similar to the response by isolated adult type II cells (38). More recently, Alcorn et al. (1) reported maintenance of SP-A mRNA contents and lamellar bodies in fetal type II cells isolated from explants and cultured on extracellular matrix-coated dishes in the presence of dibutyryl cAMP. Addition of dexamethasone along with cAMP reduced SP-A mRNA and increased SP-B gene expression, similar to the responses observed in explants. We subsequently confirmed and further characterized the important role of glucocorticoid and cAMP in maintaining differentiation of human fetal type II cells during monolayer culture (25). Moreover, a recent report describes similar effects of glucocorticoid + cAMP treatment in maintaining differentiation of type II cells isolated from rat lung (9). One limitation of these culture systems is the inability to study the differentiation process, including lamellar body genesis.

On the basis of the observation that glucocorticoid + cAMP treatment maintains surfactant production in already differentiated, isolated type II cells, we hypothesized that this treatment would induce differentiation of immature fetal lung epithelial cells in monolayer culture. However, it could not be assumed a priori that agents capable of inducing differentiation in intact tissue (i.e., explants) and maintaining differentiated function of cultured mature type II cells would necessarily be sufficient for inducing differentiation of immature cultured cells. When cells are cultured on plastic substratum, they lose possible influences of the basement membrane, contact with adjacent cells that exist in tissue, and polarization. It has been proposed that differentiation of type II cells in fetal lung involves direct contact of mesenchymal and epithelial cells as well as paracrine interactions, aspects that are preserved in explant tissue and disrupted in monolayer culture of isolated epithelial cells. Previous attempts to induce type II cell differentiation in vitro resulted in limited responses of isolated undifferentiated epithelial cells to various combinations of serum, growth factors, and use of Matrigel (17, 22, 53, 55, 60). To our knowledge, this is the first description of type II cell differentiation in vitro with fully defined culture medium and substratum conditions. A possible contribution from the contaminating mesenchymal cells is the only undefined factor in the culture system.

In vitro differentiation occurs for epithelial cells of other tissues under specific culture conditions. Primary cultures of undifferentiated intestinal cells mature in response to glucocorticoid or serum only if cocultured with fibroblasts and laminin (33) or with Matrigel (reconstituted basement membrane) (52). Mammary cells from pregnant rats differentiate morphologically and produce milk proteins during culture on Matrigel in the presence of lactogenic hormones or triiodothyronine (7). Thus the requirement for glucocorticoid + cAMP appears to be unique for lung epithelial cells among different tissues that have been examined.

Several lines of evidence support the conclusion that surfactant components are coordinately induced in this culture system. 1) Each of the SPs as well as fatty acid synthase, a key enzyme for surfactant phospholipid production (61), was induced, and pro-SP-C and pro-SP-B were fully processed to mature protein forms consistent with induction of processing enzyme(s). 2) Nkx2.1 (TTF-1), a transcription factor known to be important in the regulation of surfactant protein gene expression (14, 57), was hormonally induced. 3) Electron microscopy confirmed formation of abundant lamellar bodies with ultrastructural characteristics of lamellar bodies in type II cells of adult lung. 4) Hormone-treated cells had an increased rate of PC synthesis with a shift in composition of PC and PI toward that of mature surfactant (63), changes that are consistent with the developmental pattern during human gestation (12, 31). These responses were not secondary to alterations in the exogenous lipid supply, inasmuch as cells were cultured without serum and without lipid additives. The response was cell-type specific, inasmuch as similar changes did not occur in parallel primary cultures of fetal lung fibroblasts. Although the percentage of disaturated PC doubles with hormone treatment, the absolute percentage is low (~16 mol%) compared with isolated newborn rat type II cells (36%) (8) and human surfactant (~65%) (63), perhaps reflecting in part that not all epithelial cells produced lamel-
lar bodies. Additional studies are underway to characterize secreted surfactant.

Epithelial cells cultured as a monolayer in the absence of hormones showed no maturation, in contrast to fetal lung explant cultures (26, 46). The spontaneous maturation of type II cells in explant culture reflects, in part, increasing content of endogenous cAMP secondary to prostaglandin stimulation (5). Our findings that dexamethasone alone strongly induces SPs in lung explants (3), but not in isolated epithelial cells, highlight the critical role of cAMP. Previous studies with fetal lung explants found stimulatory effects of cAMP analogs (5) and synergism with glucocorticoids on phospholipid synthesis (27), formation of lamellar bodies (46), and induction of SPs (39, 40) and fatty acid synthase (61). Our present studies in cultured cells indicate a more extensive role of cAMP than previously surmised from explant studies. cAMP and dexamethasone showed additive or synergistic effects on most of the highly regulated genes (~75% of induced and repressed genes), suggesting that the responses to antenatal glucocorticoid treatment, given to enhance lung maturation in premature infants, may be modulated by levels of endogenous cAMP. By contrast, other known stimulatory factors of lung development, including epidermal growth factor, PTHrP, and IL-1-related genes (pro-IL-1β, IL-1 receptor antagonist, type II IL-1 receptor, and associated kinase), that were present on the DNA microarrays were unaffected after 4 days of DCI treatment and, thus, are apparently not directly involved in this in vitro differentiation process.

Our microarray data indicate that a subset (~3.7%) of expressed genes are hormone responsive (i.e., glucocorticoid and cAMP) and also identify new candidate genes that may be important in the type II cell phenotype. One highly induced gene is ABCA3, a membrane protein that transports lipids (35) and may be important for transport of lipids into type II cells and/or lamellar bodies. mRNA for cathepsin H is abundant in DCI-treated cells and may have a role in processing of SP-B/C (15, 29). The possible role of alcohol dehydrogenase in type II cells is uncertain but could involve conversion of retinol to retinoic acid, a hormone that influences SP-B/C expression (2, 45). Fibroblast growth factor (FGF) receptor 2 and its coreceptor glycopican were strongly induced (5.3- and 10.4-fold, respectively) by DCI treatment. Isforms of this receptor mediate effects of FGFs, including FGF-9 and FGF-10, which appear to be essential for normal lung morphogenesis (19, 54). Other groups of regulated genes of particular interest include transcription factors and genes related to signaling of TGF-β, a negative regulator of type II cell differentiation (11), as well as Na⁺-K⁺-ATPase and ENAC, two genes involved in water and ion transport that were recently reported to be synergistically increased in rat type II cells by dexamethasone and cAMP in vitro (20). A limitation of our experimental system is impurity of the epithelial cells as currently isolated. Accordingly, some of the regulated mRNAs, in particular repressed genes, may reflect hormone effects in fibroblasts or other pulmonary cell types.

Results of the four microarray experiments generally agreed, with some variability between the two types of chips when different target sequences were probed. The level of induction or repression on each microarray was comparable for eight genes for which probes were generated from two or more database entries (e.g., SP-A; see web site). Most genes regulated by DCI were also regulated by dexamethasone and/or cAMP alone, further supporting reliability of the chip data. Additional experiments are underway with larger-capacity chips to extend the list of regulated genes in this experimental system.

Hormonal responsiveness of new target genes tentatively identified by microarray must be confirmed (e.g., Northern, RT-PCR, and/or Western analyses). We have confirmed the responsiveness of ABCA3 (44), the protease cathepsin H (29), and TTF-1 (Fig. 9). A product of the Nkx2.1 gene, TTF-1 plays critical roles in early lung morphogenesis (34) and in later SP gene expression (14, 45). We found that TTF-1 was expressed in freshly isolated cells, as expected on the basis of its appearance in airway epithelial cells early in human lung development (57), but decreased during culture in the absence of hormones. Interestingly, DCI treatment maintained the level of TTF-1 comparable to that in freshly isolated cells, indicating that although TTF-1 may be required for SP gene expression, it is not sufficient, and that other critical transcription factors and/or coactivators are induced by hormone treatment. Alternatively, hormone treatment may cause posttranslational modifications (e.g., phosphorylation) of TTF-1, altering its enhancer function, in addition to maintaining protein production. A previous report described a stimulatory effect of cAMP on TTF-1 DNA binding activity (36). TTF-1 is likely an important mediator of the stimulatory effect of DCI in this culture system, although blocking experiments are needed to determine its role in the induction of specific genes.

This cell culture system will be useful for exploring mechanisms of the differentiation process as well as the role of newly identified proteins in surfactant production and other functions of pulmonary type II cells. Importantly, the culture system is fully defined, utilizing serum-free medium and plastic as the substratum, and the cells can potentially be transfected and transduced with constructs of interest. These and other approaches can be used for loss or gain of function experiments to evaluate the role of specific regulated proteins.

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Differential of human lung type II cells in vitro