Thyroid Transcription Factor in Differentiating Type II Cells

Regulation, Isoforms, and Target Genes

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Thyroid transcription factor-1 (TTF-1, product of the *Nkx2.1* gene) is essential for branching morphogenesis of the lung and enhances expression of surfactant proteins by alveolar type II cells. We investigated expression of two TTF-1 mRNA transcripts, generated by alternative start sites and coding for 42- and 46-kD protein isoforms in the mouse, during hormone-induced differentiation of human fetal lung type II cells in culture. Transcript for 42-kD TTF-1 was 20-fold more abundant than TTF-1₄₆ mRNA by RT-PCR. Only 42-kD protein was detected in lung cells, and its content increased during *in vivo* development and in response to *in vitro* glucocorticoid plus cAMP treatment. To examine TTF-1 target proteins, recombinant, phosphorylated TTF-1₄₂ was expressed in nuclei of cells by adenovirus transduction. By microarray analysis, 14 genes were comparably induced by recombinant TTF-1 (rTTF-1) and hormone treatment, and 9 additional hormone-responsive genes, including surfactant proteins-A/B/C, were partially induced by rTTF-1. The most highly (~10-fold) TTF-1-induced genes were DC-LAMP (LAMP3) and CEACAM6 with induction confirmed by Western analysis and immunostaining. Treatment of cells with hormones plus small inhibitory RNA directed toward TTF-1 reduced TTF-1 content by ~50% and inhibited hormone induction of the 23 genes induced by rTTF-1. In addition, knockdown of TTF-1 inhibited 72 of 274 other genes induced by hormones. We conclude that 42-kD TTF-1 is required for induction of a subset of regulated genes during type II cell differentiation.

Keywords: cyclic AMP; dexamethasone; gene expression; microarray; surfactant protein B

Thyroid transcription factor-1 (TTF-1, product of the *Nkx2.1* gene) was originally identified as one of three transcription factors that regulate thyroid-specific gene expression (1, 2). TTF-1 is a homeodomain-containing protein expressed in embryonic diencephalon, thyroid, and lung (3). In the lung, TTF-1 is essential for both early morphogenesis and branching as well as later surfactant protein (SP) expression by type II epithelial cells (3–6). TTF-1 null mice have only a rudimentary outpocketing of the early airway (4) lacking branched airways and lung parenchyma. In a human infant, haploinsufficiency of TTF-1 due to heterozygous TTF-1 gene deletion was associated with respiratory failure at birth (7).

Alveolar type II cells differentiate from precursor epithelial cells during the second half of human gestation. These cells perform a variety of functions, including production of pulmonary surfactant (8), transport of ions and water across the epithelium (9), and production of molecules that are related to local immune defense and inflammation (10); they also proliferate after lung injury to repopulate type I cells for gas exchange (11). The role of TTF-1 in most of these processes has not been investigated. We have used primary cultures of epithelial cells, isolated from mid-gestation lung tissue, as an *in vitro* model for type II cell differentiation. Differentiation of type II cells is accelerated by *in vivo* or *in vitro* exposure to glucocorticoid and/or cAMP (12). Cells cultured in the absence of serum and exposed for 4 d to dexamethasone plus cAMP develop lamellar bodies and secrete surface active surfactant. This treatment induces a subset of genes including many related to surfactant production and ion/fluid flux. Using microarray gene expression profiling, we found that ~3% of expressed epithelial cell genes were upregulated, representing a variety of categories of biological function (13). The transcriptional mechanisms responsible for these changes are only partly defined, but importantly we found that TTF-1 was induced by glucocorticoid plus cAMP. Aside from well-documented effects on morphogenesis and expression of surfactant proteins, the specific role(s) of TTF-1 in lung epithelial cell differentiation is largely uncharacterized. Consensus sequences for TTF-1 binding have been identified in one or more regions of the promoters of the surfactant proteins (SP-A, SP-B, and SP-C) (14–16), CCSP (17), and claudin 5 (18). Functional interaction of TTF-1 with other transcription factors or co-regulators of SP gene promoters has also been studied. These cofactor proteins include members of the forkhead family (HNF3 β) (14), CAAT-enhancer binding proteins (19), CBP/p300 (20), upstream stimulatory factor (USF), and Smad3 families of proteins (21), as well as retinoid receptor (22), novel binding proteins such as BR22 (23, 24), and ubiquitous factors such as SP1 and SP3 (25).

The major TTF-1 protein is a 42-kD isoform encoded by a 2.1-kb mRNA. A slightly larger TTF-1 isoform of ~46 kD, encoded by a 2.3-kb transcript, has been described in the mouse by one laboratory (26), and the two transcripts were differentially expressed during mouse embryonic lung development. The 30-amino acid extension sequence of TTF-1₄₆ is highly conserved among various nonprimate species, and multiple mRNA transcripts have also been identified in thyroid tissue, but their functions are unknown (27). The ontogeny and regulation of human TTF-1 isoforms and possible differential roles in lung development are not known and have been addressed in this study.

The culture system for *in vitro* differentiation of parenchymal epithelial cells into type II cells affords a unique system to examine hormonal regulation of TTF-1 and its isoforms in human cells. The aims of this study were to characterize the TTF-1 isoforms expressed in differentiating human fetal lung type II cells and to assess developmental and hormonal effects on

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expression. In addition, the profile of genes influenced by TTF-1 was determined by adenovirus-mediated overexpression and small inhibitory RNA (siRNA) knockdown of TTF-1. Part of this study has previously been published in preliminary form (28).

MATERIALS AND METHODS

Materials

Cell culture media, antibiotics, and fetal calf serum (FCS) were obtained from Invitrogen Inc. (Carlsbad, CA). Restriction enzymes, modifying enzymes, and other molecular biology reagents were purchased from Promega (Madison, WI) and New England Biolabs, Inc. (Beverly, MA). "Complete" Protease Inhibitor cocktail tablets were obtained from Roche Applied Sciences (Indianapolis, IN). ³⁵S-methionine was purchased from Perkin-Elmer Life and Analytical Sciences, Inc. (Boston, MA). Dexamethasone, 8-bromo-cAMP, and all other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific Inc. (Pittsburgh, PA), and H-441 and A549 cells from American Type Culture Collection (Rockville, MD).

Lung Cell Isolation and Culture

Human fetal lung and thyroid tissues of 11–22 wk gestation abortuses were obtained from Advanced Bioscience Resources (Alameda, CA) and/or the Birth Defects Laboratory of the University of Washington (Seattle, WA). Lung epithelial cells of ~88% purity (29) were isolated and cultured as previously described under approved IRB protocols of the Children's Hospital of Philadelphia (13). Briefly, the tissue was digested with trypsin, collagenase, and DNase, fibroblasts were removed by differential adherence, and nonadherent cells were plated on 60-mm plastic culture dishes in Waymouth's medium containing 10% FCS. After overnight culture (Day 1), attached cells were cultured an additional 3–9 d in 1 ml of serum-free Waymouth's medium alone (control), or with DCI (dexamethasone, 10 nM; 8-Br-cAMP, 0.1 mM; isobutylmethylxanthine, 0.1 mM), or with dexamethasone or 8-Br-cAMP/isobutylmethylxanthine separately. These concentrations maximally induce surfactant components in human lung explant cultures (30).

Cell Culture and Transfections

Human lung epithelial cells were cultured in serum-free Waymouth's medium. A549, HEK293 (for adenovirus growth), and HeLa cells were cultured in Dulbecco's modified Eagle's medium (DMEM), and lung NCI H441 cells were cultured in RPMI 1640 medium + 10% FCS. Transient transfections of HeLa and A549 cells (2×10^5) with TTF-1 expression plasmids (1–5 µg) and a reporter pEGFP expression plasmid (0.2 µg) were performed by the calcium phosphate precipitation method or with SuperFect (Qiagen Inc., Valencia, CA) or Fugene-6 (Roche Diagnostics, Basel, Switzerland) as per the manufacturer's recommended conditions.

Cloning

Expression plasmids for both TTF-1 (rTTF-1) transcripts were generated in a eukaryotic T7-tagged pcDNA3 expression plasmid at *EcoRI* and *XhoI* sites. TTF-1 short isoform (12A2) was amplified directly from lung type II cell total RNA (cDNA cloning) and the long isoform (5E) was subcloned after PCR amplification from a previously cloned adenovirus expression plasmid (26). All constructs were confirmed by restriction enzyme analyses and DNA sequencing.

Recombinant Adenovirus Construction

Recombinant sense constructs for TTF-1 (rTTF-1) of either the 12A2 (42 kD, Ad12A2) or the 5E (46 kD, Ad5E) transcript were generated using the Adeno-X Expression System as per the manufacturer's instructions (BD Biosciences, Clontech, Palo Alto, CA). Briefly, constructs were generated under the CMV promoter in a replication-deficient adenovirus H5.010 to facilitate high expression levels, subcloned into a pShuttle plasmid at *NotI* and *HindIII* sites to generate expression cassettes that were digested with *Pi-SceI*/*I-CeuI* and cloned into the adenovirus genome. After *SwaI* digestion (removes nonrecombinant virus) the recombinant adenovirus DNA was transformed with *Escherichia coli* and propagated. TTF-1 adenovirus plasmids were digested with *PacI* and

transfected into HEK 293 cells; purified adenovirus was isolated and the number of active, infective particles was determined by plaque assay. Control adenovirus containing pEGFP coding sequence under a CMV promoter was cloned into pShuttle vector and was used to facilitate visual observation of infection efficiency.

RT-PCR

Total RNA from fetal lung cells and cell lines was prepared (RNeasy spin column kit; Qiagen) and analyzed by RT-PCR (TITANIUM one-step RT-PCR kit; BD Biosciences, Clontech). The amount of input RNA (10–100 ng) and number of cycles to amplify TTF-1 were optimized in preliminary reactions using the Platinum Quantitative RT-PCR ThermoScript One-Step System (Invitrogen). Reverse transcription reaction was performed at 50°C for 1 h followed by PCR. The optimal PCR conditions to determine isoform identification were: 94°C, 5 min for 1 cycle followed by 26 cycles of 94°C, 45 s; 62°C, 45 s; and 72°C, 45 s with a final extension cycle of 72°C, 30 s. TTF-1 product was detected in a linear fashion at 26 cycles.

Immunofluorescence

Cells cultured on cover glass were fixed in 1% paraformaldehyde in PBS, permeabilized with 0.3% Triton X-100, and immunostained as described (13) using polyclonal antibodies: rabbit anti-bovine pancytokeratin (1:100; Zymed Laboratories, South San Francisco, CA); rabbit anti-bovine SP-B (1:100; Chemicon, Temecula, CA), and monoclonal antihuman DC-LAMP (1:200; Immunotech, Marseille, France) plus Cy3-conjugated or Alexa 488-tagged secondary antibodies (1:200). After cytokeratin immunostaining, sections were exposed to DAPI (0.1 µg/ml; Molecular Probes, Eugene, OR) for 10 min to stain nuclei.

Cell Extract Preparation

To prepare whole cell extracts (type II, HeLa, A549, and H441) cells were washed once with PBS, lysed by sonication in ice-cold buffer (50 mM Tris HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 1 mM EDTA, 2 mM dithiothreitol [DTT], 1 mM phenylmethylsulfonylfluoride [PMSF], 5 µg/ml leupeptin, 5 µg/ml aprotinin, and 5 µg/ml antipain), then centrifuged at $10,000 \times g$ for 10 min at 4°C and supernatants collected for Western analysis. Nuclear and cytosolic fractions were prepared from cells by the modified Dignam method (31) as described previously (32). Protein concentrations were determined by a microplate assay protocol (Bio-Rad Laboratories, Hercules, CA).

In Vitro Transcription and Translation

The coding regions for both transcripts of TTF-1, cloned into eukaryotic T7 pcDNA3 expression plasmids, were *in vitro* transcribed/translated in the presence of either ³⁵S-labeled or cold methionine and rabbit reticulocyte lysate with a T7-RNA polymerase-coupled TNT kit (Promega) according to the manufacturer's recommendations. Protein products were denatured by boiling in a sample buffer (62.5 mM Tris HCl, pH 6.8, 2% SDS, 0.72 M 2-mercaptoethanol, 10% glycerol, 0.0075% bromophenol blue, 3–5 min) and loaded onto a 12% SDS-polyacrylamide gel along with a stained molecular weight marker. The ³⁵S-labeled products were visualized by exposing the dried gel directly to X-ray film.

Western Analysis

Western blot analyses for TTF-1 and CEACAM6 were performed as described previously (13) using NuPAGE Bis-Tris gels with MOPS-SDS Running Buffer as per the manufacturer's protocol (Invitrogen). For SP-B immunoblots, samples were run on MES SDS-PAGE gels. Proteins were transferred to Duralose membrane (Stratagene, La Jolla, CA) and probed with antibodies using enhanced chemiluminescence detection (Dupont-NEN, Bradford, MA). TTF-1 monoclonal antibody (Lab Vision Corp., Fremont, CA), and two polyclonal antibodies were purchased (amino acids 1–190, SC; Santa Cruz Biotechnology Inc., Santa Cruz, CA; and anti-rat peptide, 10AA [DL]; Biopat Immunotechnologies, Caserta, Italy). Polyclonal anti-baboon TTF-1 (amino acids 1–50, CM) was a generous gift from Dr. C. Mendelson (University of Texas Southwestern, Dallas, TX). CEACAM6 (CEA/CEACAM6) monoclonal antibody (Novus Biologicals, Littleton, CO) (33), anti-β-actin (Abcam Limited, Cambridge, UK), rabbit anti-bovine SP-B (Chemicon) and anti-GAPDH (Chemicon) were purchased.

Immunoprecipitation and Phosphorylation of TTF-1

Fetal lung cells were cultured 72 h in control medium to deplete endogenous TTF-1 and then transduced with Ad12A2 (1–8 plaque-forming units [pfu]/cell) in control ($n = 1$) or cAMP+IBMX-containing ($n = 3$) medium for ~24 h. Some cells were cultured in DMEM+DCI. Two hours after virus addition, an aliquot (100 μ l) of medium containing 32 P-inorganic phosphate (300 μ Ci/dish, 1.5 ml) was added and culture continued for total 24 h. Cells were then washed and harvested for preparation of nuclear and cytosolic extracts. Immunoprecipitation was performed essentially as previously described (34) using nuclear extract (50 μ g protein, $\sim 1.5 \times 10^6$ cpm) in buffer containing phosphatase inhibitors (50 mM Na fluoride, 10 mM Na pyrophosphate, 5 mM Na vanadate), protein G-agarose to preclear and then anti-TTF-1 antibody (1 μ l of anti-TTF-1, SC; or 0.5 μ l of anti-TTF-1, DL) for overnight incubation at 4°C. After addition of protein G-agarose and washing, the agarose-antibody-antigen complex was solubilized in 40 μ l lysis buffer and aliquots applied to SDS-PAGE gels (20 μ l/well for 32 P-detection by exposure to film, 10 μ l/well for TTF-1 immunostaining).

siRNA Silencing of TTF-1

Four *for sure* TTF-1 silencing small inhibitory RNA oligonucleotides (siRNA)—TTF-1A, TTF-1B, TTF-1C, TTF-1D—were synthesized and purified by Qiagen-Xeragon (Germantown, MD). By Western analysis, oligonucleotides B and C did not effectively knockdown TTF-1 protein; however, both A and D oligonucleotides were effective. Sequences of A and D as well as control nonsilencing oligonucleotide sequences are as follows: forward TTF-1A (188–208) r(5'-GCACACGACUCCGUU CUCA)d(TT) and reverse TTF-1A r(5'-UGAGAACGGAGUCGU GUGC)d(TT) siRNAs; forward TTF-1D (817–837) r(5'-UGAAGCG CCAGGCCAAGGA)d(TT) and reverse TTF-1D r(5'-UCCUUGGC CUGCGCUUCA)d(TT) siRNAs; and control (nonsilencing) siRNA, fluorescein-labeled forward r(5'-UUCUCCGAACGUGUCACGU) d(TT), reverse r(5'-ACGUGACACGUUCGGAGAA)d(TT). The control oligonucleotides bear no homology with relevant human genes by BLAST analysis. Oligonucleotides were dissolved in buffer (100 mM potassium acetate, 30 mM HEPES-potassium hydroxide, 2 mM magnesium acetate, pH 7.4) to a final concentration of 20 μ M, heated (90°C for 60 s, and 37°C for 60 min) before use. Optimum dose was determined (1–5 μ g); no cell toxicity was observed at any dose. Fetal lung cells were cultured for 3 d in control media to deplete endogenous TTF-1 protein and transfected with 3–5 μ g of siRNA with RNAifect transfection reagent (Qiagen) as per manufacturer's instructions. After 6 h, cells were treated with DCI or control media for 72 h and sonicates were analyzed for TTF-1 RNA (RT-PCR) and protein (Western blots). No inhibition (RT-PCR, Western) of known TTF-1-dependent genes (SP-B, SP-C) was observed after transfection with the ineffective TTF-1 oligos (siTTF-1B, siTTF-1C) or the control oligonucleotide, confirming specificity of the oligos A and D. In addition, the effective oligonucleotides siTTF-1A or siTTF-1D did not inhibit CEBP δ , a TTF-1-independent gene.

For some immunostaining studies, cells were treated with siRNA oligonucleotides (200 nM) by nucleofection according to the manufacturer's protocol using epithelial cell specific buffer (AMAXA Inc., Gaithersburg, MD). Before fixation Nile Red (0.1 μ g/ml) was added to the medium of some cells to visualize lamellar bodies.

DNA Microarray Analysis

For all experiments, undifferentiated epithelial cells were isolated from lungs of 16–20 wk gestation abortuses and cultured individually for 3 d in control media to deplete endogenous TTF-1. For DCI versus control experiments (group 1, $n = 11$ lungs), medium containing DCI or diluent was added on Day 3 and cells were harvested 72 h later. Hormonal responsiveness in each experiment was established by real-time PCR assays of SP-B mRNA content. RNA was pooled (four, four, and three lungs, respectively) to create three pooled experiments for microarray analysis of DCI versus control (six chips). The time-course of gene expression was studied with pooled RNA from one of these three experiments. Cells from four lungs were cultured individually with harvest at 4, 8, 24, 48, and 72 h after DCI exposure (control = no DCI), and RNA was pooled at each time point.

Second, for TTF-1 overexpression arrays, cells (group 2, $n = 7$ lungs) were cultured 3 d in control media, then transduced (~ 5 pfu/cell) with

Ad12A2 or AdGFP (control virus expressing green fluorescent protein) and cultured an additional 72 h. TTF-1 protein expression (Western analysis) with Ad12A2 was ~ 3.5 -fold greater than the level in DCI-treated cells (tested concurrently). Two microarray experiments (four chips) were performed with pooled RNA (four and three cell preparations, respectively) as above. Cell viability (Live-Dead cell viability/cytotoxicity assay; Molecular Probes, Inc.) was high for transduced and nontransduced cells ($95 \pm 1\%$ viability at doses 1–10 pfu/cell, ~ 900 cells/group counted).

Third, for microarray analysis after silencing of TTF-1 gene expressions ($n = 3$ lungs and 6 chips, group 3 cells) cells were cultured 3 d in control media, some dishes were transfected with antiTTF-1 siRNA (at 5 μ g/35 mm dish) + RNAifect, and DCI was added on Days 3–5. RNA was analyzed by cDNA microarray (six chips). Efficiency of transfection was $> 50\%$ based on transfection with a fluorescent-tagged nonspecific oligonucleotide. Criteria for inclusion of samples in the analysis of TTF-1 knockdown were decreases in TTF-1 mRNA $> 40\%$ ($47\% \pm 7\%$, $n = 3$) and similar decreases in SP-B mRNA ($53\% \pm 6\%$, $n = 3$) by RT-PCR.

Total RNA was extracted by the acidic guanidinium isothiocyanate method with RNA Stat 60 (TelTest Inc., Friendswood, TX) (35), and RNA quality was established by gel electrophoresis on an Agilent chip (micro run). For pooled samples, equal amounts of RNA were pooled from each experiment. cDNA and biotinylated cRNA were prepared according to the instructions of the manufacturer (Affymetrix, Santa Clara, CA) as previously published (13, 29). Labeled cRNA was hybridized to Affymetrix U133A human array chips containing $\sim 22,000$ probes for $\sim 14,500$ human genes. Fluorescence was quantified using Microarray suite 5.0 software (Affymetrix, Santa Clara, CA), comparing control and treated samples. Default values provided by Affymetrix were applied to all analysis parameters. Induced genes were defined as having a ≥ 1.7 -fold increase (P value of < 0.003) in mRNA content (DCI or Ad12A2-treated versus control cells). For very low abundance mRNAs in control cells (< 20 fluorescence units), a value of 20 units was assigned in calculating fold change. Fold-stimulation results are expressed as mean $\pm$ SEM. The designated molecular function of induced genes was determined by the Gene Ontology category.

RESULTS

RT-PCR Analysis of TTF-1 Transcripts

Two TTF-1 mRNAs, products of alternative start sites, are present in mouse lung (26). To assess the presence and inducibility of these TTF-1 transcripts in human lung cells, total RNA was prepared from human fetal lung epithelial cells, two human lung cell lines (A549, H441) and a nonlung human cell line (HeLa) cultured in the absence (control) or presence of DCI. Primer locations and sequences for RT-PCR are shown in Table 1. Primers producing a 313-bp product from the coding region common to both mRNAs were used to detect total 12A2 + 5E transcripts. Primers producing a 597-bp product, for which the forward primer was complementary to a sequence in the unique 5' coding region of the 5E mRNA, were used to detect 5E mRNA.

TTF-1 mRNA (12A2 + 5E) was detected in primary lung epithelial cells and H441 cells (Figure 1A, upper panel, lanes 2–5) but was absent in A549 and HeLa cells (Figure 1A, lanes 6–9). 5E mRNA was also found in epithelial and H441 cells (Figure 1A, center panel, lanes 2–5) at an apparent lower signal intensity. Culture in DCI medium markedly increased expression of both total TTF-1 mRNA (12A2 + 5E) and 5E mRNA in primary lung epithelial cells (compare lanes 2 versus 3), with a modest induction of 5E, but not 12A2 + 5E in H441 cells (compare lanes 4 versus 5). Neither TTF-1 transcript was detected in A549 and HeLa cells (lanes 6–9).

Quantitation of TTF-1 mRNAs

The primer pairs (sets 1, 4) used in the experiments of Figure 1A produced products of unequal length, hence with different

TABLE 1. RT-PCR PRIMERS FOR THYROID TRANSCRIPTION FACTOR-1 AND β -ACTIN

Amplicon	Primer Sequence	Primer Location	Size (bp)	Product Detected
(1)	For: 5' TCGCTCGCTCATTGTG 3' Rev: 5' CCATGTTCTTGCTCACGTCC 3'	36–53 632–613	597	5E
(2)	For: 5' TCGCTCGCTCATTGTG 3' Rev: 5' CCCGCTAGCGCTGAGCTGCCGGGGCT 3'	36–53 160–142	126	5E
(3)	For: 5' CCCAAGCTTATGTCGATGAGTCCAAAG 3' Rev: 5' GCC TGT ACG CCG CCA GCG GAG 3'	173–191 299–278	126	12A2+5E
(4)	For: 5' AACAGCGGCCATGCAGCAGCAC 3' Rev: 5' CCATGTTCTTGCTCACGTCC 3'	319–330 632–613	313	12A2+5E
β -Actin	For: 5' GTGGGCCGCTCTAGGCACCAA 3' Rev: 5' CTCTTTGATGTCACGCACGATTTC 3'		540	β -Actin

ultraviolet intensity per nmole of product. Thus, two new primer sets were designed to produce products of equal length (126 bp) for both the total TTF-1 (12A2 + 5E, set 3) and 5E-specific mRNA (set 2). Amplification with both primers using genomic DNA template generated products of equivalent intensity (Figure 1C). These primer sets were used to estimate content of 12A2 mRNA by subtraction (total minus 5E mRNA). Band intensity, using RNA as template, increased linearly for the 5E primer pair (Figures 1B and 1D, *right panels*; RNA range 25–100 ng) and for the 12A2 + 5E primer pair (Figures 1B and 1D, *left panels*; 2.5–20 ng RNA) over the concentration ranges tested. Using these standard curves, the percent of total TTF-1 mRNA present as the 5E or 12A2 isoforms, by difference calculation, was determined for RNA samples and results are summarized in Figure 1F. In DCI-treated lung epithelial cells, the 5E isoform accounted for $5 \pm 2\%$ of the total TTF-1 mRNA ($n = 3$). The relative content of 5E TTF-1 transcript in fetal thyroid tissue ($\sim 9\%$ of total TTF-1 mRNA, $n = 3$) was similar to that in lung.

Expression of TTF-1 Protein Isoforms

We next investigated the content of TTF-1 isoforms by Western blot. To determine whether both isoforms were recognized by TTF-1 antibodies, we prepared *in vitro* transcription/translation products from plasmids expressing either the 12A2 (pVBA2) or the 5E (pVBE19) proteins. 35 S-methionine was included in the reactions and strong bands were evident for each product, illustrating synthesis of proteins of expected size (~ 42 kD for 12A2 plasmid and ~ 46 kD for 5E plasmid; Figure 2A, *lanes 2 and 3*, respectively). The vector-only reaction (*lane 1*) produced no radioactive product. Both *in vitro* protein products were recognized by the monoclonal TTF-1 antibody when loaded individually (Figure 2B, *lanes 2 and 3*) or combined (*lane 4*). However, this antibody detected only 42 kD protein in type II cells (Figure 2B, *lane 1*). Migration of the 42-kD *in vitro* translation product was slightly slower than the endogenous protein (compare *lanes 2* versus *1*) due to presence of a T7 tag on the plasmid product.

Three polyclonal TTF-1 antibodies (SC, CM, DL) were also used to evaluate TTF-1 protein products on Western blots (data not shown). Each of these antibodies reliably detected the 42-kD band with increased band intensity in DCI-treated cells. However, none of the antibodies reliably detected a positive band at ~ 46 kD as would be expected for a protein encoded by the 5E mRNA. Occasional faint higher weight bands (~ 44 – 47 kD) were detected, but lack of hormonal response and failure of recognition by more than one antibody suggested nonspecific binding. As nonspecific bands were present with each of the polyclonal antibodies, the monoclonal antibody (Figure 2) was used for the majority of experiments.

Although TTF-1 normally localizes to the nucleus by immunofluorescence, cytoplasmic immunoreactivity is observed under

some conditions (20, 32). To address intracellular distribution of TTF-1 isoforms in cultured fetal lung cells, Western analysis was performed with whole cell, nuclear, and cytoplasmic extracts (Figure 2C). Extract of cells transduced with Ad5E (*lane 1*) provided markers for 12A2 and 5E band locations. For both control and DCI-treated cells, only 42-kD TTF-1 was detected, being present in the nuclear (Figure 2C, *lanes 4 and 5*) but not cytoplasmic fractions (*lanes 6 and 7*).

TTF-1 Expression During Lung Development

To determine expression of TTF-1 isoforms during lung development *in vivo*, both RNA and proteins were examined. 12A2 mRNA content increased $\sim 100\%$ between 12 and 20 wk gestation (Figure 3A). The content of 5E mRNA remained constant, constituting $\sim 17\%$ of the total TTF-1 mRNA in the younger age group and $\sim 10\%$ in the older group. Nuclear proteins extracted from these lung samples showed a 50% higher content for the 42-kD protein in the later age group (Figure 3B) and 46-kD protein was not detected (not shown); results were similar with all TTF-1 antibodies (data not shown). Cytokeratin (pan) staining of Westerns showed similar band intensity at all ages, indicating similar epithelial cell contribution to the total lung protein obtained at the two ages (data not shown). Thus, expression of the 12A2 isoform is developmentally regulated.

Hormonal Effect on TTF-1 Expression

We previously reported that exposure of human fetal lung epithelial cells to DCI medium for 3 d increased both TTF-1 mRNA and nuclear protein content (13). To examine the time course of this response, cells were cultured 3 d in control medium to deplete endogenous TTF-1, then DCI or diluent was added and cells were harvested at various times. Representative time courses for TTF-1 mRNA induction (Figure 4A) and protein expression (Figure 4B) are shown. Total TTF-1 mRNA (12A2 + 5E) increased by 2 h, was maximal between 4 and 8 h, and remained constant at 3-fold versus control until 24 h (Figure 4C). The nuclear content of 42-kD TTF-1 protein showed a delayed time course relative to the mRNA, reaching a maximal level at 16 h with a 3- to 4-fold increase over control (Figure 4C). No TTF-1 protein was detected in the cytosolic fraction over this time course (data not shown).

Using DNA microarray analysis, we examined induction kinetics of known TTF-1 target genes in type II cells relative to TTF-1 expression (Figures 4D and 4E). Microarray results for TTF-1 mRNA induction by DCI (Figure 4D) were similar to those by RT-PCR (Figure 4C), with an increase at 4 h after hormone addition. By comparison, SP-B gene expression was increased at 8 h and maximally induced within 24 h (Figure 4D), whereas induction of SP-C and SP-A was delayed > 24 h. Thus, kinetics of SP induction by DCI are delayed relative to TTF-1

Figure 1. Expression of two TTF-1 transcripts (12A2 and 5E) in lung type II cells and H441 cells. (A) Total cellular RNA (25–50 ng) was used to perform nonquantitative RT-PCR analyses for primary lung cells and cell lines (H441, A549, HeLa). PCR products for total (12A2+5E) and 5E TTF-1 transcripts as well as β -actin (loading control) were separated on a 1.5% agarose gel, stained with ethidium bromide, and visualized under ultraviolet light. TTF-1 primer sets 1 and 4 (Table 1) were used. (B) Various amounts of total RNA (lung, shown here, thyroid not shown) were used to characterize semiquantitative RT-PCR analyses using primer sets (126 bp products) specific for 5E transcript or both transcripts (primer sets 2 and 3, this figure and Table 1). (C) RT-PCR products of equal length (126 bp) were generated with these primers and efficiency of transcription was equivalent with genomic human DNA as template. (D and E) Densitometric analysis of a representative RNA sample. The amounts of product for 12A2+5E transcript (D) and 5E transcripts (E) were linear with RNA added (10-fold greater RNA input for 5E). (F) Relative content of the two TTF-1 transcripts in DCI-treated lung epithelial cells and fetal thyroid tissue. 5E accounts for $5 \pm 2\%$ of the total TTF-1 mRNA in lung type II cells and $9 \pm 3\%$ in thyroid tissue. Data are mean $\pm$ SEM, $n = 4$.

Figure 2. Coupled *in vitro* TTF-1 transcription and translation. The two forms of TTF-1 (12A2 and 5E) were cloned in pcDNA3 and transcribed/translated *in vitro*. (A) Products labeled with ³⁵S-methionine were separated on a 12% PAGE gel and detected at the expected sizes of ~42 and 46 kD after exposure to X-ray film. (B) Proteins translated in the presence of unlabeled methionine were separated on SDS-PAGE and subjected to Western blot with TTF-1 monoclonal antibody, which detected both forms of TTF-1 (marked with arrows). Lane 1 contains total cell extract from type II cells as a positive control for the endogenous 42-kD isoform. *In vitro* products were detected as slightly higher molecular weight proteins compared with endogenous TTF-1 due to the presence of T7 peptide and the multiple cloning site (26 aa). (C) Intracellular distribution of TTF-1. Extracts as shown were prepared and run on SDS-PAGE and probed with TTF-1-specific Neomarker antibody. As a control, extract of type II cells transduced with Ad5E was loaded in lane 1. TTF-1 (42 kD) is more prominent in the total cell extract (lane 3) and nuclear fraction (lane 5) of the cells treated with DCI versus control (lanes 2 and 4) and is not detected in cytosolic fraction (lanes 6 and 7). TTF-1₄₆ is not detected in any fraction. GAPDH staining was strongest in cytosolic fractions, with the faint bands in the nuclear extract samples indicating slight cytoplasmic contamination of nuclear extracts.

induction, consistent with a role for TTF-1 in transcriptional activation of these genes.

Expression of recombinant TTF-1 by Adenovirus Transduction

To examine effects of increased TTF-1, in the absence of hormone treatment, freshly isolated epithelial cells were treated

Figure 3. Effect of gestational age on TTF-1 expression. (A) RT-PCR analysis. Human fetal lungs were obtained at 11–13 wk (early second trimester) and 19–21 wk (late second trimester). Total RNA (10 ng for both transcripts and 100 ng for 5E isoform), based on the relative abundance of two TTF-1 transcripts (Figure 2), was subjected to RT-PCR followed by densitometry as described in MATERIALS AND METHODS. Content of 12A2 mRNA increased between 11 and 13 wk and 19 and 21 wk gestation, whereas content of 5E mRNA remained constant. (B) Western analysis. Nuclear proteins were analyzed by Western blot analysis with Neomarker antibody, and densitometric analysis of the 42-kD band was performed. Data are mean $\pm$ SEM ($n = 4$, * $P < 0.05$).

Figure 4. TTF-1 induction by DCI. Cultured cells were analyzed for TTF-1 mRNA and protein at various times after hormone addition. (A) Total TTF-1 mRNA (12A2 and 5E) by RT-PCR. In a representative gel, content of TTF-1 mRNA increased within 2–4 h after DCI exposure and was maximal at 16–24 h. Loading control β -actin was constant. (B) In a representative Western blot, nuclear TTF-1 protein (42 kD) content appeared increased at all time points (4–24 h) after DCI exposure. (C) Densitometry. Nuclear protein content increased later (maximal at 16 h), compared with mRNA expression (maximal at 8 h). Relative increases for TTF-1 were ~3- to 4-fold for both mRNA and protein, consistent with transcriptional regulation of expression (mean $\pm$ SEM, $n = 4$, $*P < 0.05$ versus time 0). (D and E) Time course of TTF-1 and target gene induction by cDNA microarray. Total RNA was extracted from control and DCI-treated cells at various times (4–72 h) after DCI exposure. An equal amount of RNA from four experiments was pooled at each time point. TTF-1 mRNA increased by 4 h of exposure (D) and target genes were first induced at 8 h (SP-B; D) and at > 24 h (SP-A and SP-C; E).

with TTF-1-expressing adenovirus (~ 2 pfu/cell) and cultured 5 d in control medium. Both 5E and 12A2 recombinant TTF-1 isoforms were expressed in cell nuclei (Figure 5A, center panel and right panel, respectively), consistent with the intracellular localization of endogenous TTF-1 (20, 36, 37). Control cells displayed no TTF-1 immunoreactivity under the same conditions (Figure 5A, left panel). By visual inspection, efficiency of transduction was 80–90% for both 5E and 12A2 at a dose of ~ 8 pfu/cell (not shown).

TTF-1 effects in mouse lung are influenced by phosphorylation status (6). To confirm phosphorylation of recombinant TTF-1, human lung cells were transduced with Ad12A2 in the

Figure 5. Effect of TTF-1 overexpression. (A) TTF-1 Immunostaining. In this representative experiment, epithelial cells were transduced with TTF-1 adenovirus (Ad12A2 or Ad5E) and cultured for 4 d in the absence of hormones. Cells were immunostained for TTF-1 (red) and nuclei were stained with DAPI (arrowhead, blue). Nuclear localization of TTF-1 (reddish purple stain, arrow) is shown for cells transduced with Ad5E (middle panel) and Ad12A2 (right panel), whereas no nuclear staining for TTF-1 was observed in control cells (left panel). (B) Phosphorylation of endogenous and rTTF-1. Cells were treated 24 h with DCI (lane 2) or cultured without hormones and transduced with AdGFP control virus in control media (lane 1) in the presence of 32 P-phosphate for the final 22 h of culture (lane 7, control cells). Nuclear extracts were prepared and TTF-1 immunoprecipitated. Upper panel: Western blot with TTF-1 detected by immunoblotting. Lower panel: radioactivity of immunoprecipitated TTF-1 bands detected by direct blot exposure to film. There is a dose-dependent (virus pfu/cell) increase in both TTF-1 immunoreactivity and phosphorylation, with similar phosphorylation/TTF-1 protein ratios for Ad12A2-treated cells versus DCI-treated cells (ratio = 10 versus 13, respectively). No bands (TTF-1 staining or radioactivity) were detected by immunoprecipitation with nonimmune IgG (not shown). (C) Western blot for SP-B. In Ad12A2-treated cells (6 pfu/cell, lane 3), SP-B protein (8 kD) was slightly increased (~ 3 fold, $n = 3$, $P < 0.01$) compared with untransduced control (lane 1), but was low ($\sim 7\%$) compared with the value in DCI-treated cells. **Lower portion of the blot containing the 8-kD SP-B band was exposed to film for a much shorter time compared with the upper portion with the 16-, 25-, and 42-kD bands. (D) Western blot analysis for TTF-1. In the same experiment shown in C, cells transduced with Ad12A2 (lanes 3 and 4) contained more TTF-1 protein (6 ± 2 -fold in three experiments at the same dose of virus, ~ 6 pfu/cell) than DCI-treated cells (lane 2).

presence of radiolabeled phosphate. Nuclear TTF1 was immunoprecipitated and both TTF-1 protein content (Figure 5B, *upper panel*) and radioactive signals (Figure 5B, *lower panel*) were determined. Intensities of both radioactivity and TTF-1 protein were positively correlated with viral dose (1–8 pfu/cell). In four dose–response experiments, the ratio of ^{32}P signal to immunoprecipitable TTF-1 (index of phosphorylation state) was similar for cells treated with DCI versus cells transduced with Ad12A2 (ratios 2.03 versus 2.41, 1.0 versus 1.14, 0.45 versus 0.31, 10 versus 13, respectively). cAMP alone does not promote differentiation in these cells, but was included to provide optimum phosphorylation conditions. In these experiments the expression level of rTTF-1 ranged from 0.5- to 4-fold compared with endogenous TTF-1 expression in DCI-treated cells. Thus, relative phosphorylation was comparable for recombinant and endogenous TTF-1.

To examine inductive effects of rTTF-1 in lung cells, we first examined SP-B, a known TTF-1 target gene induced synergistically by dexamethasone plus cAMP. In Ad12A2-infected cells, 8 kD mature SP-B protein product was increased (Figure 5C, compare *lanes 3* versus *1*) compared with nontransduced control cells ($323 \pm 41\%$ versus control cells, $P < 0.01$, $n = 3$); however, this induced level was only $7 \pm 4\%$ of that in DCI-treated cells (Figure 5C, *lane 2*). Cells transduced with Ad5E showed a similar effect on SP-B (8 kD, data not shown). The intermediate forms of SP-B (25 kD and 16 kD) were not detected in control cells with or without Ad12A2, likely due to low SP-B expression, but these forms were present in DCI-treated cells (Figure 5C). In this experiment nuclear TTF1 content in Ad12A2-treated cells (Figure 5D, *lane 3*) was 6 ± 2 -fold higher than in DCI-treated cells. Addition of dexamethasone or cAMP individually to transduced cells did not increase SP-B expression above the ~ 3 -fold increase by rTTF-1 alone (data not shown). These data indicate that TTF-1 alone is insufficient for full induction of SP-B, consistent with role(s) for additional DCI-induced transcription factors.

Identification of TTF-1-Responsive Genes by Expression of rTTF-1 and Silencing of Endogenous TTF-1

To determine the TTF-1 target gene population among DCI-induced genes, we employed a combinatorial approach. cDNA microarray analysis was performed after either rTTF-1 overexpression in control cells or with knockdown of TTF-1 during DCI treatment. In the knockdown experiments, TTF-1 mRNA was decreased $50 \pm 10\%$ ($n = 4$) and SP-B mRNA was decreased $55 \pm 7\%$ ($n = 4$) as quantitated by RT-PCR. TTF-1 protein by Western analysis was decreased $33 \pm 5\%$ ($n = 4$, $P < 0.05$). There were 274 genes induced > 2 -fold by DCI; these were categorized into four groups based on responses to rTTF-1 and siRNA oligonucleotides for TTF-1. Group 1 (designated as TTF-1-sufficient candidate genes) contained 14 genes that were induced by rTTF-1 to $\geq 50\%$ of the DCI level and had $> 10\%$ inhibition (in two of three experiments) of DCI induction by TTF-1 knockdown (Table 2). Two genes in this group (LAMP 3 [identified as DC-LAMP by sequence] and CEACAM6) were highly induced (~ 10 -fold) with the remaining genes induced 2.5- to < 6 -fold; none of these genes are currently recognized as putative TTF-1 target genes. Another eight genes met the TTF-1 induction criteria, but were inhibited by siRNA oligonucleotides in only one of three experiments (not shown). Group 2 (designated as TTF-1-insufficient candidate genes) contained nine DCI-induced genes that were inhibited by TTF-1 knockdown, but induction by rTTF-1 (≥ 1.7 -fold) was $< 50\%$ of the DCI level (Table 3). This group includes the known TTF-1 target genes SP-A/-B/-C, which were induced by rTTF-1 at $< 10\%$ of the level achieved with DCI, ABCA3 (which is required for surfactant synthesis), and five other genes of diverse protein functions (ion/fluid transporter, a cytochrome P450 gene, two inhibitors

of signaling, a cytokine receptor) with unknown roles in type II cell differentiation. Two additional genes (solute carrier family 6, member 14, SLC6A14, and cytochrome P450, family 4B, polypeptide 1, CYP4B1) were induced by DCI (13-fold and 6.2-fold, respectively) and met the criteria for rTTF-1 induction (10 to $< 50\%$ of the DCI induction), but TTF-1 siRNA did not repress the DCI induction. Confirmation of these genes as TTF-1 targets will require additional studies by other approaches. Group 3 (designated as TTF-1-dependent candidate genes) was a larger group with 72 genes. These genes were not induced by rTTF-1 expression alone, but DCI induction was inhibited by siRNA oligonucleotides in two to three experiments, consistent with a permissive role for TTF-1 in hormone induction (Table 4). Thus, expression of 109 ($\sim 40\%$) of the DCI-responsive genes was influenced by TTF-1 expression to some extent (Groups 1, 2, and 3). PECAM and complement component 1, q subcomponent, receptor 1 (C1QR1), both endothelial cell products, were identified in this group, suggesting an indirect effect of TTF-1 on contaminating endothelial cells via an epithelial cell product. An additional 165 DCI-induced genes were not induced by rTTF-1 nor inhibited by siRNA oligonucleotides (not shown) and appear to represent TTF-1-independent genes.

Two newly identified TTF-1 target genes of particular interest were LAMP 3 (lysosomal associated membrane protein 3 or dendritic cell [DC] LAMP) and CEACAM6 (carcinoembryonic antigen cell adhesion molecule 6), which were comparably induced (~ 10 -fold) by both DCI and rTTF-1. We confirmed TTF-1 induction of these genes by immunostaining and Western analysis. CEACAM6 is a GPI-linked membrane protein that has been extensively studied in gastrointestinal cells and tumors (38) and has been previously reported in lung (33), where its function is unknown. By Western analysis, Ad12A2 (at 6 pfu/cell) increased CEACAM6 more than DCI treatment (Figure 6). Control AdGFP did not elevate CEACAM6 protein expression, indicating that adenoviral infection was not responsible for the induction. The role of TTF-1 in CEACAM6 induction remains to be determined, as putative TTF-1-responsive elements were not detected on examination of upstream sequence (10 kb) of the CEACAM6 gene start site.

DC-LAMP has been previously identified in both mouse and human lung parenchymal cells associated with lamellar bodies (39). We found that both rTTF-1 (Figure 7B) and DCI treatment (Figure 7C) markedly increased DC-LAMP expression compared with untreated cells (Figure 7A). DC-LAMP co-stained with SP-B in DCI-treated human cells (Figure 7D), consistent with lamellar body localization.

Knockdown of TTF-1 expression in DCI-treated cells changed cell morphology and decreased target protein expression. Nile Red staining showed a marked decrease in large stained vesicles (i.e., lamellar bodies) in siRNA-treated cells compared with the scrambled control (Figure 8). By immunostaining (Figure 8), knockdown of TTF-1 did not appear to decrease nuclear TTF-1 (likely due to lack of sensitivity); however, staining intensities for SP-B, DC-LAMP, and CEACAM6 were markedly decreased, consistent with decreased mRNA content in the microarray profiles.

DISCUSSION

This study describes TTF-1 expression in human fetal lung epithelial cells during hormone-induced differentiation of the type II cell phenotype *in vitro*. In this model, morphologic markers of the type II cells (i.e., lamellar bodies) as well as surfactant components (SP-A, SP-B, SP-C, SP-D, and phospholipids) are induced with hormone treatment (13) and surface-active surfactant is secreted (40). Whereas some inductive effects of DCI

TABLE 2. GENES INDUCED COMPARABLY BY rTTF-1 AND DCI: TTF-1-SUFFICIENT CANDIDATE GENES

Gene Title	Gene Symbol	Fold Induction			GO Function*
		rTTF-1 (n = 2)	DCI (n = 3)	siRNA (% inhibition) (n = 3)	
Lysosomal-associated membrane protein 3	LAMP3	13.0 ± 0	12.9 ± 5.9	14 ± 13	Cell proliferation
Carcinoembryonic antigen-related cell adhesion 6	CEACAM6	9.1 ± 0.4	10.7 ± 3.5	25 ± 9	Signal transduction, cell-cell signaling
Endothelin 3	EDN3	2.8 ± 0.3	5.0 ± 2.6	41 ± 29	Signal transduction, cell-cell signaling
Solute carrier family 39 (zinc transporter), 8	SLC39A8	2.8 ± 0.2	4.9 ± 1.6	33 ± 20	Metal ion transport
Fibulin 5	FBLN5	2.5 ± 0.2	3.7 ± 1.3	36 ± 13	Cell-matrix adhesion
Laminin, alpha 3	LAMA3	2.1 ± 0.1	3.7 ± 0.5	25 ± 17	Cell adhesion
Glutathione peroxidase 3	GPX3	3.7 ± 0.1	3.6 ± 1.6	64 ± 19	Response to lipid hydroperoxide
Calcium channel, voltage-dependent, alpha 2/ delta 2	CACNA2D2	2.4 ± 0.3	3.4 ± 0.6	32 ± 23	Ion channel, Ca
Citron (rho-interacting, serine/threonine kinase 21)	CIT	2.5 ± 0.2	2.8 ± 0.2	30 ± 24	Protein phosphorylation
Carbonic anhydrase II	CA2	5.7 ± 0.4	2.6 ± 1.0	58 ± 21	One-carbon compound metabolism
SH3-domain binding protein 5 (BTK-associated)	SH3BP5	2.9 ± 0.7	2.6 ± 0.3	45 ± 24	Signal transduction
3'-Phosphoadenosine 5'-phosphosulfate synthase 2	PAPSS2	4.6 ± 0.1	3.2 ± 0.2	27 ± 18	Nucleobase/ nucleic acid metabolism
DKFZP564O0823 protein	DKFZP564O0823	2.6 ± 1.0	2.4 ± 0.8	51 ± 8	Not determined
ATP-binding cassette, sub-family C4 (CFTR/MRP)	ABCC4	2.5 ± 0.3	2.0 ± 0.2	13 ± 9	Ion transport

Definition of abbreviations: DCI, dexamethasone + cyclic AMP + isobutylmethylxanthine; rTTF-1, recombinant TTF-1; siRNA, small inhibitory RNA; TTF-1, thyroid transcription factor-1.

Undifferentiated epithelial cells were cultured 72 h in control media (to deplete endogenous TTF-1), then transduced with Ad12A2 (~ 6 pfu/cell) or AdGFP (~ 6 pfu/cell) and cultured an additional 72 h in control media. Parallel cells were cultured 72 h in DCI. For each treatment group, RNA isolated from individual experiments with seven lungs (16–20 wk gestation) was combined into two pools, and each was analyzed by cDNA microarray analysis (n = 2). To identify genes for which TTF-1 expression may be sufficient to account for induction in the presence of DCI, induction by both rTTF-1 and DCI had to be > 2-fold, and induction by rTTF-1 had to be > 50% of the induction by DCI. In separate microarray experiments (n = 3), some cells were treated with siRNA for TTF-1 (5 µg/ml, 8 h) and then DCI for 48 h. Inhibition by the siRNA was determined as a percentage of the expression by DCI only-treated cells. All of the TTF-1-induced genes were inhibited > 10% in each of three experiments. Values are mean ± SEM (n = 3) or range (n = 2). * Gene Ontology (GO) Biological Process description from Affymetrix.

may entirely reflect primary responses mediated by GR and/or CREB, it is likely that many responses also involve secondary effects via changes in content, localization, or activity of transcription factors, co-activators, and/or co-repressors. The transcription factor TTF-1 activates the promoters of SP-A, -B, and -C (14–16, 36) and interacts functionally with a variety of cofactor proteins (14, 22, 23). Hormonal regulation of TTF-1 expression *in vitro* has been reported but not characterized (13). Our current results demonstrate induction of the 42-kD isoform TTF-1 as an early response to DCI, preceding induction of SP-B and other known candidate TTF-1 target genes. Expression of rTTF-1, in the absence of hormone treatment, fully induced only a small subset of hormone-responsive genes but partially regulated others.

Thus, TTF-1 has a central but insufficient role in mediating changes in gene expression during type II cell differentiation.

Translational and functional importance of the different TTF-1 transcripts is largely unknown. Multiple TTF-1 mRNA transcripts were reported in a thyroid cell line (26, 27) as well as in H441 cells and in mouse fetal lungs (27). In the human lung cell culture model described here, ~ 95% of the TTF-1 mRNA consisted of the 12A2 transcript (~ 5% was 5E transcript) under all treatment and developmental conditions, suggesting that a change in the TTF-1 transcript distribution (12A2 versus 5E) is not a prominent feature of either normal human lung development or precocious type II cell maturation *in vitro*. TTF-1 has generally been detected as a ~ 40-kD nuclear protein,

TABLE 3. GENES INDUCED BY DCI WITH < 50% INDUCTION BY rTTF-1: TTF-1-INSUFFICIENT CANDIDATE GENES

Gene Title	Gene Symbol	Fold Induction			GO Function
		rTTF-1 (n = 2)*	DCI (n = 3)†	siRNA (% Inhibition of DCI Induction) (n = 3)‡	
Surfactant protein C	SFTPC	2.8 ± 0.5	257 ± 29	55 ± 25	Liquid surface tension
Surfactant protein A	SFTPA	13.8 ± 5.8	208 ± 58	59 ± 16	Surface tension
Surfactant protein B	SFTPB	1.9 ± 0.1	21.7 ± 4.3	32 ± 11	Lipid metabolism
Cytochrome P450, 2, B, 6	CYP2B6	4.4 ± 3.0	16 ± 4.0	20 ± 7*	Electron transport
WNT inhibitory factor 1	WIF1	1.9 ± 0.1	15.2 ± 4.7	36 ± 23	Cell-cell signaling
ATP-binding cassette, sub-family A (ABC1), 3	ABCA3	2.3 ± 0.3	22.7 ± 8.5	34 ± 22	Transport
Aquaporin 4	AQP4	1.9 ± 0.1	8.9 ± 1.9	37 ± 23	Water transport
Homeodomain-only protein	HOP	1.7 ± 0.1	7.3 ± 2.3	38 ± 12	Regulation of transcription
Chemokine (C-X-C motif) receptor 4	CXCR4	3.2 ± 0.1	6.7 ± 2.9	33 ± 24‡	Chemokine receptor pathway

For definition of abbreviations, see Table 2.

Cells were treated as in Table 2. To identify genes induced by rTTF-1 but for which TTF-1 expression was insufficient to account for maximal induction of the genes by DCI treatment, gene expression criteria were a 1.7-fold induction by rTTF-1 versus control, but < 50% of the induction level by DCI treatment. Seven of these genes were inhibited by siRNA for TTF-1, > 10% in three experiments.

* Values are mean ± range of pooled samples (n = 2) from three and four individual lungs of 16–20 wk gestation.

† Mean ± SEM, n = 3 experiments.

‡ Mean ± range (n = 2).

TABLE 4. DCI-INDUCED GENES INHIBITED BY TTF-1 siRNA, BUT NOT INDUCED BY rTTF-1: TTF-1 REQUIRED CANDIDATE GENES

Gene Title	Gene Symbol	DCI Induction (fold)	siRNA (% Inhibition of DCI Induction)	GO Function
Progastricsin (pepsinogen C)	PGC	111.4	52 ± 9	Proteolysis and peptidolysis
Sodium channel, nonvoltage-gated 1 alpha	SCNN1A	20.1	56 ± 30	Ion transport
Claudin 18	CLDN18	16.4	66 ± 31	Cell adhesion
Interleukin 1 receptor-like 1	IL1RL1	15.2	34 ± 23	Signal transduction
Alcohol dehydrogenase 1B (class I), beta polypeptide	ADH1B	12.1	32 ± 2	Ethanol oxidation
Lipoprotein lipase	LPL	11.1	42 ± 2	Fatty acid metabolism
Hypothetical protein FLJ11127	FLJ11127	10.6	55 ± 13	—
Melanophilin	MLPH	7.5	44 ± 25	Intracellular protein transport
Sodium channel, nonvoltage-gated 1, beta	SCNN1B	7.5	48 ± 10	Ion transport
Hypothetical protein FLJ21415	IGF2	7.3	52 ± 3	Regulation of cell cycle
Aldehyde dehydrogenase 3, B1	ALDH3B1	7.0	38 ± 6	Alcohol metabolism
Claudin 5	CLDN5	5.9	32 ± 3	Cell adhesion
Nuclear receptor subfamily 4, 2	NR4A2	5.7	42 ± 23	Regulation of transcription
BENE protein	BENE	5.7	41 ± 24*	—
Pyruvate dehydrogenase kinase, isoenzyme 4	PDK4	5.5	52 ± 2	Glucose metabolism
ATPase, aminophospholipid transporter, Class I, type 8A, 1	ATP8A1	5.4	46 ± 6	Cation transport
Calcitonin/calcitonin-related polypeptide, alpha	CALCA	5.4	42 ± 12	Skeletal development
Zinc finger and BTB domain containing 16	ZBTB16	4.9	48 ± 23	Regulation of transcription
Suppressor of cytokine signaling 3	SOC3	4.8	46 ± 25	Regulation of cell growth
Hypoxia inducible factor 3, alpha subunit	HIF3A	4.8	58 ± 14	Regulation of transcription
Transmembrane protease, serine 2	TMPS2	4.8	45 ± 3	Proteolysis and peptidolysis
Chloride intracellular channel 5	CLIC5	4.5	62 ± 37	Ion transport
Mucin 1, transmembrane	MUC1	4.4	56 ± 37	—
Hypothetical protein FLJ12443	FLJ12443	4.4	44 ± 9	Metabolism
Cytochrome b-5	CYB5	4.4	50 ± 30	Electron transport
G protein-coupled receptor kinase 5	GRK5	4.2	50 ± 34	Phosphorylation
Intercellular adhesion molecule 4	ICAM4	4.2	77 ± 23	Cell-cell adhesion
Solute carrier family 1 (high affinity glutamate transporter), 1	SLC1A1	4.1	47 ± 37	Transport
Chromosome 14 open reading frame 147	C14orf147	3.9	50 ± 4	—
Fibroblast growth factor receptor 2 (KGF receptor)	FGFR2	3.7	62 ± 35	Phosphorylation
Interleukin 18 receptor 1	IL18R1	3.6	24 ± 4	Immune response
Aldehyde oxidase 1	AOX1	3.5	37 ± 17	Electron transport
Fatty acid synthase	FASN	3.5	68 ± 17	Fatty acid biosynthesis
Hypothetical protein MGC11242	MGC11242	3.5	58 ± 43	—
Inositol(myo)-1(or 4)-monophosphatase 2	IMPA2	3.4	36 ± 20	Phosphate metabolism
Phospholipase D3	PLD3	3.4	26 ± 8	Metabolism
Rap guanine nucleotide exchange factor (GEF) 5	RAPGEF5	3.4	42 ± 12	GTPase signal transduction
ATP-binding cassette, sub-family C (CFTR/MRP), member 6	ABCC6	3.4	75 ± 25	Transport
Phosphodiesterase 4D, cAMP-specific	PDE4D	3.4	34 ± 14*	Signal transduction
Growth arrest-specific 1	GAS1	3.2	43 ± 8	Cell cycle arrest
KIAA0672 gene product	KIAA0672	3.2	47 ± 26	—
Estrogen-related receptor beta like 1	ESRRB1	3.1	59 ± 38	Signaling
Platelet/endothelial cell adhesion molecule (CD31 antigen)	PECAM1	3.0	23 ± 3	Cell motility
Preylcysteine oxidase 1	PCYOX1	2.9	60 ± 16	Preylated protein catabolism
Serine (or cysteine) proteinase inhibitor, clade A,1	SERPINA1	2.9	60 ± 40	Acute-phase response
Hypothetical protein MAC30	MAC30	2.8	48 ± 34	Regulation of cell growth
CGI-146 protein	PNAS-4	2.8	45 ± 35	—
Lanosterol synthase (2,3-oxidosqualene-lanosterol cyclase)	LSS	2.7	64 ± 36	Steroid biosynthesis
Complement component 1, q subcomponent, receptor 1	C1QR1	2.7	30 ± 18	Phagocytosis
WAP four-disulfide core domain 2	WFDC2	2.7	71 ± 29	Proteolysis and peptidolysis
ATP-binding cassette, sub-family A (ABC1), member 8	ABCA8	2.5	19 ± 9	Signaling, transport
Thyroid transcription factor 1	TITF1	2.5	59 ± 21*	Regulation of transcription
24-dehydrocholesterol reductase/	DHCR24	2.3	42 ± 28*	Electron transport
Dihydropyrimidinase-like 2	DPYSL2	2.3	45 ± 10	Nucleic acid metabolism
Inhibitor of DNA binding 1	ID1	2.3	27 ± 7*	Regulation of transcription
ATPase, Na ⁺ /K ⁺ transporting, beta 1 polypeptide	ATP1B1	2.3	20 ± 6	Transport
7-Dehydrocholesterol reductase	DHCR7	2.3	49 ± 9	Cholesterol biosynthesis
Chromosome 14 open reading frame 1	C14orf1	2.2	64 ± 27	—
Protein tyrosine phosphatase type IVA, member 1	PTP4A1	2.2	58 ± 8	Dephosphorylation
Chromosome 9 open reading frame 61	C9orf61	2.2	60 ± 40	—
Cyclin D3	CCND3	2.2	57 ± 33	Regulation of cell cycle
Deleted in liver cancer 1	DLC1	2.2	71 ± 11	Cytoskeleton organization
Flotillin 1	FLOT1	2.1	42 ± 20	—
Transmembrane 7 superfamily member 2	TM7SF2	2.1	56 ± 43	Cholesterol biosynthesis
ADP-ribosylation factor-like 7	ARL7	2.1	53 ± 9	Intracellular protein transport
Diacylglycerol kinase, delta 130 kD	DGKD	2.1	47 ± 7	Immune response
Thiosulfate sulfurtransferase (rhodanese)	TST	2.0	55 ± 23	Sulfate transport
Glucuronidase, beta	GUSB	2.0	74 ± 33	Carbohydrate metabolism
Nuclear receptor co-repressor 2	NCOR2	2.0	53 ± 40	Regulation of transcription
RAB27A, member RAS oncogene family	RAB27A	2.0	40 ± 14	GTPase signal transduction
Chromosome 14 open reading frame 132	C14orf132	2.0	59 ± 41	—
KIAA1280 gene product	KIAA1280	2.0	34 ± 1	—

For definition of abbreviations, see Table 2.

Cells were treated as in Table 2. Criteria for TTF-1 involvement in the DCI-induction were ≥ 2-fold induction by DCI, no induction by rTTF-1 alone, and a > 10% inhibition of the DCI induction by TTF-1 siRNA in at least two of three experiments. Seventy-two DCI-induced genes met these criteria, with five genes being inhibited in each of the three experiments.*

Figure 6. Expression of CEACAM6. (A) Western blots. Total cell extracts from cells cultured in the absence (–) or presence (+) of DCI for 4 d were analyzed by immunoblotting with anti-TTF-1 (upper bands) or anti-CEACAM6 (lower bands) antibodies. Results from two experiments with cells from individual lungs are shown. (B) Densitometric analysis of Western blots. In four experiments, cells were transduced with either Ad12A2 (6 pfu/cell) or AdGFP in control media, or cultured with DCI for 4 d. TTF-1 and CEACAM6 were increased with both DCI treatment and rTTF-1 expression, but not with control virus (AdGFP, 6 pfu/cell). * $P < 0.05$ versus AdGFP control; ** $P < 0.05$ versus DCI-treated cells.

although larger isoforms were reported in mouse lung (26) and transformed mouse type II cells (MLE-15) (41). In thyroid cells, longer transcripts were more prevalent in proliferating, less differentiated cells (27). Using four antibodies with different TTF-1 recognition epitopes to examine TTF-1 protein expression in lung cells, we consistently detected only the 42-kD protein band, suggesting that only this isoform is translated and functional in fetal lung.

Although TTF-1 is crucial in late gestation for SP expression (6), there are few developmental studies of TTF-1 expression.

Figure 7. Expression of DC-LAMP by immunostaining. In this representative experiment, epithelial cells were cultured for 4 d and immunostained for DC-LAMP expression. Staining in control cells was faint (A), with greatly increased vesicular cytoplasmic staining in cells treated with Ad12A2 (B) or DCI (C). DC-LAMP staining (Alexa 488-tagged antibody, green) colocalized (yellow) with SP-B staining (cy3-tagged polyclonal antibody, red) in DCI-treated cells (D) in a punctate pattern consistent with lamellar body localization. Magnification: $\times 300$.

In late gestation fetal mouse lung, the 42-kD TTF-1 protein was expressed at a constant level whereas content of the 46-kD protein isoform reportedly increased (26). In a qualitative histochemical study of human fetal lung, TTF-1 staining was detected at 11 wk gestation, initially in both airway and distal parenchymal epithelial nuclei, with later restriction to more distal alveolar epithelial cell nuclei (37). Our findings of a $\sim 100\%$ increase in TTF-1 (12A2) mRNA and 42 kD protein between ~ 12 and 20 wk gestation support a model of increased TTF-1 expression in ductal airway epithelial cells coincident with type II cell differentiation that is initiated late in the second trimester.

Effects of hormones on TTF-1 expression have not been extensively explored. In mice with nitrofen-induced lung hypoplasia, TTF-1 content was much reduced, and was restored by *in vivo* dexamethasone treatment (42). Li and coworkers reported that cAMP treatment of cultured cells increased TTF-1 activity, with no change in TTF-1 protein content (36). We previously reported synergistic increase of TTF-1 gene expression by glucocorticoid and cAMP in human fetal lung epithelial cells (13). TTF-1 is likely to be hormonally regulated *in vivo* during lung maturation; however, this has not been examined.

The relatively rapid induction of TTF-1 by DCI is consistent with a direct effect of glucocorticoid and cAMP on gene expression, although a secondary inductive response is possible. Factors directly regulating the TTF-1 promoter are largely undefined, and in particular, functional GREs and CREs have not been reported. *Hoxa2*^{–/–} mice have lung dysmorphology and respiratory failure, along with absence of TTF-1; however, there is no description of direct regulation of TTF-1 expression by *Hoxa2*, which is expressed exclusively in mesenchymal cells (43). HNF3 β is expressed early in lung development along with TTF-1 (44) and *in vitro* studies have shown that HNF3 can activate the TTF-1 promoter (45), as can ubiquitous factors SP-1 and SP-3 (46). GATA-6 is co-expressed in lung epithelial cells with TTF-1 and transactivates the latter *in vitro* (47), whereas Ras elements negatively regulate TTF-1 expression (48). None of these factors are known to be responsive to glucocorticoid or cAMP. TTF-1 expression is inhibited by TGF- β , which is down-regulated by glucocorticoids (49). Thus DCI may induce TTF-1, in part indirectly, by repressing a negative regulator. Additional studies are required to elucidate the mechanism of TTF-1 induction in hormone-treated cells.

We found that treatment of lung cells with small inhibitor RNA oligonucleotides to silence TTF-1 mRNA reduced TTF-1 protein content and proportionally blocked SP-B mRNA induction by DCI. However, overexpression of rTTF-1 via adenovirus transduction in the absence of DCI minimally induced (~ 2 -fold) SP-B expression compared with the DCI response, indicating that TTF-1 is required but not sufficient for expression of SP-B. In contrast, overexpression of recombinant TTF-1 in a thyroid cell line, which expresses another key transcription factor Pax-8, successfully activated thyroid-specific gene expression (50). Thus, one or more additional gene regulatory factors are likely increased by DCI treatment to fully activate the SP-B promoter (51–53). Although a number of regulatory proteins are reported to functionally interact with TTF-1 on various gene promoters (20, 22, 23, 25, 36, 54), hormonal regulation of these proteins has not been systematically explored. CEBP family members are hormonally induced and are required for SP-A induction in human fetal lung epithelial cells (19). Notably, we found that both CEBP α and CEBP δ were induced by DCI, with no evidence for involvement of TTF-1 in this induction (data not shown).

Alternatively, we cannot rule out the possibility that rTTF-1 expressed in lung cells is not appropriately phosphorylated. TTF-1 can be phosphorylated at seven serine sites, and alteration

Figure 8. Effect of TTF-1 knockdown by siRNA treatment on cell morphology and expression of TTF-1 target genes. Cells were electroporated with TTF-1 siRNA oligonucleotides (200 nM, *upper panels*) or scrambled control oligonucleotides (200 nM, *lower panels*) and cultured 72 h in DCI. Nile Red staining was reduced in siRNA-treated (A) compared with scrambled controls (F), with fewer large intensely staining vesicles (i.e., lamellar bodies). Nuclear immunostaining for TTF-1 was only slightly reduced by siRNA treatment (B versus G), whereas there was marked reduction in cells staining intensely for SP-B (C versus H), CEACAM6 (D versus I), and DC-LAMP (E versus J), with the siRNA knockdown of TTF-1. In these experiments, TTF-1 mRNA was re-

duced 50% (qPCR assay) and TTF-1 protein was reduced 47% (Western blot analysis) by siRNA treatment. Primary antibodies used were anti-TTF-1 (Lab Vision), anti-SP-B (Chemicon), anti-CEACAM6 (Novus Biologicals), anti-DC-LAMP (Immunotech), and secondary antibodies were tagged with Alexa 488. Images are representative of duplicate experiments with similar cell plating density for all treatments.

of the phosphorylation level changes DNA binding activity, enhancer activity, and effects in lung epithelial cells (36, 55). Mutation of serine residues to eliminate phosphorylation of TTF-1 in a transgenic mouse prevented induction of most surfactant-related differentiation (6). In the current cell model, lack of phosphorylation is unlikely to account for low induction of SP-B by rTTF-1 alone, as the relative phosphorylation level of rTTF-1 was equivalent to that for endogenous TTF-1 in DCI-treated cells.

Microarray gene profiling for TTF-1 overexpression in control cells and knockdown in DCI-treated cells indicated that ~40% of the genes induced by DCI were in the TTF-1 domain of influence (Tables 2, 3, and 4). The responsive genes were categorized into three groups based on the response to rTTF-1 in comparison to the DCI effect: (1) genes induced to a similar level by rTTF-1 and DCI; (2) genes induced by rTTF-1, but to levels < 50% of the DCI level; and (3) genes not induced by rTTF-1 but inhibited by TTF-1 knockdown in DCI-treated cells. Additional studies by other approaches will be required to establish that the newly identified genes are TTF-1 regulated, as non-specific effects of siRNA oligonucleotides have been identified in some studies (56), but not others (57). The remaining 60% of DCI-induced genes were unresponsive to rTTF-1 expression or TTF-1 knockdown (i.e., TTF-1 independent).

Our findings support an important role of TTF-1 in differentiation of type II cells that extends beyond the surfactant proteins. TTF-1 knockdown also affected induction of genes of particular interest including ABCA3 (a putative phospholipid transporter of lamellar bodies) (58), LAMP3 and CEACAM6 (proteins associated with lamellar bodies) fatty acid synthase (the key regulated lipogenic enzyme) (13, 59), pepsinogen C (a type II cell-specific protease likely involved in SP-B processing) (60), claudin 18 (a tight junction protein important in transepithelial cell resistance) (18, 61), and sodium channels α and β (mediators of ion and fluid flux across the epithelium) (12). Development of lamellar bodies was also depressed by TTF-1 knockdown, as shown by decreased Nile Red staining of large intracellular vesicles.

We propose that Group 1 genes are regulated by nuclear levels of TTF-1 and are likely not influenced directly by glucocorticoids and cAMP. The action of TTF-1 could be direct, enhanc-

ing transcription by binding to response sequences, or indirect, mediated by TTF-1-induced transcriptional regulators.

Group 2 genes, which are modestly induced by rTTF-1 compared with the DCI response, likely represent regulation by TTF-1 interacting with other transcriptional regulators that are induced by glucocorticoid and/or cAMP (independent of TTF-1). These additional regulatory factors may include CEBP α , CEBP δ , HNF-3, Transcription factor 21, DSIP1, PLAGL1, ASCL1, and STAT3, as identified by responsiveness to DCI (29). Alternatively, DCI could decrease levels of transcriptional repressors that modulate expression of genes in this category. Notably, Group 2 includes SPs A/B/C, which are known to be regulated by a variety of factors acting in concert with TTF-1 (14, 20, 22, 62, 63).

The largest group of putative TTF-1 target genes, Group 3, was not induced by overexpression of TTF-1 alone; however, TTF-1 silencing consistently reduced induction by DCI. Expression of Group 3 genes presumably requires the presence of TTF-1, or TTF-1-induced factor(s), at promoter regulatory elements where other hormonally regulated factors act to enhance transcription.

In Groups 1–3, TTF-1 influenced 19 of the 25 most highly DCI-induced genes (29) underscoring the importance of this transcription factor in type II cell differentiation. Approximately 60% of the identified DCI-induced genes were regulated independently of TTF-1 (i.e., neither induced by rTTF-1 nor repressed by anti-TTF-1 siRNA oligonucleotides). Thus, TTF-1 is necessary but not sufficient for induction of ~30% of genes induced during hormone-mediated differentiation. SP-D was induced by DCI, consistent with previous reports (64, 65), but was independent of the TTF-1 level in these studies, in contrast to *in vitro* activation of the SP-D promoter (66). Thus, glucocorticoids and cAMP promote differentiation-linked changes in this epithelial cell model via both TTF-1-dependent and -independent mechanisms.

A gene of particular interest identified by the microarray studies as highly induced by both DCI and rTTF-1 expression was CEACAM6, a GPI-anchored membrane glycoprotein present in a number of gastrointestinal cells and tumors (33, 67). Western blot analysis confirmed induction of this protein in cells cultured in DCI or expressing rTTF-1. Analysis of ~10 kb of the upstream sequence of the CEACAM6 promoter failed to

identify a consensus TTF-1-binding site, thus induction of CEACAM6 may be indirect via as yet unidentified transcription factor(s). CEACAM6 was previously identified in human lung, perhaps in type II cells (33), but its role in the lung is speculative and under current investigation.

Induction of DC-LAMP by rTTF-1 was confirmed by immunofluorescence staining, and costaining with SP-B antibody localized DC-LAMP to lamellar bodies. The role of this protein in lamellar bodies and type II cell function remains to be studied. These findings increase the number of hormonally responsive, TTF-1-dependent, lamellar body proteins to 4 (SP-B, SP-C, ABCA3, DC-LAMP). Further studies of TTF-1 regulation, induction, and interaction with cofactors in this culture model of type II cell differentiation should provide important insights into the role of TTF-1 and its target genes in type II cell biology.

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