Lipidomics of cellular and secreted phospholipids from differentiated human fetal type II alveolar epithelial cells[S]

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Abstract **Maturation of fetal alveolar type II epithelial cells in utero is characterized by specific changes to lung surfactant phospholipids. Here, we quantified the effects of hormonal differentiation in vitro on the molecular specificity of cellular and secreted phospholipids from human fetal type II epithelial cells using electrospray ionization mass spectrometry. Differentiation, assessed by morphology and changes in gene expression, was accompanied by restricted and specific modifications to cell phospholipids, principally enrichments of shorter chain species of phosphatidylcholine (PC) and phosphatidylinositol, that were not observed in fetal lung fibroblasts. Treatment of differentiated epithelial cells with secretagogues stimulated the secretion of functional surfactant-containing surfactant proteins B and C (SP-B and SP-C). Secreted material was further enriched in this same set of phospholipid species but was characterized by increased contents of short-chain monounsaturated and disaturated species other than dipalmitoyl PC (PC16:0/16:0), principally palmitoylmyristoyl PC (PC16:0/14:0) and palmitoylpalmitoleoyl PC (PC16:0/16:1). Mixtures of these PC molecular species, phosphatidylglycerol, and SP-B and SP-C were functionally active and rapidly generated low surface tension on compression in a pulsating bubble surfactometer.** JLR **These results suggest that hormonally differentiated human fetal type II cells do not select the molecular composition of surfactant phospholipid on the basis of saturation but, more likely, on the basis of acyl chain length.**—A. D. Postle, L. W. Gonzales, W. Bernhard, G. T. Clark, M. H. Godinez, R. I. Godinez, and P. L. Ballard. **Lipidomics of cellular and secreted phospholipids from differentiated human fetal type II alveolar epithelial cells.** ***J. Lipid Res.*** **2006.** 47: **1322–1331.**

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Although all differentiated cells in vivo maintain a specific membrane phospholipid composition optimized for their individual functions, little is known about the precise functional role of any individual phospholipid molecular species. An exception perhaps is the role of phosphatidylcholine (PC) molecular species in the action of pulmonary surfactant within the lung alveoli and airways. The function of dipalmitoyl PC (PC16:0/16:0, characterized by the presence of two saturated fatty acids) as the major surface-active agent of lung surfactant is arguably the best-defined role for any individual phospholipid species. Pulmonary surfactant is secreted from the type II epithelial cell of the alveolus, and the ontogeny of both surfactant and the type II cell is well defined. From ~22 weeks of human gestation, type II cells progressively acquire cytoplasmic lamellar bodies, which are the sites for storage of surfactant lipids and proteins in anticipation of postnatal life. The fractional content of PC16:0/16:0 in fetal lung increases over this time period in utero, from ~25 mol% total PC at 15 weeks of gestation to 45 mol% at term birth (1).

Intriguingly, comparative analysis from a wide range of mammals and marsupials indicates that PC16:0/16:0 is not always the major PC component of lung surfactant (2). Other short-chain species, typically palmitoylmyristoyl PC (PC16:0/14:0) and palmitoylpalmitoleoyl PC (PC16:0/16:1), predominate in a number of fast-breathing animals. Additionally, the composition of surfactant PC changes considerably during postnatal development; the content of PC16:0/16:0 is lower in surfactant isolated from children (3, 4) compared with adults (5) and is decreased significantly in the neonatal pig (6) and rat (7) with a

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Abbreviations: DCI, dexamethasone + 8-bromo-cAMP + isobutylmethylxanthine; ESI-MS, electrospray ionization mass spectrometry; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; SP-A, -B or -C, surfactant proteins A, B or C.

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[S] The online version of this article (available at http://www.jlr.org) contains additional six tables.

concomitantly increased content of PC16:0/14:0. The mechanisms regulating such compositional changes to surfactant PC are not clear. Presumably, the molecular specificities of the associated synthetic enzymes and transport proteins are not developmentally regulated, suggesting that a combination of poorly defined physiological and nutritional factors may be major mediators of altered surfactant PC composition.

Here, we present a lipidomic analysis of the molecular specificity of the phospholipid composition of surfactant isolated from human fetal lung type II epithelial cells to help address this question. Isolated immature fetal lung epithelial cells, when cultured on plastic in serum-free medium in the presence of dexamethasone, 8-bromo cAMP, and isobutylmethylxanthine, undergo an apparent differentiation into cells with characteristics of type II alveolar epithelial cells at a greatly accelerated time course compared with in vivo (8). They acquire cytoplasmic lamellar bodies and express surfactant protein A (SP-A), SP-B, and SP-C mRNA and protein. Moreover, this apparent differentiation is associated with significant modulation to the compositions of both PC and phosphatidylinositol (PI) in whole cell extracts. This culture system provides an ideal model to investigate the interaction between phenotypic expression and culture conditions in the regulation of surfactant phospholipid composition. Consequently, in this study, we report the effect of hormonal treatment on the composition and surface tension function of surfactant secreted by fetal type II epithelial cells. As secreted material is one product from a single cell type, this analysis also overcomes the problems of mixed populations of cells and in effect accesses a defined intracellular compartment of differentiated alveolar type II epithelial cells.

MATERIALS AND METHODS

Isolation and preparation of cells

Isolated enriched populations of undifferentiated epithelial cells and fibroblasts were prepared from second trimester human fetal lung under institutional review board-approved protocols as reported previously (8). Briefly, after overnight culture as explants, tissue was digested with trypsin, collagenase, and DNase; fibroblasts were removed by differential adherence and cultured for 4–6 days in serum-free Waymouth's medium (9). Nonadherent cells were plated on 60 mm plastic culture dishes in Waymouth's medium containing 10% fetal calf serum. After overnight culture and adherence, some cells were harvested for analysis (day 1 cells), and the remaining attached cells were cultured for an additional 4–6 days in 1 ml of serum-free Waymouth's medium alone (control) or with dexamethasone (10 nM) plus 8-bromo-cAMP (0.1 mM) and isobutylmethylxanthine (0.1 mM), a combination that is referred to as DCI. The purity of the epithelial cell preparation assessed by cytokeratin staining was 83 ± 2%. This hormonal treatment routinely resulted in up to 40% of epithelial cells strongly staining positive with Nile Red, a lipophilic stain known to stain lamellar bodies.

Secretion

To examine phospholipid secretion, epithelial cells were cultured for 4 days without (control) or with DCI, their intracellular choline was depleted by adding choline-free medium for 2 h, and their PC was labeled overnight with [^{3}H]choline (10 μCi/ml in choline-free Waymouth's medium). After washing cells to remove unincorporated label, we treated some dishes with a combination of secretagogues (10 nM tetradecanoyl phorbol acetate, 100 nM Ca^{2+} ionophore A23187, and 10 nM terbutaline in Waymouth's medium containing 1.8 mM choline). Media and cells were collected at 0, 1, 2, and 4 h. Basal (no secretagogues added to the cells) and secretagogue-stimulated secretions were determined by extracting total lipid from medium and cells, purifying total PC by thin-layer chromatography, and determining radioactivity by liquid scintillation counting (10).

To collect the large amounts of secreted surfactant required for surface tension measurements and lipid compositional analysis, 25–30 dishes (60 mm) of cells from each of the control and DCI-treated groups were analyzed. On day 6, fresh medium (control or DCI) without secretagogues was added to cells for a 10 h period of basal secretion. These media were collected, pooled, and centrifuged at 27,000 *g* for 1 h to recover a large aggregate surfactant fraction pellet. Fresh medium containing the secretagogue cocktail was added to both control and DCI-treated cells for a further 10 h secretion period. The media were collected, pooled, and centrifuged as described above. The large aggregate surfactant pellets were suspended in small volumes of buffer and used for analysis of surface tension, total phospholipid (10) and protein, surfactant proteins, and individual molecular species of PC and PI (see below).

Surface activity

Phospholipids were extracted from an aliquot of large aggregate surfactant secreted from DCI-treated cells using chloroform and methanol according to Bligh and Dyer (11) and quantified as phospholipid phosphorous (10). The phospholipid concentration of the remaining volume of intact surfactant was adjusted to 1.5 mg/ml by the addition of buffer (154 mM NaCl, 10 mM Tris-HCl, pH 7.4, and 1.5 mM $CaCl_2$), and surface tension was measured at 37°C in humidified air on a pulsating bubble surfactometer (Electronetics, Buffalo, NY). A bubble radius of 0.4 mm was maintained for 10.6 s and then varied between 0.4 and 0.55 mm at a frequency of 0.33 Hz for 5 min, and minimal surface tension was recorded. The coefficient of variation for surface tension determination was 6.0%.

Electrospray ionization mass spectrometry of phospholipid molecular species

Cell phospholipids were extracted with chloroform and methanol according to Bligh and Dyer (11) after adding the following internal standards (nmol/10^7 cells): PC14:0/14:0 (15 nmol), PE14:0/14:0 (4 nmol), PG14:0/14:0 (2 nmol), and PS14:0/14:0 (2 nmol). Electrospray ionization mass spectrometry (ESI-MS) of phospholipids in cell extracts was performed on a Micromass Quattro Ultima triple quadrupole mass spectrometer (Micromass, Wythenshaw, UK) equipped with an electrospray ionization interface. Samples were dissolved in methanol-chloroform-water (7:2:1, v/v) and introduced into the mass spectrometer by nanoflow infusion. PC species were preferentially detected using positive ionization, whereas phosphatidylethanolamine (PE) and acidic phospholipids were quantified under negative ionization conditions. After fragmentation with argon gas, PC molecules produced a fragment with *m/z* +184 (12) corresponding to the protonated phosphocholine head group, and parent scans of the *m/z* 184 moiety provided diagnostic determination of PC. Phosphatidylglycerol (PG) and phosphatidic acid (PA) species were detected by precursor ion scans that generated a common glycerophosphate fragment of *m/z* −153, phosphatidylserine (PS)

species by neutral loss scans of serine (m/z −87), and PI species by precursor ion scans of the common dehydrated inositol phosphate fragment with m/z −241 (13). Because this region of the spectrum contained no interfering ion peaks, PG, PA, PI, and PE species were quantified directly from the ESI^- spectra and PS was quantified from the m/z −87 neutral loss scan. Data were acquired and processed using MassLynx NT software. After conversion to centroid format according to area and correction for ^{13}C isotope effects and for reduced response where appropriate of tandem MS scans with increasing m/z values, the phospholipid species were expressed as percentages of their respective totals present in the sample. The predominant molecular species present for each ion peak resolved was determined by analysis of fatty acyl ion fragments generated by collision gas-induced fragmentation under negative ionization. The compositions are reported here for the species that individually contributed >1 mol% to the total phospholipid of each respective phospholipid class.

Immunodot assay for surfactant protein content

Previously described rabbit polyclonal antibodies against human SP-A and SP-B (14, 15) were used for immunodot assays at dilutions of 1:10,000 and 1:5,000, respectively. Antibody to mature SP-C was provided by Byk-Gulden (Konstanz, Germany) and was generated against recombinant human SP-C containing phenylalanine substituted for cysteine at residues 3 and 4. This antibody recognizes mature SP-C of 3.7 kDa and has very weak immunoreactivity for other forms of SP-C. The antibody was used at a dilution of 1:4,000 for immunodot assay. Secondary antibodies and ECL reagent were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA) and Pierce Biotechnology, Inc. (Rockford, IL). Serial dilutions of cell sonicates and large aggregate surfactant were spotted on nitrocellulose (Duralose; Stratagene, La Jolla, CA) and were exposed to anti-SP-A, anti-SP-B, or anti-SP-C antibody with ECL detection, as described previously (16). We used a pooled sample of human bronchoalveolar lavage as a standard for interassay comparisons, Infasurf as a standard for SP-B and SP-C, and purified SP-A from alveolar proteinosis fluid as an SP-A standard. Signal intensity was quantified by scanning densitometry.

Preparation of surfactant constructs and analysis of surfactant function

Lung lavage fluid was obtained from adult pigs by instillation of 4 × 1 liter of 0.9% (w/v) NaCl and used as a source of SP-B and SP-C. Briefly, lung lavage fluid was centrifuged at 27,000 *g* for 2.5 h at 4°C, the pellet was extracted with chloroform and methanol (11), and the extract was applied to a Sephadex LH20® (Pharmacia Biotech, Uppsala, Sweden) column (120 × 2.5 cm). The protein fraction eluted with chloroform-methanol-0.1 N hydrochloric acid (47.5:47.5:5%, v/v; 0.75 ml/min), as determined by absorbance at 240 nm, was repurified by the same procedure to remove residual phospholipid. Protein concentration was determined according to Bohlen et al. (17) and adjusted to 0.985 mg/ml with chloroform-methanol (7:3, v/v). Solutions of synthetic PC16:0/16:0, PC16:0/14:0, PC16:0/16:1, PC16:0/18:1, and palmitoyloleoyl PG (PG16:0/18:1) were adjusted to 1 μmol/ml with chloroform-methanol (7:3, v/v), and the purity of individual components was confirmed by ESI-MS/MS. Cholesterol was dissolved in chloroform-methanol to a final concentration of 1 mg/ml. These stock solutions were mixed to give constructs with constant basal concentrations of SP-B/C and cholesterol (37.5 and 60 mg/μmol phospholipids, respectively) and PG16:0/18:1 and PC16:0/18:1 (each 10 mol% of total phospholipid). Constructs were formed in which a variable composition of PC16:0/16:0, PC16:0/14:0, and PC16:0/16:1 constituted the remaining 80 mol% of total phospholipid. After evaporation of organic solvent under nitrogen gas, constructs were resuspended in sterile buffer (154 mM NaCl and 1.5 mM $CaCl_2$) by vigorous vortexing after the addition of sterile glass beads. These preparations were adjusted to 3.5 μmol phospholipid/ml, and surface tension function of the constructs was assessed as described above.

Statistics

Comparisons between the various groups were made using Student's independent *t*-test. $P < 5\%$ was taken as significant for the secretion analysis. Because of the inherent problem of comparing multiple groups of large data sets, $P < 1\%$ was used for all of the phospholipid molecular species analyses (see supplementary data).

RESULTS

Characterization of fetal alveolar epithelial cells differentiated in culture

As demonstrated previously, culture of undifferentiated human fetal lung epithelial cells in the presence of DCI for 4 days induced an apparent phenotypic maturation (8, 9). Cells exhibited extensive lamellar body production, characteristic of the mature surfactant system, and showed apical/basal polarity with lamellar bodies being concentrated near the apical plasma membrane. Here, we provide further evidence of the differentiation state of hormone-treated cells by examination of material secreted in the presence and absence of a secretagogue mixture used to induce maximal secretion (18). Cells cultured in the presence of DCI exhibited a time-dependent increase of secretion of newly synthesized PC in response to secretagogues (**Fig. 1A**) that reached ~2.5% of labeled cellular PC at 4 h. Basal secretion was similar for control and DCI-treated cells at <0.5% of labeled PC content. Released surfactant, pelleted at high speed from the pooled medium of secretagogue-stimulated DCI-treated cells, decreased surface tension to 3.6 ± 1.8 mN/m (n = 6) by bubble surfactometry, whereas material secreted during the previous 10 h (basal secretion) did not achieve low surface tension values (17.6 ± 1.3 mN/m; n = 6) (Fig. 1B).

The composition of this pelleted material secreted from DCI-treated cells over 10 h before and after secretagogue exposure is shown in **Table 1**. The high percentage of phospholipid in secreted material was in agreement with other studies of secretion from lungs in vivo (3) and with recent studies with adult type II alveolar epithelial cells in vitro (19, 20). The ratios of SP-A and SP-B to phospholipid secreted from these cells were similar to those in tracheal aspirate samples from premature infants. However, the fractional content of SP-C in surfactant secreted from isolated cells was lower than that found for tracheal aspirate samples, probably as a result of the slower induction of synthesis of the mature SP-C protein by cells differentiating in vitro (9). Secretagogues stimulated secretion of total phospholipid by 5.4- ± 2.6-fold (n = 4, $P < 0.05$), calculated relative to basal secretions over the 10 h before the addition

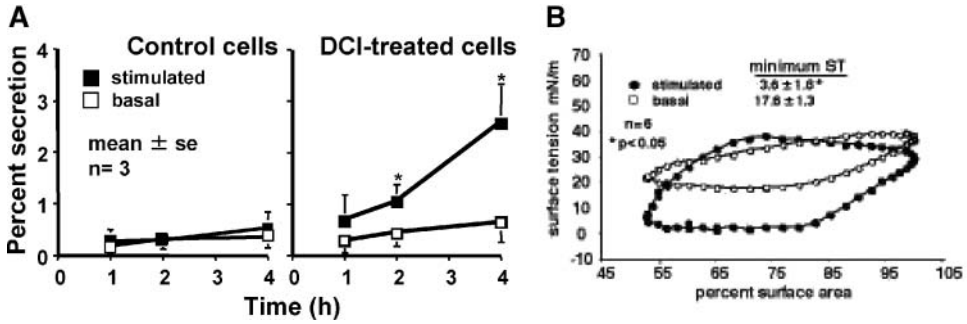

Fig. 1. Stimulated secretion of surface-active material from human fetal lung epithelial type II cells plated on plastic. A: Undifferentiated human fetal lung epithelial cells were isolated and cultured for 4 days in the absence (control; left panel) or presence of dexamethasone + 8-bromo-cAMP + isobutylmethylxanthine (DCI; right panel). Cells were labeled overnight with [^{3}H]choline (10 μCi/ml), unincorporated label was removed, and then a mixture of secretagogues (10 nM tetradecanoyl phorbol acetate, 100 nM Ca^{2+} ionophore A23187, and 10 nM terbutaline) was added to some cells and the dishes were harvested at various times (1–4 h). Basal secretion was determined from media of cells not treated with secretagogues. Values are means ± SEM of experiments with three cell preparations from different lungs. * $P < 0.05$ versus basal. B: Undifferentiated cells were isolated and differentiated in culture by DCI treatment for 6 days. After 10 h of stimulation in the presence of secretagogues, large aggregate surfactant was pelleted from the pooled medium (27,000 *g*, 1 h) and analyzed in a pulsating bubble surfactometer at 1.5 mg/ml phospholipid concentration. Surfactant recovered after secretagogue stimulation reached low surface tension (ST) values (3.6 ± 1.8 mN/m; $P < 0.05$ vs. basal), but basally secreted material did not (17.6 ± 1.3 mN/m). Values are means ± SEM of six experiments analyzing six pooled cell preparations from individual experiments.

of secretagogues, but had no significant effect on total protein release (Table 1). Secretagogues had different effects on the secretion of SP-A compared with SP-B and SP-C. Basal secretion of SP-A (as a percentage of total SP-A on the dish) was higher than that for either SP-B or SP-C, and secretagogues did not significantly increase SP-A release. In contrast, secretagogues increased the secretion of SP-B and SP-C by 11- and 18-fold, respectively (Table 1).

Phospholipid composition of cultured fetal lung cells

The concentrations of individual phospholipid classes in whole cell extracts were calculated as the sums of their individual molecular species determined by ESI-MS and expressed as the fraction of total phospholipid (**Fig. 2A**). There was no significant difference in phospholipid class distributions between epithelial and fibroblast cells in the presence or absence of hormones. Importantly, given its unique abundance in mature surfactant composition, the contribution of PG did not increase with hormonal treatment of epithelial cells. PC, calculated as the mean of all culture conditions, was consistently the major component (62.0 ± 4.1%), followed by PE (26.5 ± 4.8%), PS (5.4 ± 1.9%), PI (5.0 ± 0.8%), PG (0.7 ± 0.1%), and PA (0.5 ± 0.1%). Total phospholipid per epithelial cell increased from 27.6 ± 5.6 nmol/10^6 cells after 1 day to a mean of 61.9 ± 15.5 nmol/10^6 cells after 4 days in culture ($P < 0.05$) (Fig. 2B). This increase was independent of hormonal treatment and probably reflected an increased surface area of cells attached to plastic. By contrast, there was no difference in fibroblast total phospholipid concentration between days 1 and 4 in culture (Fig. 2B).

Phospholipid molecular species compositions of fetal lung cells in culture

Although there was no change in the distribution of phospholipid classes, differentiation of fetal lung epithe-

TABLE 1. Composition of secreted surfactant from isolated cells treated with secretagogues compared with infant tracheal aspirate samples

Component	Basal Conditions (10 h before Secretagogue Addition)	Stimulation with Secretagogues for 10 h				Infant TA[a]
	ng/10^7 cells/h		*fold increase*	*% of total mass*	*% of phospholipid*	
Phospholipid	984 ± 318	3,400 ± 1048	5.4 ± 2.6[b]	61.2 ± 6.4	—	—
Protein	648 ± 264	1,908 ± 507	3.3 ± 1.0	34.8 ± 6.8	—	—
SP-A	55 ± 26	137 ± 39	4.2 ± 1.9	3.0 ± 1.0	4.7 ± 1.4	7.1 ± 1.9
SP-B	3.9 ± 2.3	28.2 ± 7.4	11.3 ± 4.8[b]	0.6 ± 0.16	1.1 ± 0.4	1.8 ± 0.2
SP-C	3.5 ± 0.5	22.3 ± 3.1	18.4 ± 10.5[b]	0.5 ± 0.04	0.7 ± 0.1	4.6 ± 0.8

SP-A, surfactant protein A. All results are presented as means ± SEM of four cell preparations from individual lungs (13.5–20 weeks of gestation). All cells were treated for 6 days with dexamethasone + 8-bromo-cAMP + isobutylmethylxanthine before stimulation of secretion for 10 h. Basal values are derived from secretions collected for the 10 h before the addition of secretagogues.

[a] TA, tracheal aspirate samples from 35 premature infants (days 7–56 of life) with normal surface tension (minimum < 1 mN/m) by pulsating bubble surfactometer (32). Analyses were performed on large aggregate surfactant fractions (27,000 *g*, 1 h) prepared from both TA and media from cells.

[b] $P < 0.05$ for stimulated cells compared with basal conditions.

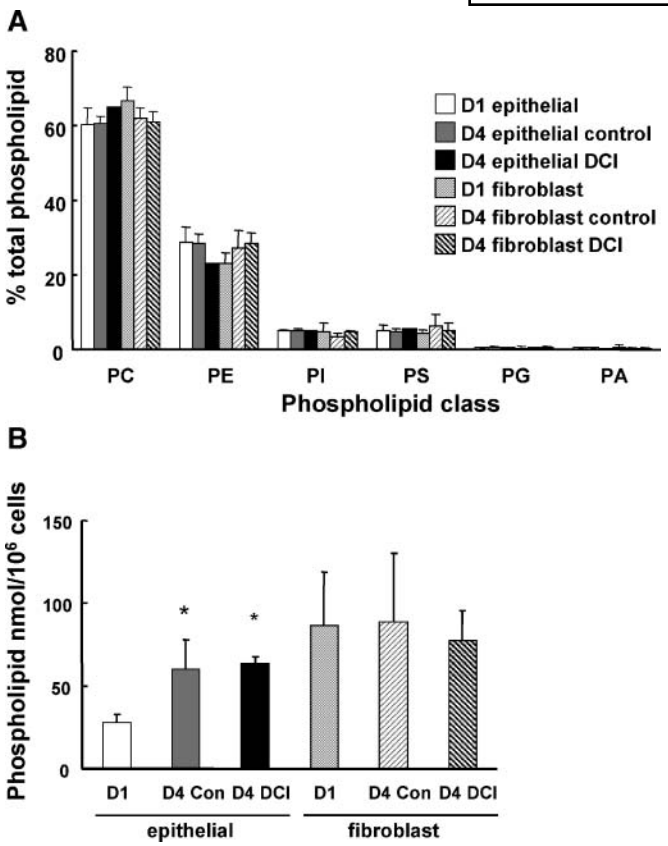

Fig. 2. Phospholipid composition of cultured human fetal lung cells. A: The relative distributions of the phospholipid classes phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), phosphatidylserine (PS), phosphatidylglycerol (PG), and phosphatidic acid (PA) were determined by electrospray ionization mass spectrometry (ESI-MS) analysis as the sums of individual molecular species from each phospholipid class and are expressed as means ± SD of four experiments for cells under the indicated culture conditions. B: Total phospholipid concentrations were calculated as the sum of each phospholipid class concentration and are expressed as means ± SD. * $P < 0.05$ for epithelial or fibroblast cells cultured for 4 days in the absence (D4 Con) or presence (D4 DCI) of DCI hormones compared with epithelial cells cultured for 1 day (D1).

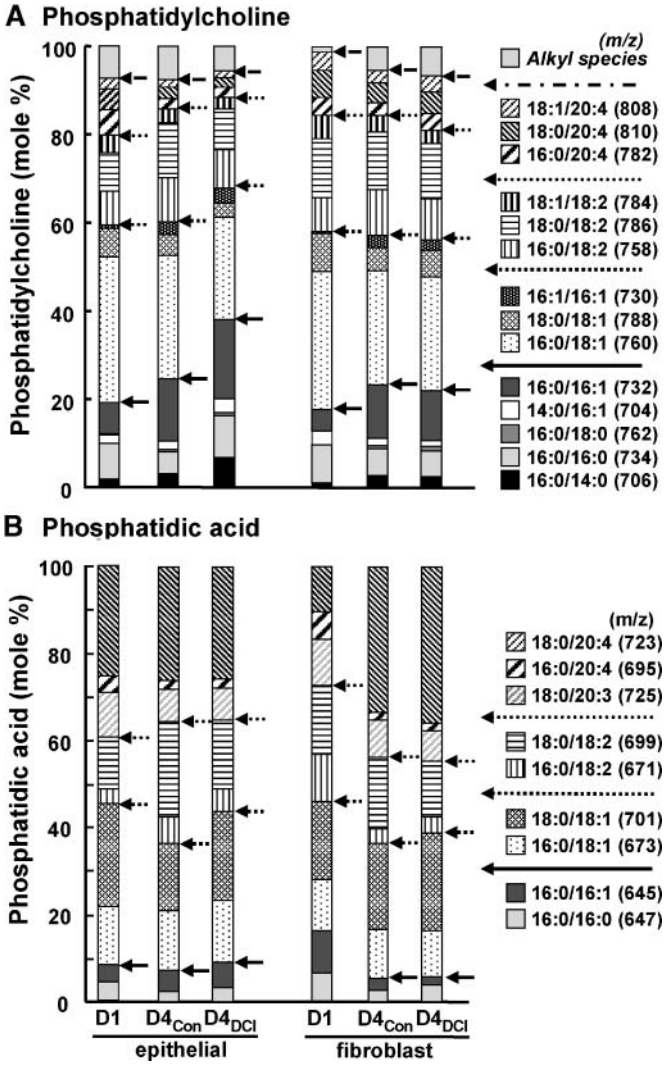

Fig. 3. Molecular species compositions of PC and PA in epithelial and fibroblast cells. Individual molecular species of cellular PC and PA were analyzed by ESI-MS for epithelial and fibroblast cells cultured under the conditions indicated for Fig. 2. Results are presented as means of four experiments. Molecular species are grouped first as the short-chain moieties characteristic of lung surfactant in vivo and then by degree of unsaturation.

lial cells was accompanied by alterations to phospholipid molecular species composition that were directly related to comparable observations in developing human fetal lung. PC, PA, PG, PI, PE, and PS species compositions are summarized in Figs. 3–5 for fetal lung epithelial and fibroblast cells cultured for 4 days in serum-free medium in the absence and presence of added hormones. The detailed compositions are tabulated in the supplementary data, but variations were generally low, with standard deviations typically 10% for PC species and no more than 20% for molecular species from any other phospholipid class.

The most significant change in response to DCI hormone treatment was to epithelial cell PC, which exhibited a significantly increased content of the short-chain PC species characteristic of lung surfactant in other studies (principally PC16:0/14:0, PC16:0/16:0, and PC16:0/16:1) (**Fig. 3A**). The sum of these short-chain PC species increased on day 4 from the control value of 24.3 ± 2.1% to 37.4 ± 3.3% ($P < 0.01$); corresponding values for epithelial cells on day 1 and for fibroblasts on day 4 in the absence and presence of hormones were 18.8 ± 2.6%, 22.6 ± 1.0%, and 21.5 ± 1.7%, respectively, and were no different from control epithelial cell values on day 4. The increased PC after DCI hormone treatment of epithelial cells was greatest for PC16:0/14:0 (2.24-fold) and somewhat less for PC16:0/16:0 (1.84-fold), PC14:0/16:1 (1.72-fold), and PC16:0/16:1 (1.26-fold). The effect of hormones on fetal lung epithelial cells was cell type-specific, as DCI treatment had no effect on the PC composition of fibroblasts cultured from the same fetal lungs. Intriguingly, the fractional concentrations of the three arachidonoyl PC species were all significantly greater ($P < 0.05$) in cultured fibroblasts (11.0 ± 2.0%) compared with epithelial cells

(6.3 ± 0.7%), irrespective of hormonal treatment of either cell type.

PA is a key intermediate in the synthesis of PC, but the molecular species composition of fetal lung epithelial cell PA did not alter significantly in response to DCI hormones (Fig. 3B). Species characteristic of surfactant PC were only minor components of epithelial cell PA; the sum of PA16:0/16:0 and PA16:0/16:1 was 7.3 ± 3.2% in the absence and 9.0 ± 2.6% in the presence of hormones. Species containing two or more double bonds accounted for some 60% of total PA. Comparison of PA and PC compositions of DCI-differentiated epithelial cells supports previous reports that acyl remodeling and sorting mechanisms are responsible for the acquisition of the more saturated nature of cell PC (Fig. 3A).

Apart from PC, PI was the other phospholipid class in which hormonal treatment caused compositional changes that reflected lung surfactant composition, which is uniquely enriched in monounsaturated species (predominantly PI16:0/18:1, PI18:1/18:1, and PI 18:0/18:1), in contrast to the polyunsaturated nature of PI analyzed in all other human cells ex vivo (21, 22). DCI hormone treatment of epithelial cells increased the content of these less unsaturated components (51.4 ± 5.5%) compared with control epithelial cells on day 4 (39.0 ± 4.7%; $P < 0.05$) at the expense of decreased concentrations of the arachidonoyl-containing PI species (**Fig. 4A**). As with PC, these changes were not a consequence of some general hormonal effect, as they were not observed for the fibroblast cultures. Indeed, fibroblast PI composition was very different from that of fetal epithelial cells, with very low amounts of monounsaturated species, and DCI treatment of fibroblasts tended to increase (68.9 ± 19.3% compared with 54.4 ± 19.8%) rather than decrease the sum of the concentrations of PI16:0/20:4, PI18:1/20:4, and PI18:0/20:4.

The other phospholipid classes exhibited only minor variations in response to DCI hormone treatment for either epithelial or fibroblast cells. PG was essentially totally monounsaturated (Fig. 4B), PS was also predominantly unsaturated but with a substantial component of polyunsaturated species (**Fig. 5A**), and PE contained a high proportion of both polyunsaturated and ether-linked (probably alkenyl-acyl) species (Fig. 5B). DCI treatment tended to increase the content of monounsaturated PS and PE species, but this was not significant.

Phospholipid compositions of secretions from cultured fetal lung cells

The phospholipid composition of surfactant secreted by fetal lung epithelial cells showed very clearly the effects of phenotypic maturation. Molecular species compositions of PC and PI from secreted material are compared in **Fig. 6** with comparable analyses of DCI-treated cells. Compared with whole cell PC composition, surfactant secreted from DCI-treated fetal epithelial cells was substantially enriched in the short-chain disaturated and monounsaturated PC species PC16:0/14:0, PC14:0/16:1, and PC16:0/16:1 (all $P < 0.01$) but intriguingly not in PC16:0/16:0 (Fig. 6A).

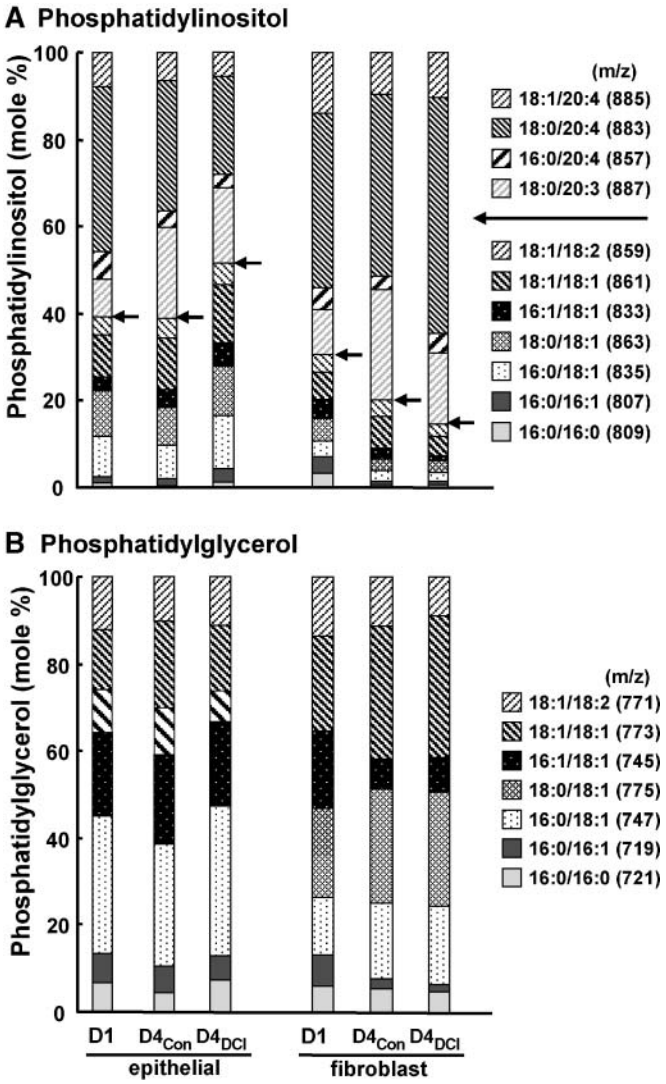

Fig. 4. Molecular species compositions of PI and PG in epithelial and fibroblast cells. Individual molecular species of cellular PI and PG were analyzed by ESI-MS for epithelial and fibroblast cells cultured under the conditions indicated for Fig. 2. Results are presented as means of four experiments. PI molecular species are grouped first as the saturated and monounsaturated moieties characteristic of lung surfactant in vivo and then as the polyunsaturated species typical of cell membranes. All PG species were typical of those present in lung surfactant and so have not been grouped.

These increases were at the expense of longer chain PC species, principally PC16:0/18:1, PC18:0/18:1, PC18:1/18:1, PC18:0/20:4, and PC18:1/20:4. The PI composition of surfactant secreted from DCI-treated cells compared with control cells exhibited similar changes, with increased fractional contents of species with shorter, monounsaturated fatty acids (PC16:0/18:1 and PC18:1/18:1; $P < 0.01$) at the expense of species containing longer chain polyunsaturated fatty acids (PI18:0/20:4; $P < 0.01$) (Fig. 6B). PC and PI compositions of material recovered from the medium of control cells were essentially identical to those of whole cell extracts (results not shown), suggesting that this material was most likely derived from cell membrane fragments.

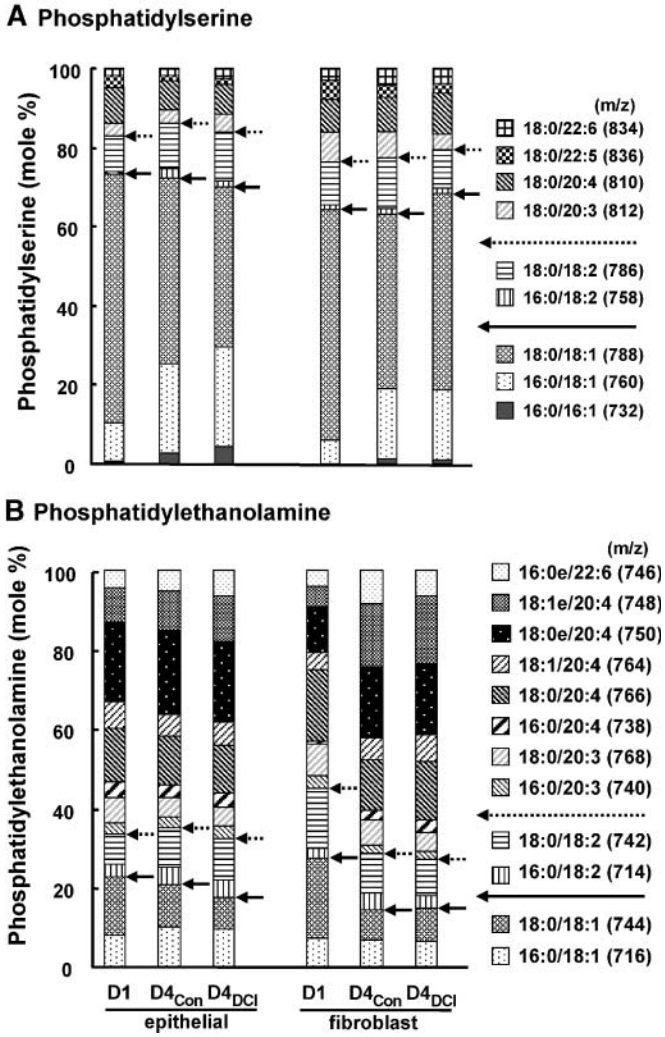

Fig. 5. Molecular species compositions of PS and PE in epithelial and fibroblast cells. Individual molecular species of cellular PS and PE were analyzed by ESI-MS for epithelial and fibroblast cells cultured under the conditions indicated for Fig. 2. Results are presented as means of four experiments. PS and PE molecular species are both grouped by degree of unsaturation.

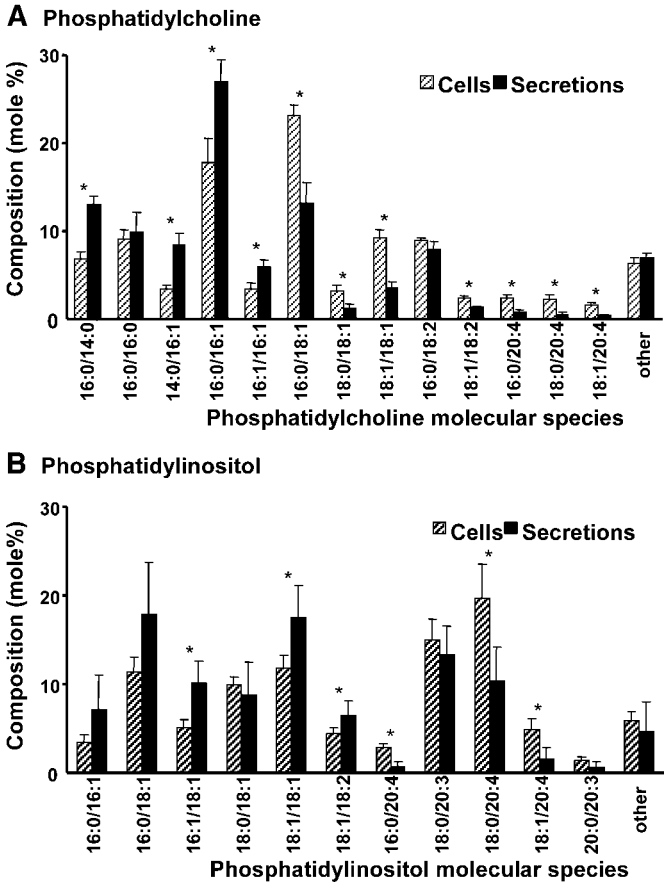

Fig. 6. Comparison of PC and PI molecular species compositions of cells and of secreted material. Cells were cultured for 6 days in the presence of DCI hormones and then treated with secretagogues (tetradecanoyl phorbol acetate, Ca^{2+} ionophore A23187, and terbutaline) for 10 h to promote secretion. Media were collected and pooled from 25–30 dishes per experiment. Lipid extracts of the large aggregate surfactant (27,000 *g*, 1 h) isolated from pooled media and from DCI-treated cells were analyzed by ESI-MS for PC (A) and PI (B). Results are presented as means ± SD for four experiments. * $P < 0.01$ for secretions compared with cells after DCI treatment.

Functional assessment of phospholipid-apoprotein constructs

To extend the observation in Fig. 1B that secretions from differentiated type II cells can generate low surface tension values on compression despite a low concentration of PC16:0/16:0 (Fig. 6A), we assessed the surface activities of a variety of phospholipid mixtures complexed with cholesterol and SP-B/C (**Fig. 7**). Constructs containing 80% PC16:0/16:0 reached values of 0–5 mN/m within 10 pulsations, and substitution of 30% of PC16:0/16:0 with either PC16:0/16:1 or PC16:0/18:1 gave essentially identical results. Replacement of PC16:0/16:0 by 30% PC16:0/14:0 alone delayed the achievement of near-zero minimal surface tension values. By contrast, constructs with a PC composition comparable to that of secretions from differentiated fetal type II cells, containing both PC16:0/14:0 and PC16:0/16:1 at the expense of PC16:0/16:0, reached surface tension values near 0 mN/m even more rapidly (three pulsations) than did the other constructs.

DISCUSSION

The results presented here represent the first comprehensive lipidomic analysis of a human fetal cell type differentiated in culture. They demonstrate clearly that phenotypic differentiation of immature human fetal epithelial cells into functionally active type II alveolar epithelial cells is characterized by significant modulations to phospholipid compositions of both whole cells and secreted surfactant. Moreover, the effect of hormonal exposure was cell type-specific, as the shift toward monounsaturated and disaturated species of PC (Fig. 3A) and monounsaturated species of PI (Fig. 4A) was not observed for fibroblasts

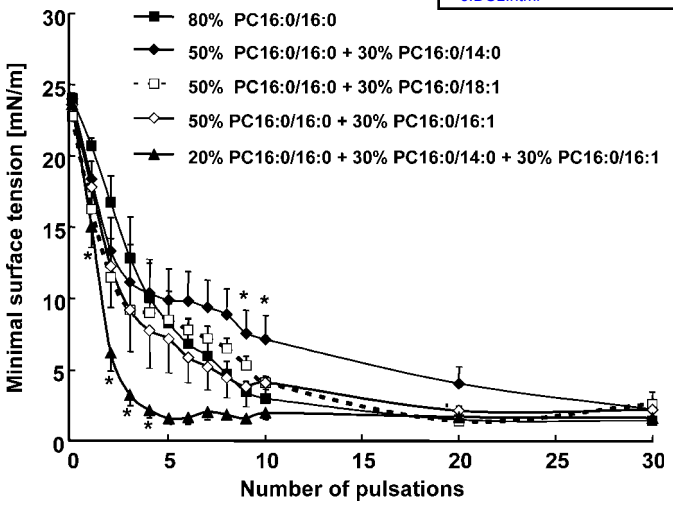

Fig. 7. Influence of PC composition on surface tension function under dynamic conditions. Minimal surface tension of surfactant mixtures with varying concentrations of PC species, but constant in PG16:0/18:1, cholesterol, and surfactant proteins B and C, were measured as indicated in Materials and Methods. Results are means ± SEM of three to six experiments. * $P < 0.05$ versus 80% PC16:0/16:0.

cultured from the same lungs. To our knowledge, these results are the first description of a differential response of phospholipid composition to hormonal treatment by two primary human fetal cell types of different lineage that in each case can be linked to cell function in vivo.

The details of the enrichment of PC species in secreted material compared with the intact cell were intriguing (Fig. 6A). Instead of an increased content of PC16:0/16:0, secretions from hormonally treated cells were enhanced in a subset of PC species containing a combination of saturated and monusaturated fatty acids with short chains of 14 or 16 carbon atoms, principally PC16:0/14:0 and PC16:0/16:1. Previous studies analyzing phospholipid fatty acid compositions in explant cultures of human fetal lung attributed increased fractional concentrations of myristate ($C_{14:0}$) and palmitoleate ($C_{16:1}$) to artifacts of the culture system (23, 24), but more recent studies suggest that this is unlikely to be the only explanation and that these molecular species are integral components of lung surfactant. Analysis of PC synthesis by mouse lungs in vivo demonstrated clearly that PC16:0/14:0 and PC16:0/16:1 were synthesized and secreted at comparable rates to PC16:0/16:0, and these rates were more rapid than for other PC species containing longer chain fatty acids (25). PC16:0/14:0 and PC16:0/16:1 are major components of purified human pulmonary surfactant; indeed, PC16:0/14:0 exhibited the greatest fractional increase of any PC species in human fetal lung tissue near term gestation (1). Analysis of the molecular specificity of surfactant from a wide range of animal species has shown a considerable variation in PC16:0/16:0 content that may be inversely related to ventilation frequency (2). Importantly, decreased surfactant PC16:0/16:0 in such analyses is always compensated by the increased contents of either PC16:0/14:0 or PC16:0/16:1, precisely the same species enriched in surfactant secreted from DCI-treated cells in this study. Finally, the content of PC16:0/16:0 was considerably decreased in neonatal pig (6) and rat (7) surfactant, and again PC16:0/14:0 was increased in compensation. It is somewhat difficult to reconcile the increased PC16:0/14:0 in secreted surfactant from DCI-treated fetal alveolar type II cells with its suggested role in compensating for increased surface curvature during the process of alveolarization in vivo (7). Obviously, this cannot be the explanation for flat cells cultured on plastic and suggests that multiple physiological mechanisms may interact to determine the final composition of surfactant PC.

The surface tension analysis (Fig. 1B) clearly shows that secretagogue-stimulated large aggregate material from differentiated fetal type II cells was functionally active in vitro despite its relatively low content of PC16:0/16:0 (Fig. 6A). The finding that reconstituted surfactant enriched in PC16:0/14:0 and PC16:0/16:1 rapidly reached low surface tension values in a pulsating bubble surfactometer (Fig. 7) further demonstrated that these two PC molecular species could account for the functional activity of the surfactant secreted from the fetal type II cells.

Although the PC result was to some extent predicted, based on previous studies of fetal lung explants measuring either disaturated PC (26) or PC fatty acids (27), the PI response was unexpected. PI from human lung surfactant has a uniquely monounsaturated composition compared with the high content of the 1-stearoyl-2-arachidonoyl species characteristic of PI in rodent surfactant or in most other cell membranes in vivo (21, 22). DCI treatment not only increased the monounsaturated PI species characteristic of human surfactant in fetal epithelial cells but, in contrast, also increased the enrichment of PI18:0/20:4 in fetal fibroblasts. This observation is potentially important both because PI18:0/20:4 is the precursor for polyphosphoinositides involved in cell signaling (28) and because it demonstrates that both cell types were sensitive to DCI treatment but with opposite responses. The relatively decreased arachidonoyl content of fetal epithelial cells after DCI treatment may be related to the provision of arachidonate substrate for the production of endogenous eicosanoids previously related to cAMP production and differentiation of fetal lung explants (29).

The lack of any change to PG species clearly dissociated any direct connection between its increased concentration in fetal lung in late gestation and the morphological maturation of the type II cell, processes that are temporally associated in vivo. To some extent, this lack of change to PG may have been partly attributable to a relatively high concentration of *myo*-inositol in the culture medium, as PI can substitute for PG in surfactant function, for instance, after inositol feeding (30). Moreover, preterm infant surfactant that is deficient in PG has normal minimal surface tension in vitro (31).

The precise mechanisms regulating the specificity of PC synthesis by type II cells are not fully established, but they are thought to involve a combination of acyl-remodeling reactions, based on phospholipase and acyltransferase en-

zymes, translocation through the cell, and selective transport across membranes. Mechanisms regulating the molecular specificity of synthesis of surfactant PI have not been investigated. Whatever the precise details of these regulatory mechanisms, our results strongly suggest that molecular size rather than individual molecular structures is the major determinant regulating PC and PI compositions of lung surfactant, because for both phospholipids the shorter chain molecular species (PC16:0/14:0, PC16:0/16:1, PC16:0/16:0, and PI16:0/18:1) were preferentially packaged into material secreted from DCI-treated epithelial cells at the expense of longer chain species (PC16:0/18:1, PC18:1/18:1, PC18:0/18:1, and PI18:0/20:4). They also strongly suggest that a disaturated structure is not a major criterion governing the uptake of PC species into lamellar bodies. One major implication of this conclusion is that factors external to the type II cell must be significant determinants of the molecular specificity of surfactant PC composition in vivo. One possibility is that the extensive intra-alveolar recycling and uptake of surfactant by type II cells in vivo may contribute significantly to the specificity of surfactant PC synthesis. Evidently, such surfactant recycling is unlikely to contribute substantially to the specificity of PC synthesis by monolayer cultures of type II cells, given the large dilution effect of culture medium volume.

In conclusion, this study presents a paradigm for the interaction of genotypic expression and environmental factors in regulating both phenotype and physiological function. The results show clearly that differentiation has a profound and essential, but not necessarily paramount, effect on the phospholipid composition of defined cellular membrane components. This interaction in vivo probably serves to fine-tune surfactant phospholipid composition for the precise physiological requirements of the lungs, for instance, at different postnatal ages or in response to respiratory demands. This is an attractive concept as it would allow such adaptations to occur without having to alter the differentiation state or gene expression of the type II alveolar epithelial cell and is possibly a mechanism that also applies to the differentiation of other stem cells.

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