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In Vitro Transdifferentiation of Human Fetal Type II Cells Toward a Type I–like Cell

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ABSTRACT: For alveolar type I cells, phenotype plasticity and physiology other than gas exchange await further clarification due to *in vitro* study difficulties in isolating and maintaining type I cells in primary culture. Using an established *in vitro* model of human fetal type II cells, in which the type II phenotype is induced and maintained by adding hormones, we assessed for transdifferentiation in culture toward a type I-like cell with hormone removal for up to 144 h, followed by electron microscopy, permeability studies, and RNA and protein analysis. Hormone withdrawal resulted in diminished type II cell characteristics, including decreased microvilli, lamellar bodies, and type II cell marker RNA and protein. There was a simultaneous increase in type I characteristics, including increased epithelial cell barrier function indicative of a tight monolayer and increased type I cell marker RNA and protein. Our results indicate that hormone removal from cultured human fetal type II cells results in transdifferentiation toward a type I-like cell. This model will be useful for continued *in vitro* studies of human fetal alveolar epithelial cell differentiation and phenotype plasticity. **(*Pediatr Res* 61: 404–409, 2007)**

The distal alveolar epithelium contains type I and II cells. Type II cells comprise 75% of distal lung epithelium, 7% of the surface area, and produce surfactant. Type II cells have many well-characterized markers, including lamellar bodies, surfactant proteins A, B, C, and D (SP-A, B, C, and D) (1), and the aspartic protease pepsinogen C (PGC) (2).

Type I cells, although less numerous, cover 93% of adult lung surface area and provide the gas exchange surface (1). Other type I functions, such as ion transport and fluid homeostasis, are less known. Type I cells form a tight monolayer with low permeability (3). However, the utility of previously described type I biochemical markers (4) has been limited by variable reproducibility and expression between species.

Challenges in isolation and culture have limited progress in type I cell biology. Despite isolated reports (5,6), there are no widespread primary culture models. Instead, rat type II cells transdifferentiated toward a type I cell on tissue culture plastic (7) have served as a proxy for type I cells (8,9). There are limited data with regards to transdifferentiation in adult human alveolar epithelium (10), with no previous *in vivo* descriptions in human fetal lung. Rodent model transdifferentiation and the observation that type II cells serve as progenitors of type I cells after alveolar injury in mature lung (11) have led to the assumption that type I cells are derived from type II cells. However, the possibility that both are derived from a common precursor and retain some degree of plasticity has not been examined in the human fetal lung.

Because of its potential plasticity, fetal alveolar epithelium provides unique opportunities to study differentiation/transdifferentiation pathway(s) in the developing lung. We previously demonstrated that type II cells from human fetal lung can maintain a differentiated phenotype with dexamethasone, cAMP, and isobutylmethylxanthine (DCI) (12) and that these conditions induce differentiation of type II cells from naive human fetal lung epithelium (13). Here, we demonstrate that DCI withdrawal from cultured human fetal type II cells results in transdifferentiation toward type I-like cells. Transdifferentiation is associated with diminution of type II morphology, decreased expression of type II markers, and induction of type I cell markers. Importantly, transdifferentiated cells behave like type I cells, with decreased permeability and increased transepithelial resistance (TER), indicating a tight type I cell monolayer. Our data establish transdifferentiation as a tool for future study of human alveolar type I cell biology.

METHODS

Reagents. Dexamethasone, isobutyl methylxanthine, and 8-bromo-cAMP were obtained from Sigma Chemical Co. (St. Louis, MO). Carboxyfluorescein and Texas Red dextran were from Molecular Probes (Eugene, OR). All other reagents were purchased from Fisher (Fair Lawn, NJ), Pierce (Rockford, IL), or Invitrogen (Carlsbad, CA).

Antisera used herein included cytokeratin (DAKO, Carpinteria, CA), Ki-67 (Vision Biosystems, Norwell, MA), vimentin (US Biologicals, Swampscott, MA), zonula occludens-1 (ZO-1) (Zymed, South San Francisco, CA), SP-B (Chemicon, Temecula, CA), caveolin-1α (Cav -1) (Santa Cruz Biotechnologies, Santa Cruz, CA), claudin 7 (Zymed), SP-A (US Biologicals), PGC (Abcam, Cambridge, UK), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Chemicon), and plasminogen activator inhibitor-1 (PAI-1) (BD Transduction Laboratories, Lexington, KY). An epitope-specific pro-SP-C rabbit antiserum (NPRoSP-C; Met^{10}-Glu^{23} of rat proSP-C) was donated by Dr. Michael Beers.

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Abbreviations: Cav-1, caveolin-1; **DCI,** dexamethasone, cAMP, and isobutylmethylxanthine; **GAPDH,** glyceraldehyde-3-phosphate dehydrogenase; **PAI-1,** plasminogen activator inhibitor-1; **PGC,** pepsinogen C; **SEM,** scanning electron microscopy; **SP-A, SP-B, SP-C,** surfactant proteins A, B, C; **TEM,** transmission electron microscopy; **TER,** transepithelial resistance; **ZO-1,** zonula occludens-1

Cell culture. Human fetal lung from 14- to 18-wk therapeutic abortions came from the Birth Defects Laboratory in the Department of Pediatrics, University of Washington Medical Center (Seattle, WA). All tissue was obtained and handled under protocols approved by the Committee for Human Research, Children's Hospital of Philadelphia.

Fetal lung epithelial cells were prepared as described (13). Cells were cultured on tissue culture plastic for up to 9 d in 10 nM dexamethasone, 0.1 mM 8-bromoadenosine-3′,5′-cyclic monophosphate, and 0.1 mM isobutyl methylxanthine (DCI, or "hormones") to induce and maintain type II cell differentiation. Transdifferentiation was accomplished by hormone withdrawal after the type II cell phenotype was established on d 4.

Epithelial cell viability and purity assays. The LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes) was used to determine cell viability. This two-color fluorescent viability assay relies on the retention of calcein AM in live cells (red) and infiltration of ethidium homodimer (EthD-1) into damaged cells (green). Cytokeratin (to identify epithelial cells) and vimentin (to identify fibroblasts) immunostaining were used to assess epithelial cell purity. Proliferation was characterized by Ki67 immunostaining.

Ultrastructural studies. Transmission electron microscopy (TEM) samples were prepared and imaged as previously described (14), with the addition of polycarbonate filters for cross-sectional images. For scanning electron microscopy (SEM), cells were cultured on glass and subjected to hexamethyldisilazine for critical point drying. Samples were sputter coated with gold palladium target and representative images captured with a Philips XL 20 scanning microscope at 5 kV, with a working distance at 100 μm. All TEM supplies were from Electron Microscopy Sciences (Fort Washington, PA) or Polysciences (Warrington, PA).

Alveolar epithelial barrier studies and indirect immunofluorescence. Barrier properties were assessed by fluorescent dye diffusion measurement across cell monolayers plated on Transwell permeable membranes with 0.4-μm pores (Corning, Corning, NY), as detailed elsewhere (15). Filtered fluorophore concentrations were assessed using a SpectraMAX Gemini microplate fluorimeter (Molecular Devices, Sunnyvale, CA). Barrier function was also characterized by measuring TER with an ohmmeter (World Precision Instruments, Sarasota, FL). With all barrier studies, a confluent monolayer with tight junctions was confirmed using indirect immunofluorescence for the tight junction protein ZO-1 (16).

Western immunoblotting. Cells were harvested in lysis buffer (1% Triton X-100, 150 mM NaCl, 50 mM Tris-HCL, 5 mM ethylenediamine tetraacetic acid, 5% glycerol, pH 8.0) mixed with 1 × protease inhibitor (Roche, Indianapolis, IN). Westerns were performed using NuPAGE Bis-Tris gels with MES running buffer (Invitrogen) and transferred to polyvinylidine difluoride membrane. Primary antibodies used were claudin 7, Cav-1, PAI-1, and NProSP-C at 1:1000, SP-A at 1:2500, SP-B at 1:4000, PGC at 1:5000, and GAPDH at 1:20,000. Blots were probed with secondary antibodies conjugated to Alexa Fluor 680 (Molecular Probes) or IRdye 800 (Rockland, Gilbertsville, PA) at a dilution of 1:10,000 for all primary antibodies. Blotted proteins were detected and analyzed using the Odyssey infrared imaging system (Li-Cor, Lincoln, NE).

Real-time reverse transcriptase polymerase chain reaction (RT-PCR). Total cellular RNA was isolated with RNA STAT-60 Reagent (Tel-Test, Friarswood, TX). Purity was verified by the OD 260:280 ratio. Integrity was screened using the eukaryote total RNA nano assay on an Agilent 2100 bioanalyzer (Agilent, Palo Alto, CA). Real-time RT-PCR reactions using a singleplex strategy were performed using an ABI Prism 7900 system [Applied Biosystems (ABI), Foster City, CA]. The two-step PCR protocol was performed as detailed elsewhere (2). All reagents/probes were obtained from ABI. Fluorescence intensity was recorded during the annealing step of each cycle. The following primer/probe sets, as listed on the ABI Web site (http://www.allgenes.com), were used: SP-A1 Hs00831305, SP-C Hs00161628, SP-B Hs00167036, PGC Hs00160052, claudin 7 Hs00600772, Cav-1 Hs00184697, aquaporin 5 Hs00387048, PAI-1 Hs00167155, and GAPDH Hs99999905. All assays were determined to be in the linear amplification range by using cDNA standards created from RNA from alveolar epithelial cells cultured for 4 d in DCI (type II cells) or from banked frozen adult lung tissue (type I cells).

Statistical analysis. Results are given as mean ± standard error of the mean (SEM). *t* tests (for lamellar body quantification) or analysis of variance (ANOVA) (all other studies) were performed using GraphPad Prism 4.00 for Macintosh (GraphPad, San Diego, CA). All protein and RNA results were normalized to GAPDH.

RESULTS

Cell viability and proliferation. We examined cell purity, proliferation, and viability in differentiating (d 4 and 7 in DCI) and transdifferentiating (4 d DCI followed by 3 d Waymouth's) culture conditions (three experiments, five random fields per condition). Cell cultures were ≥91% epithelial cells by cytokeratin and vimentin immunostaining. No epithelial cells were proliferating, as indicated by absent Ki67 staining. There were no differences in cell viability between groups (4d DCI: 94 ± 0.4%, 7d DCI: 92.6 ± 1.0%, 4d DCI/3 d Waymouth's: 92.0 ± 1.0%, p = not significant).

Morphologic studies. Effects of transdifferentiation on morphology were examined by TEM and SEM. Naive epithelial cells exhibited no lamellar bodies. By 4 d in DCI (4 d DCI), lung epithelial cells demonstrated hallmarks of differentiated type II cells, including lamellar bodies and microvilli (Fig. 1*A,C*), as previously noted (13). SEM supported the TEM phenotype, with microvilli visible as abundant, short cytoplasmic projections (Fig. 1*E*). Transdifferentiating cells demon-

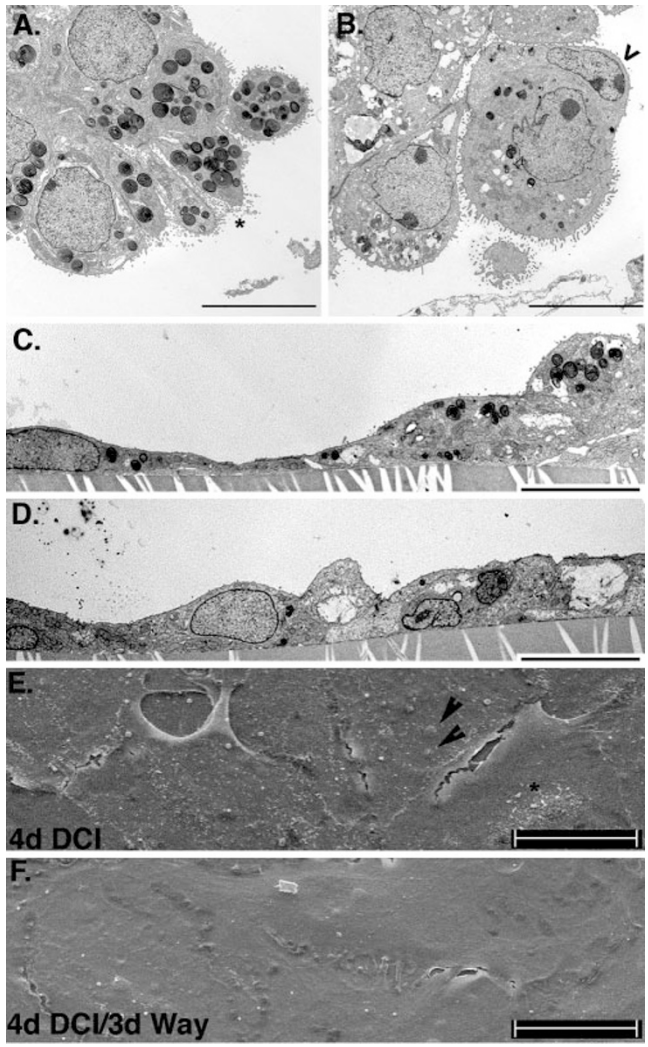

Figure 1. Transdifferentiation *via* hormone withdrawal alters type II cell morphology and surface characteristics. Representative electron micrographs of human fetal lung epithelial cells treated with DCI for 4 d (4 d DCI) (*A,C,E*), or 4 d DCI followed by 72 h without hormones (4 d DCI/3 d Waymouth's) (*B,D,F*). TEM of cell pellets (*A,B*) and cross-sectional slices of plated cells (*C,D*) at ×5000. SEM images of plated cells (*E,F*) at ×1000. (*A,E*): Microvilli indicated by *asterisk*. (*B*) Blunting of microvilli indicated by *open arrowhead*. (*E*) Lamellar bodies indicated by *bold arrowhead*. (*A–D*) Bars = 10 μm. (*E,F*) bars = 20 μm.

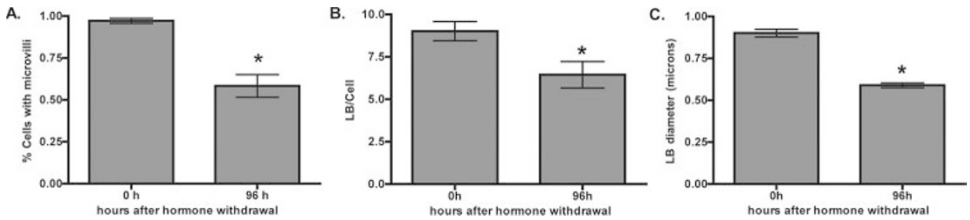

Figure 2. Quantitative morphology changes with transdifferentiation. TEM samples from three experiments were evaluated at ×5000 after 4 d DCI (0 h) and 96 h after hormone withdrawal from 4 d DCI-treated cells (4 d DCI/4 d Waymouth's). (*A*) Percentage of cells exhibiting microvilli (*$p < 0.001$). (*B*) Lamellar bodies per cell (*$p < 0.05$ *vs* 4 d DCI). (*C*) Lamellar body diameter ($p < 0.001$).

strated fewer lamellar bodies and blunting of microvilli by TEM (Fig. 1*B*), despite maintaining a confluent monolayer (Fig. 3*D*). This was evidenced on SEM as a loss of surface projections (Fig. 1*F*).

Field counting supported these changes [three experiments, seven to eight high-power fields (hpf) per treatment group with 6.0 ± 0.5 cells/hpf]. Of 4 d DCI cells, 97 ± 0.1% had microvilli. Withdrawal of hormones was associated with only 58.4 ± 0.1% of cells exhibiting microvilli (Fig. 2*A*) ($p < 0.001$). Lamellar bodies were also diminished (Fig. 2*B*) after hormone withdrawal (6.4 ± 0.8 lamellar bodies/cell), compared with 4 d DCI type II controls (9.0 ± 0.6 lamellar bodies/cell) ($p < 0.05$). Remaining lamellar bodies after hormone withdrawal were smaller (Fig. 2*C*), with a mean diameter of 0.6 ± 0.01 μm *versus* 0.9 ± 0.02 μm in 4 d DCI cells ($p < 0.001$). Together, these data show that transdifferentiation is associated with diminution of type II cell morphology.

Alveolar epithelial cell barrier function. We assessed epithelial barrier function by dye filtration and transepithelial resistance (Fig. 3). The concentration of both carboxyfluorescein (Fig. 3*A*) and Texas Red dextran (Fig. 3*B*) filtered into the bottom chamber was decreased in cells after hormone withdrawal ($n = 9–11$, $p < 0.05$ *versus* 4 d and 7 d DCI). Rat type II cells routinely demonstrate TER of 500–1000 Ω × cm^2 (17). Human differentiated type II cells (4 d DCI) demonstrated a mean TER of 347 ± 14 Ω × cm^2 (Fig. 3*C*). Mean TER increased with transdifferentiation, to 446 ± 20 Ω × cm^2 ($n = 8$, $p < 0.01$ *versus* 4 d and 7 d DCI) 3 d after hormone withdrawal. Together, these data indicate tighter barrier function with transdifferentiation.

Disappearance of type II cell markers. Real-time RT-PCR (Fig. 4*A*) revealed significant reduction of type II markers after hormone withdrawal. All markers were decreased to less than 50% of controls within 24 h ($n = 6–7$, $p < 0.01$ *versus* 4 d DCI) and continued to decline throughout the subsequent 5 d ($n = 3–7$, $p < 0.01$ *versus* 4 d DCI, except SP-B at 144 h, for which $p < 0.05$). Only a small amount of SP-B mRNA was present in nonhormone-treated controls (d 4 Waymouth's) (6.4 ± 2.3% of d 4 DCI values). Similar to our previous work (2), immunoblotting revealed that hormone withdrawal resulted in progressive decline of all type II markers studied, with disappearance of SP-A, NPro SP-C, and PGC by 72 h and SP-B by 120 h (Fig. 4*B*).

Increased expression of type I cell markers. Real-time RT-PCR (Fig. 5*A*) revealed expression of all type I markers examined in 4 d Waymouth's–treated controls, particularly PAI-1. Expression of the type I cell markers Cav-1 (mean 2.3 ± 0.4-fold), aquaporin 5 (mean 2.7 ± 0.6-fold), and PAI-1 (mean 5.1 ± 0.7-fold) increased with transdifferentiation over 24–96 h after hormone withdrawal, compared with type II controls ($n = 7$, $p < 0.05$). There was no induction of claudin 7 mRNA. At 144 h after hormone withdrawal, PAI-1 remained significantly induced at 4.3 ± 1.3-fold *versus* 4 d DCI ($n = 3$, $p < 0.05$). By immunoblotting, there were increases in the type I markers Cav-1 and PAI-1, with no change in claudin 7 (Fig. 5*B*). Although PAI-1 protein appeared earlier than Cav-1, both were induced by 72 h after hormone withdrawal. The type I cell marker aquaporin 5, induced in our mRNA studies, was also probed *via* immunoblotting, but only nonspecific banding patterns were visible using a variety of commercial antibodies (data not shown).

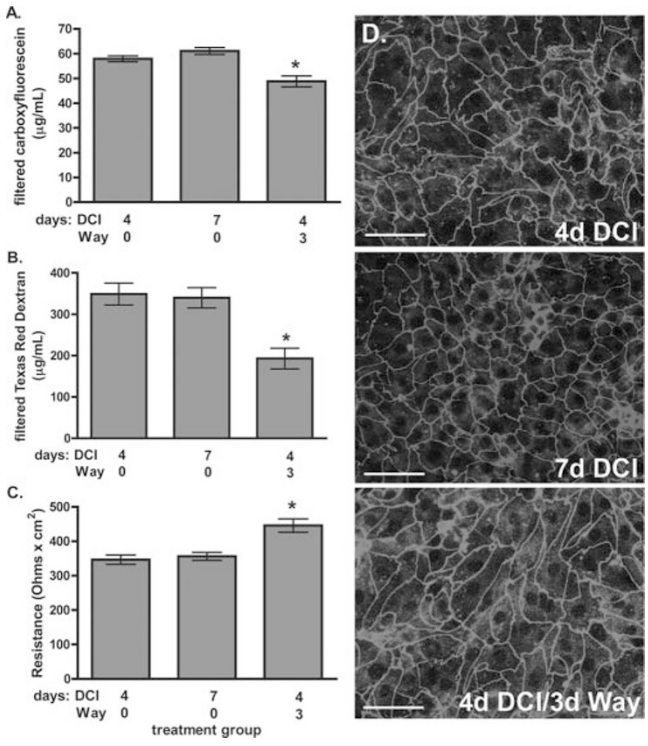

Figure 3. Increased barrier function with transdifferentiation. Epithelial barrier function was examined in cells cultured on permeable supports with 4 d DCI, 7 d DCI, or 4 d DCI followed by hormone withdrawal (4 d DCI/3 d Waymouth's). (*A*) Carboxyfluorescein filtration (*$p < 0.05$). (*B*) Texas Red dextran filtration (*$p < 0.05$). (*C*) TER (*$p < 0.01$). (*D*) Cells immunostained for ZO-1 at ×20 demonstrate confluent monolayers. Bars = 20 μm.

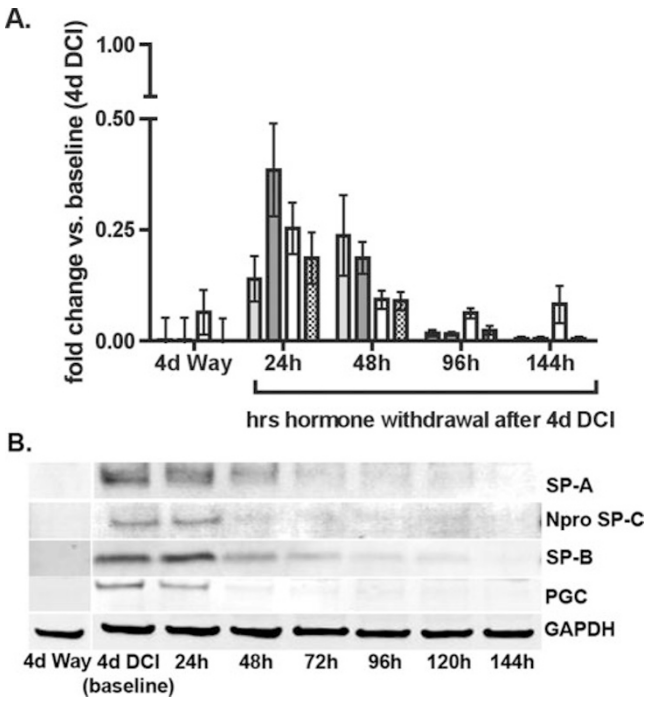

Figure 4. Type II cell markers decrease with transdifferentiation. The type II cell markers SP-A, SP-C, SP-B, and PGC were assessed by real-time RT-PCR (*A*) and immunoblotting (*B*) for up to 144 h after hormone withdrawal from 4 d DCI–treated cells. SPA (*lightly shaded columns*), SP-C (*darkly shaded columns*), SP-B (*open columns*), PGC (*hatched columns*).

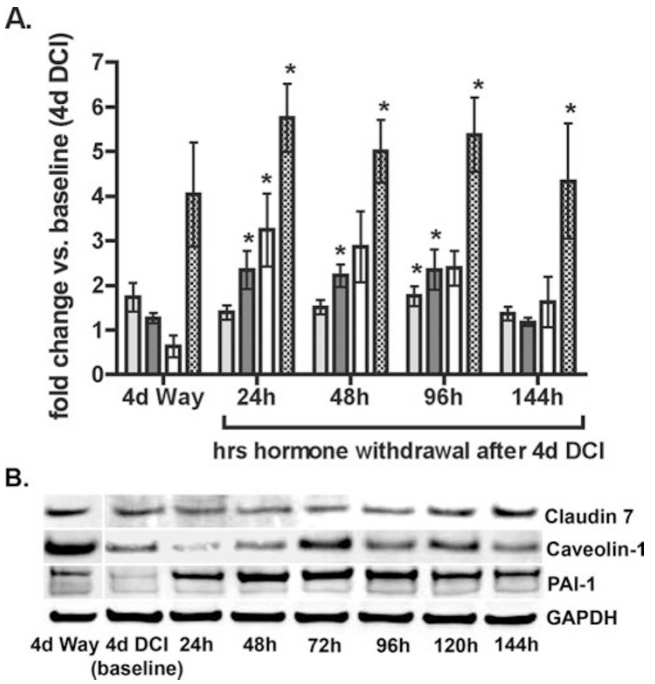

Figure 5. Type I cell markers increase with transdifferentiation. Type I cell markers claudin 7, Cav-1, aquaporin 5, and PAI-1 were assessed by real-time RT-PCR (*A*) and immunoblotting (*B*) for up to 144 h after hormone withdrawal from 4 d DCI–treated cells. Claudin 7 (*lightly shaded columns*), Dark gray: Cav-1 (*darkly shaded columns*), aquaporin 5 (*open columns*), PAI-1 (*hatched columns*) ($*p < 0.05$).

DISCUSSION

Difficulties in isolating and maintaining type I cells in culture, limited functional assays, and a paucity of cross-species reproducible markers have limited investigations of type I cell functions beyond gas exchange. Although investigators have used transdifferentiation as a model of adult type I cells (9,18), this is the first report of transdifferentiation leading to a type I–like cell from human fetal alveolar epithelium. Transdifferentiated fetal cells lost type II cell characteristics. Importantly, transdifferentiated fetal cells demonstrated not only a functionally tight monolayer consistent with type I cell behavior, but increased expression of a battery of type I cell markers. The ability to elicit transdifferentiation in human fetal alveolar epithelium provides investigators with a unique opportunity to explore the mechanisms of fluid balance, epithelial cell lineage, and phenotype plasticity in the developing human lung.

Isolation and culture of type I cells has been difficult for several reasons. Type I cell numbers are decreased relative to type II cells (1), resulting in low yield. They require higher digestive enzyme concentrations to isolate them from surrounding matrix (9). Investigators have used techniques including tissue enzyme digestion, followed by density gradient centrifugation methods (9) or, alternatively, combinations of gradient centrifugation and magnetic beads to remove type II cells from type I cells (6). Such methods generally necessitated immediate analysis of the isolated type I cells. There are few reports of long-term culture of type I cells (5). This has prompted searches for alternative models. Most popular has been a model of transdifferentiation, based on the repeated observation that adult type II cells on tissue culture plastic lose the type II cell phenotype (19) and gain type I cell markers (3,10). The model outlined in this report is the first description of successfully applying this technique to human fetal cells.

Poor functional markers have also complicated studies of type I cell biology. Although recent reports suggested that type I cells are an important source of antioxidants (20,21), two established physiologic functions of the type I cell are gas exchange and serving as a barrier to fluid transport. Barrier function has been evaluated by characterizing ion and water channels (22,23), micromolecular permeability (24), transepithelial resistance (8), and electrophysiologic measurement of ion channel activity (25). Claudins are an important mediator of this barrier function (26). Human fetal lung expresses claudins 1, 3, 4, 5, 7, and 18 (16), and changes in claudin expression and cellular localization correlate with changes in alveolar epithelial cell permeability (15). In our model, claudin 7 expression did not significantly change with transdifferentiation. The observed increase in barrier function may be mediated by changes in claudin 7 localization or by other claudins and/or junctional proteins not yet examined. It is important to note that the observed tighter alveolar epithelial barrier with transdifferentiation concurs with rat transdifferentiation models (27). Although our measured TERs were lower than in the rat studies, they are similar to TER measurements from cultured human airway epithelial cells (28).

Studies of type I cell biology have been hampered by a lack of reliable markers that distinguish type I cells from type II cells. Recently, newer specific type I cell markers have come from adult rodent (9,18) and adult human (29) models. In our studies, multiple type I markers were induced with transdif-

ferentiation from the type II cell to the type I cell phenotype, with Cav-1 and PAI-1 being the most robust indicators of transdifferentiation. However, not all purported type I cell markers were induced in our model. Although we establish that a reliable panel of markers signifying a type I cell–like phenotype is expressed in human transdifferentiating cells, our results suggest that studies of type I cells should incorporate multiple markers as no single marker may accurately reflect changes in phenotype.

Cav-1 is a component of caveolae, plasma membrane invaginations that are lipid rich and involved in signaling, intracellular trafficking, and protein sorting (30). It exists in two isoforms, alpha and beta (31). In the mouse lung, the alpha isoform is present in both endothelium and type I epithelial cells. Our model concurs with other nonhuman models in which Cav-1 appears with transdifferentiation toward a type I cell–like phenotype (10,18). However, our model differs with respect to two previous observations. Other investigators noted no Cav-1α in fetal or neonatal alveolar epithelium (32), and no Cav-1 in adult rat type II cells (33). We observed expression of Cav-1α in all treatment groups, albeit with the highest expression seen in the type I–like transdifferentiated cells. Although this may reflect species-specific differences, our studies suggest that fetal alveolar epithelium is more multipotential than previously suspected.

PAI-1 was the most induced type I cell marker in our model. It is a serine protease inhibitor participating in fibrinolysis in many tissues, including the lung. Hyperoxic injury up-regulates PAI-1 (34) in murine models and mice lacking the PAI-1 gene have a decreased fibrotic response to bleomycin (35). PAI-1 is elevated in preterm infants with respiratory distress syndrome (RDS) and may play a contributory role in bronchopulmonary dysplasia (36). The robust up-regulation of PAI-1 with transdifferentiation seen here supports the likely important role of PAI-1 in tissue repair in the alveolar microenvironment.

The transition between epithelial cell phenotypes in our model was not complete by the end of the culture period, and there was baseline expression of many markers by untreated control cells. With transdifferentiation, type II markers were still evident, albeit reduced to less than 10% of controls. The incompletely flattened appearance (Fig. 1D) of our transdifferentiated cells may be due to culture conditions and agrees with previous reports (3). Not all microvilli and lamellar bodies were lost by 96 h, as in prior reports. Thus, transdifferentiated cells, although type I–like, have a somewhat intermediate phenotype. Work by others suggests differences between freshly isolated type I cells and transdifferentiated cells (21). It is important to acknowledge that transdifferentiation is an imperfect model for studying type I cells, but remains a powerful tool for initial investigations into type I cell biology, until more reliable primary culture methods for type I cells are available.

Our data have important implications regarding the developmental lineage of type II and type I cells. Evidence from adult animal models suggests that type I cells are derived from type II cells (19,37), as type II cells are progenitors of type I cells after alveolar injury in mature lung (11) and isolated type II cells in culture tend to lose characteristics of type II cells, adopting a more type I–like appearance (7). However, *in vitro* (38) and *in vivo* (39) evidence supports the plasticity of alveolar epithelial cell phenotype (38,40). Our data support the prevailing concept that human fetal type II cells can give rise to type I–like cells. Our data also support the notion of epithelial cell plasticity; given the observation of some type I cell markers in our type II cells. However, our findings of type I cell markers with selected type II cell markers in untreated cells imply that our starting cell population is far from undifferentiated and advocates a role for greater plasticity in fetal alveolar progenitor epithelial cells than has been classically appreciated.

In summary, we have shown that under defined conditions, human fetal alveolar type II cells transdifferentiate, with regression of type II cell morphologic and phenotypic markers. Transdifferentiation is further associated with increased expression of type I cell markers and increased alveolar epithelial cell barrier function. This model will serve as a useful tool for further study of human fetal alveolar epithelial cell differentiation and type I cell biology.

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