ORIGINAL RESEARCH

Breaking the *In Vitro* Alveolar Type II Cell Proliferation Barrier while Retaining Ion Transport Properties

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Abstract

Alveolar type (AT)I and ATII cells are central to maintaining normal alveolar fluid homeostasis. When disrupted, they contribute to the pathogenesis of acute lung injury (ALI) and acute respiratory distress syndrome. Research on ATII cells has been limited by the inability to propagate primary cells *in vitro* to study their specific functional properties. Moreover, primary ATII cells *in vitro* quickly transdifferentiate into nonproliferative "ATI-like" cells under traditional culture conditions. Recent studies have demonstrated that normal and tumor cells grown in culture with a combination of fibroblast (feeder cells) and a pharmacological Rho kinase inhibitor (Y-27632) exhibit indefinite cell proliferation that resembled a "conditionally reprogrammed cell" phenotype. Using this coculture system, we found that primary human ATII cells (*1*) proliferated at an exponential rate, (*2*) established epithelial colonies expressing ATII-specific and "ATI-like" mRNA and proteins after serial passage, (*3*) up-regulated genes important in cell proliferation and migration, and (*4*) on removal of feeder cells and Rho kinase inhibitor under air–liquid interface conditions, exhibited bioelectric and volume transport characteristics similar to freshly cultured ATII cells. Collectively, our results demonstrate that this novel coculture technique breaks the *in vitro* ATII cell proliferation barrier while retaining cell-specific functional properties. This work will allow for a significant increase in studies designed to elucidate ATII cell function with the goal of accelerating the development of novel therapies for alveolar diseases.

Keywords: proliferation; transdifferentiation; ion transport

Clinical Relevance

We describe a novel coculture technique that breaks the *in vitro* alveolar type II cell proliferation barrier while retaining cell-specific functional properties. This technique increases the supply of human primary alveolar type II cells and allows for additional studies to be performed focused on important biological and functional processes relevant to the physiology and pathophysiology of alveolar lung diseases.

It is critical that the alveolar epithelium maintains a thin liquid layer lining to promote proper surface tension, gas exchange, and protection from inhaled toxins and pathogens. Alveolar type (AT)I and ATII epithelial cells are damaged during inflammation associated with acute lung injury (ALI) and acute respiratory distress syndrome (1–4). Resolution of ALI through removal of alveolar edema fluid has been the focus of many *in vitro* and *in vivo* studies (5–7). However, one consistent limitation of these studies has been that ATII cells *in vitro* quickly transdifferentiate (i.e., lose their ATII cell–specific markers and gain "ATI-like"–specific markers) and do not proliferate under traditional culture conditions (8–10). The failure of human primary ATII cells to proliferate greatly limits the number of studies designed to

elucidate the pathogenesis of human alveolar diseases.

Previous attempts have been made to promote ATII cell proliferation *in vitro*. *In vivo*, fibroblasts reside beneath alveolar lung epithelia and make contact with ATI and ATII cells through gaps within the basement membrane (11). Fibroblasts secrete important soluble factors (e.g., keratinocyte growth factor [KGF]) that have been reported to limit transdifferentiation and to promote ATII cell proliferation (11–15). ATII cells cocultured with human or rat fibroblasts or exposed to KGF exhibit surfactant mRNA expression *in vitro* for extended periods, suggesting that KGF (added or presumably secreted by fibroblasts) was in part responsible for retaining ATII cell differentiation *in vitro* (12). These studies also reported that KGF could stimulate ATII cell proliferation (14, 16, 17), which could be antagonized by the transforming growth factor (TGF)-β (18). However, a precise genetic characterization of the proliferating cells was not reported, nor were KGF-treated ATII cells amendable to serial passage and expansion.

Recent published work has demonstrated the ability of human cells (e.g., keratinocytes and airway epithelial cells) to proliferate indefinitely, without the transduction of exogenous viral or cellular genes, by addition of a pharmacological inhibitor of the Rho kinase signaling pathway (Y-27632, "Y") (19–22) in the presence of mouse feeder cells (23). These "conditionally reprogrammed cells" were shown to exhibit a stem cell–like phenotype with an up-regulation of adult stem cell genes (e.g., α6/β1 integrin, ΔNp63α) (24). Passaged CRCs could revert to their original epithelial cell phenotype on removal of the feeder cells and "Y." This recently developed cell culture technology has the potential to accelerate alveolar epithelial research by expanding the availability of human alveolar cells and, thus, increasing cell-specific studies designed to target therapeutics against ALI and acute respiratory distress syndrome.

We tested the hypothesis that primary human ATII cells cocultured with feeder cells and "Y" would exhibit a break in the *in vitro* ATII cell proliferation barrier and undergo serial passage and expansion. Due to the ATI transdifferentiation that affects most ATII cells *in vitro*, we genetically characterized the expanded cells on tissue culture plastic and after culture on an air–liquid interface by mRNA sequencing analyses. Finally, using electrophysiological and imaging techniques, we explored whether human ATII cells that have undergone serial passage and removal of feeder cells and whether "Y" would be useful for studies of ion transport processes that mediate normal and alveolar fluid balance and responses to pulmonary edema.

Materials and Methods

Additional experimental details are available in the online supplement.

Human ATII Isolation
Primary ATII cells were isolated from human lungs as previously described with slight modifications (29).

Cell Culture
The coculturing technique used in this study was as previously described (23) with slight modifications.

ATII Proliferation Assay
Freshly isolated or passaged ATII cells were grown with feeder cells and "Y" on rat tail collagen-1–coated 12- or 24-well plates in "base media" as described above. After 72 hours, cells were incubated with the Click-iT EdU labeling reagent, and the cells were fixed and imaged following the manufacturer's protocol (Invitrogen, Carlsbad, CA).

RT-PCR
Total RNA was extracted from freshly isolated, freshly cultured, or passaged ATII cells under cocultured conditions as previously described (29).

Immunofluorescence
Freshly isolated or passaged ATII suspensions (cytospins) were used for immunocytochemistry analysis as described previously (29). Cells were probed with antibodies against the HTII-280 protein (a gift from Dr. Leland Dobbs, San Francisco, CA), AQP-5 (Santa Cruz Biotechnology, Santa Cruz, CA), SP-A (Santa Cruz), SP-C (Santa Cruz), or podoplanin (Santa Cruz).

Alveolar Surface Liquid Height Measurements
Alveolar surface liquid (AvSL) was imaged and calculated as previously described (29).

Data Expression and Statistical Analysis
All experiments were performed in triplicate, and cells were obtained from at least three different patient specimens. Data are presented as means ± SD and were analyzed by one-way ANOVA. Differences were considered significant if $P < 0.05$.

Results

Feeder Cells and Rho Kinase Inhibitor Induce ATII Cell Proliferation and Expansion *In Vitro*
Figure 1A depicts the *in vitro* coculture cell model in which isolated primary human ATII cells from rejected donated lungs were cultured with irradiated feeder cells (1:3) and "Y" and expanded on rat tail collagen-1–coated plastic dishes. To determine the contribution of each component to the culture process, ATII cells were plated with base media alone (Figure 1Bi), with "Y" (Figure 1Bii), with feeder cells (Figure 1Biii), or with the combination of feeder cells and "Y" (Figure 1Biv) and imaged 4 days after seeding. Human ATII cells plated with base media alone did not proliferate and formed large, round, flat, "ATI-like" cells, as previously reported (25). In contrast, ATII cells plated with the combination of feeder cells and "Y" generated islands of epithelial-like colonies surrounded by feeder cells. ATII cells plated with feeder cells or "Y" alone did not establish the well-formed epithelial colonies observed when grown under the feeder and "Y" combination.

Human ATII cells plated with base media alone (Figure 1Ci) or with the combination of feeder cells and "Y" were imaged 8 days after seeding (Figure 1Cii). The coculture technique, but not base media alone, gave rise to a marked increase in the number of epithelial-like cells within 8 to 10 days. Cells were trypsinized at approximately 90% confluence and replated (passaged) on rat tail collagen 1–coated plastic dishes in the presence of feeder cells and "Y" for serial passage (p1–p3) (Figure 1Ciii–v), and cell number was recorded approximately 8 to 10 days after passaging. Initially, 2.5×10^5 ATII cells were plated on each p60 plastic dish, and approximately 4.0×10^6 cells were counted 8 to 10 days later. Approximately the same number of cells was counted after each passage (Figure 1D). A plot of
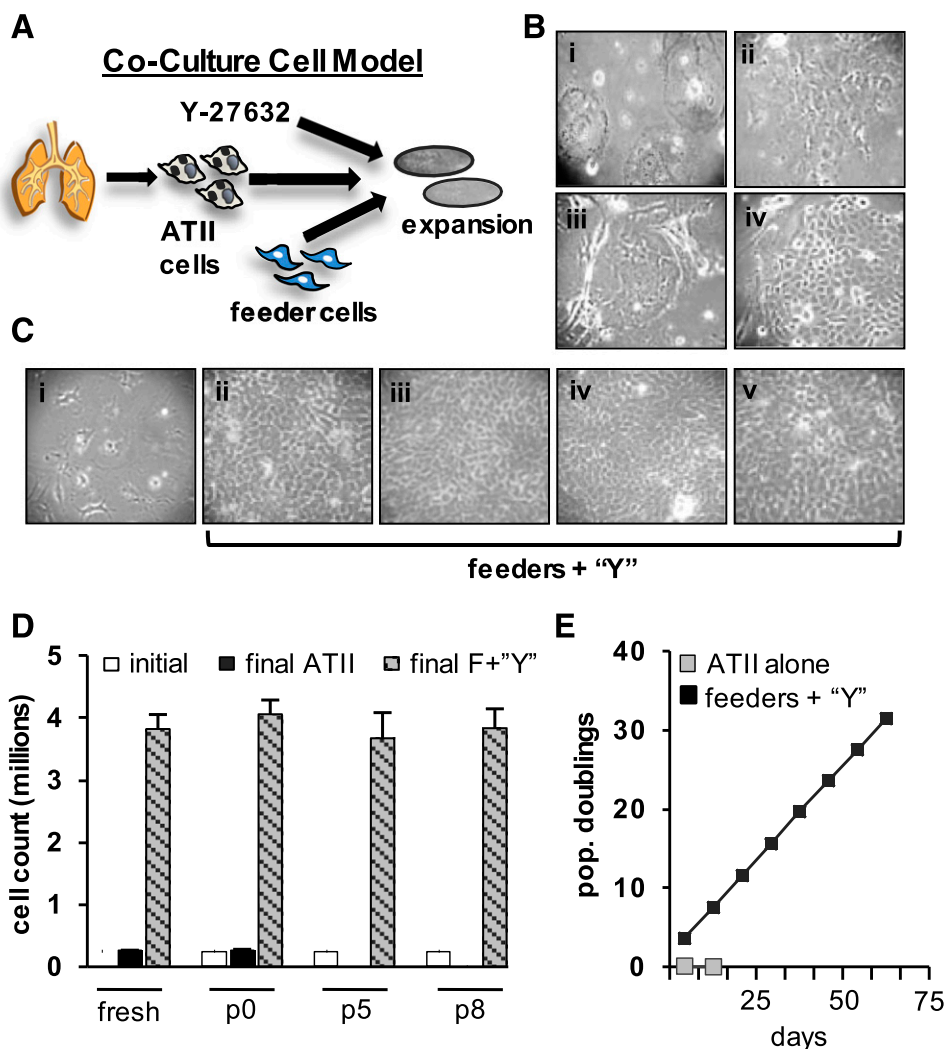
Figure 1. Expanding primary human alveolar type (AT)II cells using feeder cells and a pharmacological inhibitor of the Rho kinase signaling pathway (Y-27632, "Y"). (A) Schema illustrates the recently developed coculture. (B) ATII cells (2.5×10^5) were grown with base media alone (i), with "Y" (10 μM) (ii), with feeder cells (7.5×10^5) (iii), or with combination of feeder cells and "Y" (iv) and imaged 4 days after seeding. (C) Fresh ATII cells were grown on rat tail collagen-1–coated plastic dishes with base media alone (i) or with feeder cells and "Y" (ii) and imaged 10 days after seeding. Cells were trypsinized and further passaged with feeder cells and "Y" (p1–p3; iii–v). (D) Total cell count was recorded for ATII cells plated alone (final ATII) or in combination with feeder cells + "Y" (final F + "Y") up to passage 8 (p8), and a plot of population doublings versus time (days) was constructed (E). All images were taken using phase contrast microscopy under 20× objective. Values are mean ± SD ($n \geqslant 4$ different lungs).

population doublings for ATII cells grown with base media alone or with feeder cells and "Y" revealed that ATII cells grown with feeder cells and "Y" reached approximately 31 population doublings by 63 days after original seeding (Figure 1E).

Characterization of Proliferating Human Primary ATII Cells

The Click-iT EdU cell proliferation assay was used to identify newly synthesized cellular DNA. ATII cells were plated onto 12-well dishes with base media alone (Figure 2Ai) or with feeder cells and "Y" (Figure 2Aii). Cell proliferation (green) and

nuclei (blue) were imaged with fluorescence microscopy 72 hours after seeding. ATII cells grown in base media alone did not show evidence for cell proliferation, whereas a significant population of ATII cell colonies cocultured with feeder cells and "Y" stained positive for the proliferation marker.

To analyze the phenotype of the proliferating cells, the Click-iTEdU assay was combined with an antibody targeted against an apical epitope on ATII cells (HTII-280) (26) and imaged using confocal microscopy. Fresh ATII cells plated with base media alone failed to exhibit evidence

of cell proliferation (green) despite the presence of HTII-280–positive (red) ATII cells (Figure 2Bi). In contrast, a significant portion of freshly isolated ATII cells plated with feeder cells and "Y" dually stained for DNA synthesis and HTII-280 (Figure 2Bi).

Further attempts were made to characterize the passaged (p2) ATII cells that were proliferating under these coculture conditions. Using a pan-cytokeratin antibody to identify epithelial cells, the majority of the proliferating cells stained positive for the pan-cytokeratin antibody (Figure 2Ci). Using three different antibodies (HTII-280, SP-A, or SP-C) to identify the presence of
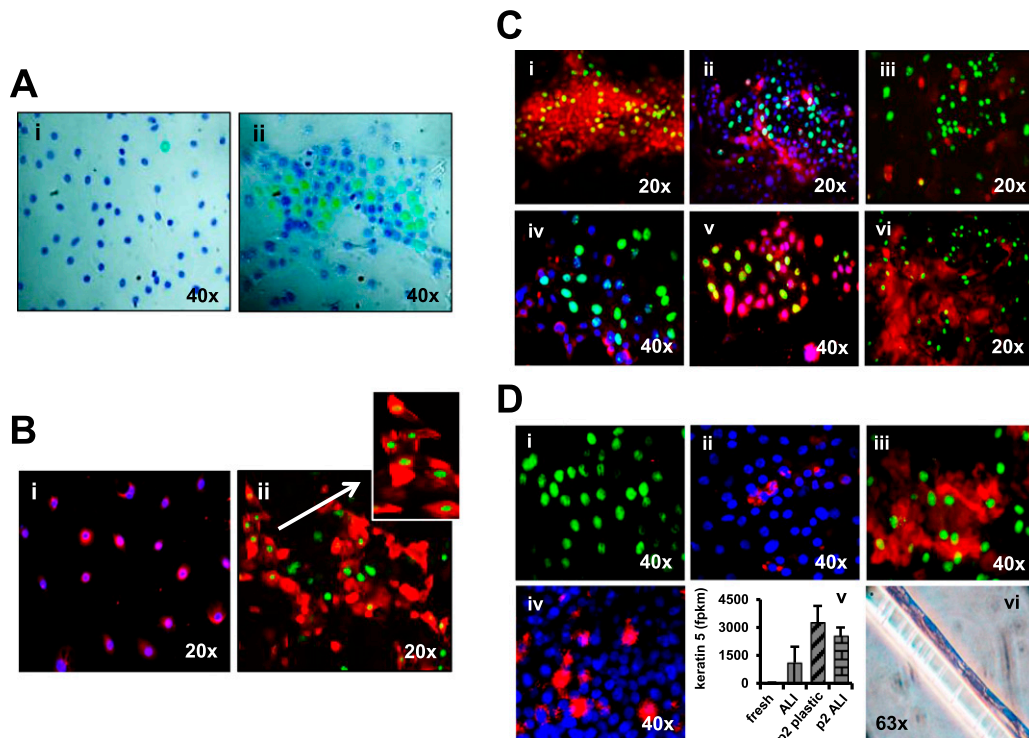
Figure 2. Feeder cells and "Y" promote ATII cell DNA synthesis. ATII cell characterization and DNA synthesis using feeder cells and "Y" coculture conditions. Newly synthesized DNA was examined using the Click-iT EdUAlexa Fluor 488 cell proliferation kit. (*A*) Freshly isolated human ATII cells were plated onto rat tail collagen-1 plates with base media alone (i) or with feeder cells and "Y" (ii) for 72 hours, and proliferating cells (*green*) and cell nuclei (*blue*) were imaged using confocal microscopy under 40× objective. (*B*) Freshly isolated ATII cells, treated with the Click-iT EdU Assay, were probed for an ATII membrane protein (HTII-280, *red*) by antibody detection to identity proliferating cells (*green*) in base media alone (i) or grown with feeder cells and "Y" (ii). (*C*) Passaged (p2) ATII cells expressed proliferating positive cells (*green*) and were colabeled with a pancytokeratin antibody (i), antibodies against ATII cell markers (HTII-280 [ii], SP-A [iii], SP-C [iv]), or antibodies against ATI markers (podoplanin [v], AQP-5 [vi]). (*D*) ATII cells were probed using an antibody for keratin 5 under the following conditions: freshly isolated with feeder cells and "Y", (ii) freshly cultured ATII cells under air-liquid interface conditions, (iii) p2 ATII cells with feeder cells and "Y", or (iv) p2 ATII cells cultured under air-liquid interface conditions. RNA deep sequencing analyses (FPKM value) for keratin 5 expression of all four conditions (v). Hematoxylin and eosin staining was performed on p2 ATII air-liquid interface cultures revealing flat nonciliated single cell monolayers (vi). Values are mean ± SD (*n* ⩾ 3 different lungs).

proliferating ATII cells at p2, we noted that very few of the proliferating cells were positive for ATII cell markers (Figure 2Cii–iv), suggesting that the transdifferentiation process may be retained in passaged cells. To test this hypothesis, we colabeled p2 ATII cell feeder plus "Y" cultures with two different antibodies against ATI markers (podoplanin and aquaporin-5 [AQP-5]) and imaged cells using fluorescence microscopy. A significant number of the proliferating positive cells also stained positive (red) for podoplanin and AQP-5 (Figures 2Cv and 2Cvi), providing evidence that these cultures (p2) had transdifferentiated and that ATI-like cells were presumably the cells that were proliferating. Transdifferentiated ATI-like cells did not proliferate when grown with base media alone (Figure 2D).

Recent evidence from Kumar and colleagues (27) demonstrated that distal airway stem cells can lead to alveoli lung structure regeneration. These p63/keratin 5–expressing distal airway stem cells were shown to assemble into "pods" that exhibited expression of markers typical of alveolar epithelial cells. To explore expression of keratin 5 in proliferating ATII cells, we measured RNA and protein expression in freshly isolated, passaged (p2), and air-liquid interface differentiated ATII preparations. First, we observed that freshly isolated primary ATII cells that stained positive for proliferation (Click-iT EdU positive [green]) were negative for keratin 5 (Figure 2Di), suggesting the absence of contaminating basal cells in the initial ATII isolation preparations. We identified an increase in keratin 5–positive cells when ATII cells were cultured under air-liquid interface conditions (Figure 2Dii), in passaged (p2) ATII cells grown with feeder cells

and "Y" (Figure 2Diii), and in passaged (p2) cells cultured under air-liquid interface conditions (Figure 2Div). Deep sequencing analyses of RNA isolated from all four conditions mimicked the protein expression pattern of keratin 5 (Figure 2Dv). Hematoxylin and eosin staining was performed on fixed passaged (p2) ATII cells cultured on membrane supports for 5 days under air-liquid interface conditions (Figure 2Dvi). No ciliated cells were observed in these monolayers.

Coculture Conditions Do Not Inhibit ATII Cell Transdifferentiation

Semiquantitative PCR analyses were performed on mRNA from freshly isolated ATII cells and from passaged ATII cells (p0–p2) grown with feeder cells and "Y" (Figure 3A). Surfactant protein (SP)-A mRNA was expressed in freshly isolated
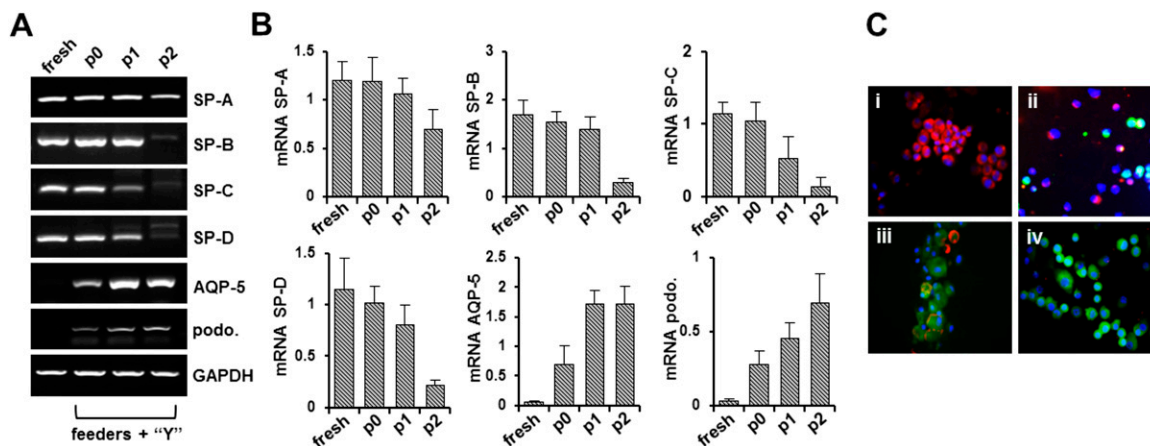
Figure 3. Fresh or passaged human ATII cells mRNA and protein expression. (*A*) mRNA expression of ATII cell–specific surfactant proteins (SP-A, -B, -C, -D) and markers for ATI /"ATI-like" cells (AQP-5 and podoplanin) were examined by semiquantitative PCR. (*B*) Quantitation of gene expression profiles by ImageJ analysis was performed in freshly isolated or passaged ATII cultures grown with feeder cells and "Y" (normalized against glyceraldehyde 3-phosphate dehydrogenase [GAPDH]). (*C*) Immunocytochemistry was performed on cytospin preparations of fresh (i), freshly cultured (ii), or passaged ATII cells grown with feeder cells and "Y" (p1; iii) or (p2; iv). Cells were probed using an ATII-specific antibody (HTII-280, *red*), an ATI/"ATI-like" cell-specific antibody (AQP-5, *green*), and nuclei stain (Hoechst, *blue*) to characterize cells at each stage by confocal microscopy using a 40× objective. Values are mean ± SD ($n \geqslant 3$ different lungs).

ATII cells and appeared to decrease slightly with cell passaging. SP-B, -C, and -D mRNA levels were also expressed through early cell passage (p1) but decreased through additional cell passing (p2). In parallel, we examined the expression of RNA transcripts identified as present in ATI and "ATI-like" cells (28). AQP-5 and podoplanin mRNA expression were absent in the freshly isolated ATII preparations, suggesting little or no ATI cell contamination. However, after ATII cells were passaged once under the coculture condition, AQP-5 and podoplanin expression was observed and increased as cells underwent serial passage. Expression

of RNA was performed by RT-PCR within freshly isolated and passaged ATII cells and confirmed these relationships (Figure 3B). Moreover, a more quantitative approach was explored using deep sequencing analyses. The expression profiles revealed similar patterns for freshly isolated ATII cells versus passaged (p2) cells (Table 1).

Cytospin preparations of freshly isolated ATII cells and cells grown with feeder cells and "Y" (p0–p2) were examined by immunocytochemistry using antibodies against the ATII cell membrane protein (HTII-280) and the ATI cell marker (AQP-5). Freshly isolated cells (Figure 3C,

i) expressed the HTII-280 (red) ATII cell surface protein, which decreased on serial passage. In contrast, AQP-5 (green) was not expressed in freshly isolated ATII cells. However, expression increased as cells underwent serial passage (Figure 3Cii–iv). This pattern was recapitulated in the deep sequencing analyses (Table 1). Collectively, these data suggest that the ATII cell transdifferentiation process persisted in the presence of feeder cells and "Y."

Gene Expression Profile by RNA Deep Sequencing Analyses
To further characterize the effects of feeder cells and "Y" at the gene expression level

Table 1: Differentially Expressed Genes by RNA Deep Sequencing Analyses

Gene ID	Gene Description	Fresh ATII Cells	Air-Liquid Interface Cultures	Passaged (p2) ATII Cells	Passaged (p2) Air-Liquid Interface Cultures
SFTP-A1*	Surfactant, pulmonary-associated protein, A1	7,879 ± 2,887[†]	4,275 ± 2,078	259 ± 244	22 ± 9
SFTP-A2*	Surfactant, pulmonary-associated protein, A2	9,872 ± 4,049	3,664 ± 2,830	63 ± 29	126 ± 59
SFTP-B*	Surfactant, pulmonary-associated protein, B	33,827 ± 28659	5,025 ± 1,853	158 ± 142	42 ± 28
SFTP-C*	Surfactant, pulmonary-associated protein, C	14,082 ± 10,060	110 ± 57	68 ± 47	7.1 ± 6.6
SFTP-D	Surfactant, pulmonary-associated protein, D	811 ± 238	109 ± 28	17 ± 14	2.8 ± 1.9
AQP-5	Aquaporin-5	26 ± 8.6	30 ± 11	286 ± 78	107 ± 19
PDPN	Podoplanin	8.2 ± 4.3	30 ± 12	37 ± 10	35 ± 15
CFTR	Cystic fibrosis transmembrane conductance regulator	8.6 ± 3.9	7.5 ± 2.0	7.5 ± 5.7	3.4 ± 0.3
SCNN1α	Sodium channel, non–voltage-gated 1α subunit	114 ± 21	279 ± 63	84 ± 3.8	111 ± 20
SCNN1β	Sodium channel, non–voltage-gated 1β subunit	21 ± 5.6	78 ± 16	6.1 ± 0.6	12 ± 1.4
SCNN1γ	Sodium channel, non–voltage-gated 1γ subunit	4.1 ± 1.2	11 ± 3.7	0.4 ± 0.1	1.6 ± 0.1

Definition of abbreviations: ATII, alveolar type II; p2, passage 2.
*Due to fragment per kilobase of exon per million fragments mapped (FPKM) values above limit of detection, reads per kilobase of transcript per million mapped (RPKM) values are presented.
[†]Values are average FPKM or RPKM.

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of human ATII cells, RNA deep sequencing analyses were performed to identify and compare gene expression profiles from the lungs of three separate individuals in (1) freshly isolated ATII cells, (2) freshly isolated ATII cells grown on porous supports for 5 days under air-liquid interface conditions (no feeder cells and no "Y"), (3) passaged (p2) ATII cells grown on plastic with feeder cells and "Y," and (4) passaged (p2) ATII cultures grown under air-liquid interface conditions for 5 days with no feeder cells and no "Y" (Figure 4B).

The number of genes expressed in ATII cells was filtered by analyzing only signals with a fragment per kilobase of exon per million fragments mapped (FPKM) $\geq$ 10. Using these parameters, over 9,000 total genes were identified. As a test of our freshly isolated ATII cells, the 25 most abundant genes expressed in our ATII cells (see Table E2 in the online supplement) were tabulated and compared with a previous report (28), with reasonable concordance ($\sim$ 60%).

Hierarchical clustering heat maps of all expressed genes revealed a clear pattern (Figure 4A). Overall, fresh ATII cells (cultured on plastic or grown under air-liquid interface conditions) clustered together relative to feeder cell and "Y" passaged (p2) cells grown on plastic or air-liquid interface, indicating a large overall effect of feeder cells and "Y" on global gene expression. For fresh ATII cells, air-liquid interface cultures clustered together and were distinguishable from freshly isolated cells. However, for p2 cultures, the gene expression clustered by individual donor specimen, not plastic versus air-liquid interface culture condition, suggesting that the feeder cells and "Y" culture system dominated over culture condition.

To gain insight into which cell phenotype (i.e., fresh ATII versus "ATI-like" after transdifferentiation) proliferated in feeder cells and "Y" media, Pearson product-moment correlation coefficients for gene expression were measured among the four different cell culture conditions (Figure 4B). We observed that (1) fresh ATII cells cultured under air-liquid interface conditions were correlated with freshly isolated ATII cells at an 0.82 level, the difference reflecting "ATI-like" cell transdifferentiation; (2) passaged (p2) cells grown on plastic were more like freshly isolated ATII cells under air-liquid interface conditions (0.77) than freshly isolated ATII cells (0.65); and (3) passaged (p2) cells under air-liquid interface conditions were more like fresh ATII cells under air-
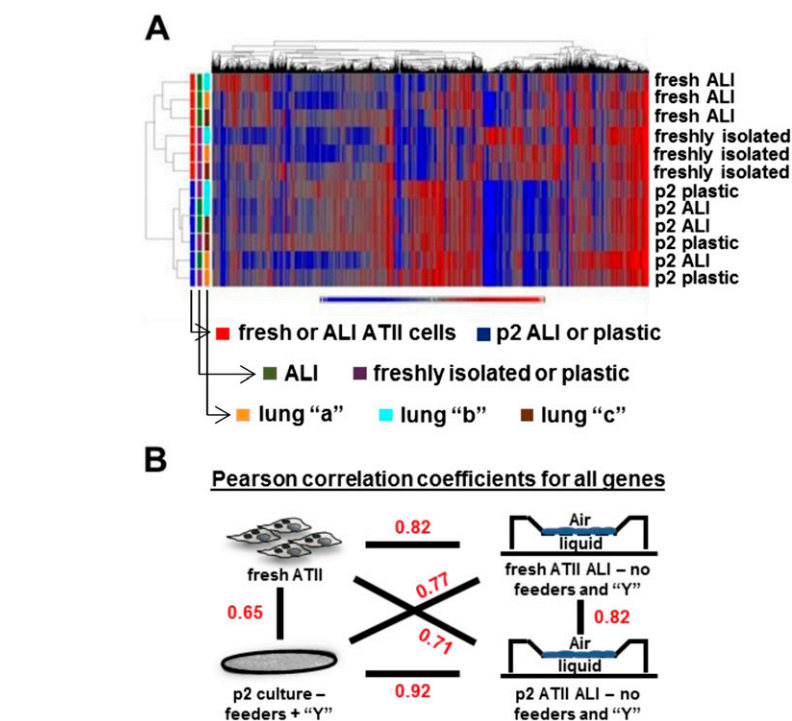

Figure 4. RNA deep sequencing analyses of human ATII cells. (A) A heat map of differentially expressed genes is displayed between fresh ATII cells versus passaged (p2) ATII cells (with feeder cells and "Y") and fresh versus p2 air-liquid interface cultures 5 days after seeding. (B) Pearson correlation coefficients for all genes between all four conditions were calculated.

liquid interface conditions (0.82) than freshly isolated ATII cells (0.71). The principal component analysis for all conditions (see Figure E1) demonstrated similar relationships. These data suggest that the p2 cells on plastic with feeder cells and "Y" and the p2 cells under air-liquid interface conditions were more like "AT-I like" transdifferentiated cells rather than freshly isolated ATII cells.

Enrichment pathway analyses for passaged (p2) ATII cells grown on plastic with feeder cells and "Y" versus freshly isolated ATII cells revealed up-regulation of cellular pathways involved in epithelial development, adhesion pathways, signaling, and epithelial cell differentiation in p2 cells (Table E2). A few of the top differentially expressed genes within these pathways included CD24, CDK6, CDK8, CDK18, EPH-A1, EPH-B2, MMP-2, MMP-14, and ADAM-9. These molecules are associated with stemness, growth control, cell cycle progression, cell migration, cell–cell interaction, and remodeling, all indicative of the highly proliferative and migratory behavior of the passaged ATII cells. In addition, we identified the top down-regulated biological processes between passaged (p2) ATII cells versus

freshly isolated ATII cells. The lipid metabolism pathway (a highly relative process for ATII cells) was significantly down-regulated (P value, $5.49E^{-05}$) in p2 ATII cells grown on plastic with feeders plus "Y" compared with freshly isolated ATII cells. In addition, gene expression analyses (FPKM average values) for markers of ATI cells, as well as cystic fibrosis transmembrane conductance regulator (CFTR) and subunits of ENaC (α, β, γ), were performed (Table E1) for all four groups and overall agreed with the RT-PCR data (Figure 3).

Concurrently, we investigated epithelial stem cell marker expression in fresh ATII cells versus passaged ATII cells grown with feeder cells and "Y." Recent work demonstrated a rapid induction of epithelial stem cell markers when primary cells were cultured under these novel coculture conditions. The $\alpha6$ and $\beta1$ integrins, reported to play a significant role in colony forming efficiency and fast attachment to the basal membrane matrix (24), were expressed 13-fold and 4.4-fold higher in ATII cells cultured with feeder cells and "Y," respectively, than in freshly isolated ATII cells. The $\Delta Np63\alpha$ transcription factor (required for the maintenance of basal stem cells) was up-regulated 48-fold (Table 2).

Passaged ATII Cells Retain Ion Transport Function

Fresh isolated (red, "p0") or passaged ATII cells cultured with feeder cells and "Y" (black, "p2") were plated onto rat tail collagen-1–coated supports and grown under air-liquid interface conditions without feeder cells and without "Y." Basal transepithelial resistances (TER, ~ 450 $\Omega \cdot cm^2$) and short-circuit currents (I_{sc}, ~ 15 $\mu A/cm^2$) were measured 5 days after seeding and were found to be similar between fresh and passaged ATII–air-liquid interface cultures (Figure 5A).

Different sequences of administration of Na^+ and Cl^- transport inhibitors were used to investigate the components of I_{sc}. The ENaC inhibitor amiloride (10^{-4} M) was used to measure the basal contribution of ENaC-mediated Na^+ absorption (Figure 5B), and the inhibitor $CFTR_{inh}172$ (10 μM) was used to estimate basal CFTR-mediated Cl^- secretion (Figure 5D). Like freshly cultured ATII cells, passaged (p2) ATII cells under air-liquid interface conditions exhibited significant amiloride-sensitive currents (Figure 5C). Basal CFTR Cl^- secretion constituted a smaller component of basal I_{sc} in fresh and passaged ATII cultures (Figure 5E).

Uridine-5′-triphosphate (UTP), a major endogenous regulator of ATII ion transport (29), was added to amiloride-pretreated freshly cultured and passaged ATII cells (Figure 5B). UTP (10 μM) stimulated a transient Cl^- secretion in both cell types (Figure 5C). When cells were pretreated with the CFTR inhibitor, UTP-stimulated Cl^- secretion was inhibited, suggesting that CFTR mediated UTP-stimulated Cl^- secretion in both culture conditions (Figure 5E). To identify the potential role of Ca^{+2}–activated chloride channels in Cl^- secretion, a nonselective Cl^- transport inhibitor (trifluoromethylphenol, 50 μM), was added

to fresh and passaged ATII–air-liquid interface cultures (Figures 5B and 5D) after CFTR inhibition. A modest inhibition in I_{sc} was detected in fresh and passaged ATII cultures (Figures 5C and 5E).

To investigate whether ENaC-mediated Na^+ transport could be regulated in passaged ATII cells as previously reported in fresh ATII cultures (29), passaged cells were treated with dexamethasone (1 μM) for 24 hours before Ussing chamber analysis (Figure E2). Like fresh ATII cells, basal I_{sc} was significantly higher in passaged ATII cells treated with dexamethasone compared with nontreated passaged ATII cells.

To evaluate the gene expression profiles of selected Na^+ (α, β, and γ subunits of ENaC) and Cl^- channels (CFTR), mRNA was isolated from fresh and passaged ATII cells and semiquantitative RT-PCR analysis was performed (Figure 5F). Except for a modest reduction in β- and γ-ENaC at p2, the levels of ENaC and CFTR RNAs expressed in ATII cells cultured with feeder cells and "Y" was similar to freshly isolated ATII cells.

AvSL Regulation in Passaged ATII Cells

Passaged (p2) ATII cells or freshly isolated ATII cells were seeded on supports with no feeder cells and no "Y," subjected to air-liquid interface conditions, and challenged with an apical bolus (30 μl) addition of a 70-kD Texas-red dextran/PBS solution. Images obtained by xz scan confocal microscopy after 24 hours revealed two key observations. First, the rate of liquid absorption was similar for both cell types. Second, both cell types achieved a similar 24-hour steady-state AvSL height (29), suggesting that the homeostatic mechanisms that regulate AvSL balance in fresh ATII air-liquid interface cultures were preserved in passaged ATII cultures (Figures 6A and 6B).

To evaluate extracellular nucleotide contributions to AvSL homeostasis, passaged

(p2) cells were grown under air-liquid interface conditions without feeder cells and without "Y" and treated with UTP (100 μM, luminal), and changes in AvSL height were imaged (Figures 6C and 6D). Passaged (p2) ATII cells exhibited an increase in AvSL height 10 minutes after UTP addition, a secretory response similar to previous reports of freshly cultured ATII cells (29).

Discussion

Over the past 25 years, there has been the consistent observation that primary adult human ATII cells do not proliferate *in vitro* and within a few days in culture transdifferentiate into "ATI-like" cells (8, 10, 30). Little progress has been made on "breaking" the ATII cell proliferation barrier, and the transdifferentiation process can only be partially slowed by growing primary ATII cells under air-liquid interface conditions. Although the transdifferentiation process clearly reduces the capacity for studies of surfactant metabolism (i.e., SP-C, SP-D), it may not affect the ion transport characteristics to the same degree; gene expression profiles of ion transport related genes of freshly isolated versus Day 5 air-liquid interface ATII cells were quite similar (Figure 5F).

To break the ATII/"ATI-like" cell proliferation block, primary human ATII cells ($\sim 2.5 \times 10^5$) were plated with a combination of irradiated feeder cells and "Y", as outlined by Liu and colleagues (23). ATII cells formed proliferating epithelial colonies throughout the dish surrounded by the feeder cells, yielding approximately 4×10^6 cells per passage from 2.5×10^5 originally plated cells (Figure 1D). ATII cells plated under the coculture conditions underwent approximately 31 population doublings within 63 days, whereas ATII cells

Table 2: Differentially Expressed Genes Identified as Markers for "Conditionally Reprogrammed Cells"

Gene ID	Gene Description	Fresh ATII Cells	Passaged (p2) ATII Cells	Passaged (p2) Air-Liquid Interface Cultures
ITGB1	Integrin, β1	73.7 ± 18.3*	313.3 ± 52.6	258.6 ± 89.4
ITGA6	Integrin, α6	8.3 ± 2.6	354.0 ± 40.6	82.7 ± 37.5
ΔNp63α	Tumor protein p63	1.2 ± 0.4	105.3 ± 17.9	34.0 ± 8.9
CD44	CD44 (Indian blood group)	153.3 ± 8.1	118.7 ± 7.1	108.4 ± 31.6
Notch1	NOTCH1	11.4 ± 0.4	18.2 ± 1.9	9.7 ± 1.2
DLL-1	Delta-like 1 receptor	1.5 ± 0.3	11.0 ± 2.9	4.5 ± 1.3

Definition of abbreviations: ATII, alveolar type II; p2, passage 2.
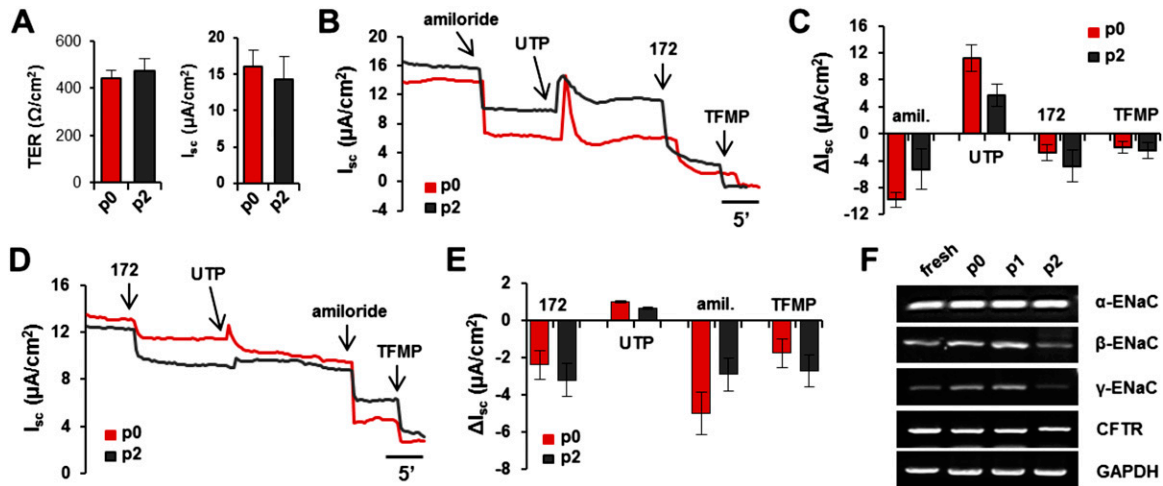*Values are fragment per kilobase of exon per million fragments mapped average values ± SE from three different lungs.

Figure 5. Passaged ATII cultures maintain ion transport properties. (*A*) Passaged (p2) ATII cells in the absence of feeder cells and "Y" exhibited transepithelial resistances (TER, $\Omega \cdot cm^2$) and basal short circuit currents (I_{sc}, $\mu A/cm^2$) similar to fresh (p0) ATII cells. (*B*) I_{sc} of fresh (p0) ATII cultures and passaged (p2) ATII cells were recorded after exposure to the ENaC inhibitor (amiloride, 10^{-4} M), followed by exposure to uridine-5'-triphosphate (UTP) (100 μM), the cystic fibrosis transmembrane conductance regulator (CFTR) inhibitor (172, 10 μM, luminal), and a nonspecific Cl^- channel inhibitor (trifluoromethylphenol [TFMP], 50 μM) in Ussing chambers. (*C*) The changes in I_{sc} (ΔI_{sc}) are summarized. (*D*) ATII cultures (p0 and p2) were treated with the CFTR inhibitor followed by UTP (100 μM), amiloride (10^{-4} M), and TFMP (50 μM). (*E*) ΔI_{sc} are summarized. (*F*) Semiquantitative PCR analysis was performed to identify expression of α-, β-, and γ-ENaC and CFTR in fresh and passaged (p0–p2) ATII cells cocultured with feeder cells and "Y." Values are mean $\pm$ SD ($n \geqslant 3$ different lungs).

plated in base media lacked the capacity to proliferate (Figure 1E).

ATII cells plated with feeder cells and "Y" were positive for the Click-IT EdU proliferation probe (Figure 2A, ii, *green*). ATII cells grown under coculture conditions retained surfactant in RNA expression through p2. Immunofluorescence studies confirmed that a number of the proliferating cells in freshly plated cultures stained positive for an ATII cell membrane protein, HTII-280 (Figure 2Bii). However, ATII cells undergoing serial passage (p2) exhibited a relative decrease in HTII-280 (ii)–, SP-A (iii)–, and SP-C (iv)–positive proliferating cells (Figure 2Bi and ii). Although our data strongly support the notion that the proliferation barrier for ATII cells *in vitro* was "broken" under these coculturing conditions, the proliferating cells with serial passage showed decreased ATII markers, suggesting that transdifferentiated "ATI-like" cells dominated later passages. Indeed, we observed that a significant population of proliferating passaged (p2) ATII cells stained positive for ATI markers (podoplanin and AQP-5), suggesting that the "ATI-like" cell phenotype dominated the proliferation process (Figure 2Cv and vi). Cells plated directly onto plastic dishes that promoted the "ATI-like" cell transdifferentiation did not proliferate, suggesting these coculture conditions can induce proliferation of "ATI-like" cells.

We also noted the appearance of keratin 5 expression in our cultures. Keratin 5 expression first appeared in freshly isolated ATII cultures under air-liquid interface conditions and became more prominent in passaged (p2) cells grown on plastic and under air-liquid
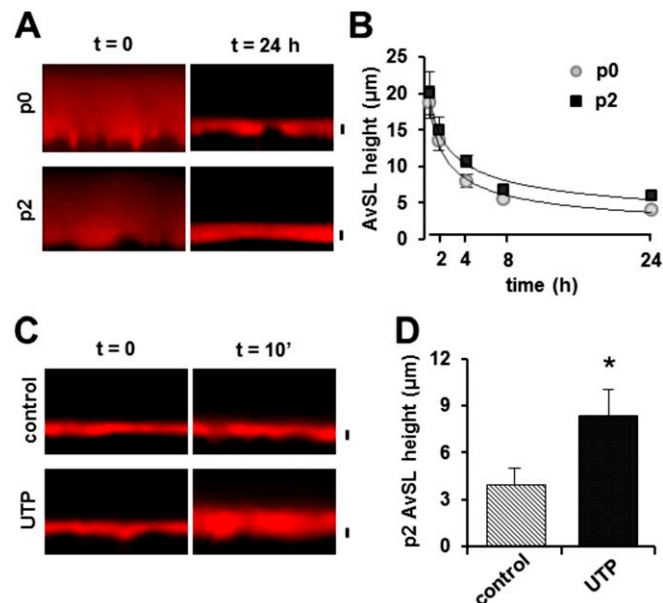

Figure 6. Freshly cultured or passaged ATII cell alveolar surface liquid (AvSL). (*A*) Freshly cultured (p0) and passaged (p2) ATII cells grown on porous supports were exposed to a bolus of a 70-kD dextran fluorophore (Texas red)/PBS solution (30 μl) at 37°C, and AvSL height was measured and imaged (original magnification: ×63) by xz scan confocal microscopy. (*B*) The changes in AvSL height were plotted over 24 hours after the bolus was added. (*C*) Passaged (p2) ATII cells were labeled with a 70-kD dextran fluorophore (Texas red)/PBS solution for 30 minutes at 37°C. The solution was aspirated, and AvSL was imaged (original magnification: ×63) in the absence or presence of UTP (100 μM, luminal) at 0 and 10 minutes by xz scan confocal microscopy. (*D*) Changes in AvSL height were quantitated and plotted. Values are mean $\pm$ SD ($n \geqslant 3$ different lungs) *$P \leqslant 0.05$.

interface conditions. We do not know whether keratin 5 expression reflects "ATI-like" differentiation or is an alveolar stem cell marker. Histological studies confirmed that keratin 5 expression did not reflect contaminating airway basal cells with a capacity for differentiation into ciliated cells (Figure 2Dvi).

RT-PCR analysis demonstrated that fresh ATII cells started losing ATII-specific genes (SP-A, -B, -C, -D) and started to express ATI makers (AQP-5, podoplanin) as early as 5 days under air-liquid interface conditions. This observation was confirmed in the immunofluorescence studies where expression of the ATII cell–specific marker HTII-280 decreased and the ATI cell–specific marker AQP-5 increased with air-liquid interface cultured fresh ATII cells (29). Hierarchical clustering heat maps from RNA deep sequencing analyses revealed that gene expression in cells grown with feeder cells and "Y" was systematically different from gene expression in freshly isolated and cultured cells (Figure 4A). Pearson's coefficients revealed that passaged (plastic and air-liquid interface) cells correlated more tightly with transdifferentiating ATII cells grown at air-liquid interface for 5 days than freshly isolated ATII cells (Figure 4B). From these observations, we conclude that it is likely that the feeder cell and "Y" approach has "broken" the cell cycle block in ATII cells and ATII cells that had transdifferentiated into "ATI-like" cells.

Previously, we demonstrated that freshly isolated human ATII cultures in vitro have the capacity to actively absorb (ENaC-mediated) or secrete (CFTR-dependent) ions and liquid, respectively, in response to local conditions and stimuli (29). We wanted to explore whether ATII cells that have undergone serial passage retained ATII cell–specific activity. Our data revealed that passaged (p2) ATII cells formed confluent monolayers under air-liquid interface conditions with basal resistances of

approximately 450 Ωcm^2 and an I_{sc} of approximately 15 $\mu A/cm^2$, similar to freshly cultured human ATII cells (Figure 5A). Approximately 60% of the basal I_{sc} of freshly cultured ATII cells was sensitive to the epithelial Na^+ channel inhibitor amiloride, whereas approximately 40% of the basal I_{sc} in passaged ATII cells was sensitive to amiloride (Figure 5C). This slightly lower Na^+ current in passaged (p2) cells was restored when these cells were cultured with dexamethasone for 24 hours (Figure E2).

Similar basal CFTR-mediated Cl^- transport rates were observed in freshly cultured and passaged ATII cells (Figures 5C and 5E). UTP-regulated Cl^- secretion in passaged (p2) ATII cells was also similar to freshly cultured ATII cells. The nucleotide-mediated increase in I_{sc} in both cell types was highly dependent on the function of CFTR. The functional data were paralleled by RNA measurements. ENaC subunit mRNA expression (α-, β-, and, γ-ENaC) was expressed similarly in both conditions (Figure 5E and Table E3). We previously demonstrated by mRNA analyses that active Cl^- transport in primary ATII cells is dominated by CFTR (29), and CFTR mRNA levels were similar in freshly isolated and passaged (p2) cells (Figure 5F).

It remains difficult to understand the relationship between ion transport measured in Ussing chambers and the surface liquid physiology within an intact alveolus. Using confocal microscopy, we previously reported that primary human ATII cells balance absorption and secretion to maintain a steady-state surface liquid height of approximately 4 to 5 μm (29). When freshly cultured and passaged (p2) ATII cells were allowed to equilibrate after the addition of a liquid bolus, they exhibited similar AvSL heights at 24 hours (Figures 6A and 6B), suggesting that passaged ATII cells retained the capacity to balance Na^+ versus Cl^- transport to maintain physiologic AvSL

heights. A key luminal regulator of respiratory epithelial surface liquid homeostasis (i.e., the nucleotide UTP) produced AvSL secretion in passaged (p2) feeder cells and "Y" grown cells similar to freshly cultured human ATII cells (Figures 6C and 6D). Finally, passaged ATII cultures exhibited the ability to absorb a "flooded volume" with similar absorptive kinetics compared with freshly cultured cells (Figure 6B). Overall, passaged ATII cultures appear to be an excellent tool to study the processes that regulates alveolar fluid physiology and pathophysiology.

In conclusion, we used a recently developed cell culture technique combining feeder cells with a pharmacological inhibitor of the Rho kinase signaling pathway to "break" the ATII cell cycle block and stimulate primary human ATII cell proliferation for serial passage. Passaged ATII cells formed confluent monolayers under air–liquid interface conditions with basal resistances, Na^+ and Cl^- ion transport properties, and AvSL regulation comparable to freshly cultured cells. This technique will increase the supply of human primary ATII cells and will allow for additional studies to be performed focused on important biological and functional processes relevant to the physiology and pathophysiology of alveolar lung diseases. ■

References

1. Ware LB, Matthay MA. The acute respiratory distress syndrome. *N Engl J Med* 2000;342:1334–1349.
2. Matthay MA, Zimmerman GA, Esmon C, Bhattacharya J, Coller B, Doerschuk CM, Floros J, Gimbrone MA , Jr, Hoffman E, Hubmayr RD, *et al*. Future research directions in acute lung injury: summary of a National Heart, Lung, and Blood Institute working group. *Am J Respir Crit Care Med* 2003;167:1027–1035.
3. Matthay MA, Zimmerman GA. Acute lung injury and the acute respiratory distress syndrome: four decades of inquiry into pathogenesis and rational management. *Am J Respir Cell Mol Biol* 2005;33:319–327.
4. Matthay MA, Ware LB, Zimmerman GA. The acute respiratory distress syndrome. *J Clin Invest* 2012;122:2731–2740.
5. Sartori C, Matthay MA, Scherrer U. Transepithelial sodium and water transport in the lung: major player and novel therapeutic target in pulmonary edema. *Adv Exp Med Biol* 2001;502:315–338.

6. Berthiaume Y, Folkesson HG, Matthay MA. Lung edema clearance: 20 years of progress: invited review: alveolar edema fluid clearance in the injured lung. *J Appl Physiol (1985)* 2002;93:2207–2213.

7. Matthay MA, Fukuda N, Frank J, Kallet R, Daniel B, Sakuma T. Alveolar epithelial barrier: role in lung fluid balance in clinical lung injury. *Clin Chest Med* 2000;21:477–490.

8. Dobbs LG. Isolation and culture of alveolar type II cells. *Am J Physiol* 1990;258:L134–L147.

9. McElroy MC, Pittet JF, Hashimoto S, Allen L, Wiener-Kronish JP, Dobbs LG. A type I cell–specific protein is a biochemical marker of epithelial injury in a rat model of pneumonia. *Am J Physiol* 1995;268:L181–L186.

10. Cheek JM, Kim KJ, Crandall ED. Tight monolayers of rat alveolar epithelial cells: bioelectric properties and active sodium transport. *Am J Physiol* 1989;256:C688–C693.

11. Griffin M, Bhandari R, Hamilton G, Chan YC, Powell JT. Alveolar type II cell–fibroblast interactions, synthesis and secretion of surfactant and type I collagen. *J Cell Sci* 1993;105:423–432.

12. Shannon JM, Pan T, Nielsen LD, Edeen KE, Mason RJ. Lung fibroblasts improve differentiation of rat type II cells in primary culture. *Am J Respir Cell Mol Biol* 2001;24:235–244.

13. Qiao R, Yan W, Clavijo C, Mehrian-Shai R, Zhong Q, Kim KJ, Ann D, Crandall ED, Borok Z. Effects of KGF on alveolar epithelial cell transdifferentiation are mediated by JNK signaling. *Am J Respir Cell Mol Biol* 2008;38:239–246.

14. Mason RJ, Lewis MC, Edeen KE, McCormick-Shannon K, Nielsen LD, Shannon JM. Maintenance of surfactant protein A and D secretion by rat alveolar type II cells *in vitro. Am J Physiol Lung Cell Mol Physiol* 2002;282:L249–L258.

15. Fehrenbach A, Bube C, Hohlfeld JM, Stevens P, Tschernig T, Hoymann HG, Krug N, Fehrenbach H. Surfactant homeostasis is maintained *in vivo* during keratinocyte growth factor–induced rat lung type II cell hyperplasia. *Am J Respir Crit Care Med* 2003;167:1264–1270.

16. Portnoy J, Curran-Everett D, Mason RJ. Keratinocyte growth factor stimulates alveolar type II cell proliferation through the extracellular signal-regulated kinase and phosphatidylinositol 3-OH kinase pathways. *Am J Respir Cell Mol Biol* 2004;30:901–907.

17. Yano T, Mason RJ, Pan T, Deterding RR, Nielsen LD, Shannon JM. KGF regulates pulmonary epithelial proliferation and surfactant protein gene expression in adult rat lung. *Am J Physiol Lung Cell Mol Physiol* 2000;279:L1146–L1158.

18. Zhang F, Nielsen LD, Lucas JJ, Mason RJ. Transforming growth factor-beta antagonizes alveolar type II cell proliferation induced by keratinocyte growth factor. *Am J Respir Cell Mol Biol* 2004;31:679–686.

19. Chapman S, Liu X, Meyers C, Schlegel R, McBride AA. Human keratinocytes are efficiently immortalized by a Rho kinase inhibitor. *J Clin Invest* 2010;120:2619–2626.

20. Emre N, Vidal JG, Elia J, O'Connor ED, Paramban RI, Hefferan MP, Navarro R, Goldberg DS, Varki NM, Marsala M, *et al.* The ROCK inhibitor Y-27632 improves recovery of human embryonic stem cells after fluorescence-activated cell sorting with multiple cell surface markers. *PLoS ONE* 2010;5:e12148.

21. Gauthaman K, Fong CY, Bongso A. Effect of ROCK inhibitor Y-27632 on normal and variant human embryonic stem cells (hESCs) *in vitro*: its benefits in hESC expansion. *Stem Cell Rev* 2010;6:86–95.

22. Pakzad M, Totonchi M, Taei A, Seifinejad A, Hassani SN, Baharvand H. Presence of a ROCK inhibitor in extracellular matrix supports more undifferentiated growth of feeder-free human embryonic and induced pluripotent stem cells upon passaging. *Stem Cell Rev* 2010; 6:96–107.

23. Liu X, Ory V, Chapman S, Yuan H, Albanese C, Kallakury B, Timofeeva OA, Nealon C, Dakic A, Simic V, *et al.* ROCK inhibitor and feeder cells induce the conditional reprogramming of epithelial cells. *Am J Pathol* 2012;180:599–607.

24. Suprynowicz FA, Upadhyay G, Krawczyk E, Kramer SC, Hebert JD, Liu X, Yuan H, Cheluvaraju C, Clapp PW, Boucher RC Jr, *et al.* Conditionally reprogrammed cells represent a stem-like state of adult epithelial cells. *Proc Natl Acad Sci USA* 2012;109: 20035–20040.

25. Gonzalez RF, Allen L, Dobbs LG. Rat alveolar type I cells proliferate, express OCT-4, and exhibit phenotypic plasticity *in vitro. Am J Physiol Lung Cell Mol Physiol* 2009;297:L1045–L1055.

26. Gonzalez RF, Allen L, Gonzales L, Ballard PL, Dobbs LG. HTII-280, a biomarker specific to the apical plasma membrane of human lung alveolar type II cells. *J Histochem Cytochem* 2010;58: 891–901.

27. Kumar PA, Hu Y, Yamamoto Y, Hoe NB, Wei TS, Mu D, Sun Y, Joo LS, Dagher R, Zielonka EM, *et al.* Distal airway stem cells yield alveoli *in vitro* and during lung regeneration following H1N1 influenza infection. *Cell* 2011;147:525–538.

28. McElroy MC, Kasper M. The use of alveolar epithelial type I cell-selective markers to investigate lung injury and repair. *Eur Respir J* 2004;24:664–673.

29. Bove PF, Grubb BR, Okada SF, Ribeiro CM, Rogers TD, Randell SH, O'Neal WK, Boucher RC. Human alveolar type II cells secrete and absorb liquid in response to local nucleotide signaling. *J Biol Chem* 2010;285:34939–34949.

30. Dobbs LG, Williams MC, Brandt AE. Changes in biochemical characteristics and pattern of lectin binding of alveolar type II cells with time in culture. *Biochim Biophys Acta* 1985;846:155–166.