Type I Alveolar Epithelial Phenotype in Primary Culture

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Type I alveolar epithelial cells (ATIs) are very large, thin cells, which extend across several air sacs and cover more than 95% of the alveolar surface area. ATIs are the target of many insults, including ventilator-induced lung injury, and are generally considered terminally differentiated cells arising from type II cell (ATII) lineage. ATIs have proven difficult to harvest and maintain in primary culture, which is why much of ATI biology has been inferred from studies on *ex vivo*, ATII-derived, so-called ATI-like cells. We report on a modified approach to rat ATI harvest and primary culture, which yielded the following observations: (1) rat ATI can be harvested and maintained with a high degree of purity in primary culture; (2) *in vitro* growth characteristics of primary ATIs differ from those of ATII-derived ATI-like cells; ATIs, but not *ex vivo*, ATII-derived ATI-like cells, are capable of cell division; (3) ATIs readily repair plasma membrane wounds without the subsequent loss of their ability to divide; (4) ATI monolayers heal scratch wounds primarily by cell spreading and migration. Although the ability of ATIs to divide may be limited to the *in vitro* environment, we do believe that their role in alveolar wound repair deserves to be revisited, and the molecular control of ATI–ATII plasticity further explored.

Keywords: alveolar epithelial type I cell; primary culture; phenotype; proliferation; injury and repair

Type I alveolar epithelial cells (ATIs) are very large, thin cells, which extend across several air sacs and cover more than 95% of the entire alveolar surface area (1, 2). ATIs are considered terminally differentiated cells, with a life span of approximately 120 days (3). It is not known how ATIs are repopulated in the normal lung, but it is generally accepted that alveolar epithelial wounds are repaired by alveolar type II cell (ATII) hyperplasia and subsequent transdifferentiation of ATII to an ATI phenotype (4, 5). Consequently, the newly generated alveolar epithelium expresses ATI-specific proteins, including T1- α (aka, podoplanin), receptor for advanced glycation end-products, aquaporin-5, and caveolins (6–10). ATIs seem to be particularly vulnerable to wounding by deforming stress, as evident in patients with the adult respiratory distress syndrome and in animal models of ventilator-induced lung injury (11, 12). However, until recently, research on mechanotransduction and repair responses of ATIs has been hampered by challenges in harvesting and maintaining these cells with sufficient purity and physiologic phenotype in primary culture (13–17). To date, most research on alveolar epithelial mechanotransduction has been performed on airway or ATII-derived cell lines. A few groups have investigated the effects of stretch on stress response

CLINICAL RELEVANCE

Although the ability of type 1 alveolar epithelial cells (ATIs) to divide may be limited to the *in vitro* environment, we do believe that their role in alveolar wound repair deserves to be revisited, and the molecular control of ATI–ATII plasticity further explored.

and cell remodeling pathways, on transdifferentiation mechanisms, as well as on wounding and plasma membrane repair responses in ATII-derived ATI-like alveolar epithelial cells (18–23). However, there is increasing concern that ATII-derived ATI-like cells in liquid culture differ in significant ways from freshly isolated ATIs (3, 24–26).

Because of the potential importance of ATI wounding in the pathogenesis of ventilator-associated lung injury, we have slightly modified a previously described approach to rat ATI cell harvest and report on ATI cell behavior in primary culture (27). We confirm that it is possible to harvest and culture rat ATIs with a high degree of purity, and that these cells are capable of repairing plasma membrane wounds *in vitro*. Moreover, we confirm that ATI cells form confluent monolayers, and that they, distinct from ATII and ATII-derived ATI-like cells, readily divide and proliferate *ex vivo* (25, 26).

MATERIALS AND METHODS

Miscellaneous Reagents

A list of reagents, cell lines, and their commercial sources has been provided in the online supplement.

Mouse Anti-Rat T1- α Antibody Preparation

Mouse anti-rat T1- α is an E11 hybridoma cell-derived monoclonal antibody directed at the ATI-specific apical surface antigen T1- α (E11 cells were a generous gift from Dr. Ramirez, Boston University School of Medicine, with permission of Dr. A. Wetterwald, University of Berne, Switzerland, who had originally generated the cell line). The clonal expansion and antibody purification approach are described in the online supplement.

ATI Isolation

Rat lungs were harvested, digested, minced, and filtered as previously described (13, 27). Cells were incubated in rat IgG-coated Petri dishes in RPMI basal medium for 1 hour at 37°C. Unattached cells were collected and incubated with 5 μ g/ml mouse anti-rat T1- α antibody in RPMI with 1% FBS and 0.15% DNase I for 45 minutes at 4°C on a rotator. To remove any unbound T1- α antibody and DNase I, cells were washed three times with RPMI supplemented with 1% FBS and then incubated with pan-mouse IgG-coated magnetic beads (100–150 μ l/rat) in 0.5% BSA at 4°C for 30 minutes on a rotator. The cell-bead mixture was attached to a magnet for 5 minutes, and the supernatant was collected as ATII, whereas ATI was assumed attached to the beads. After five washes, ATIs were separated from the beads by incubating the cell-bead mixture at 4°C with the “releasing buffer” DNase I, which is supplied with the kit. The “releasing procedure” was repeated five times, thereby generating five batches of cells with increasing ATI purity (decreasing ATII contamination). The first and

(Received in original form September 30, 2009 and in final form May 25, 2010)

This work was supported by National Institutes of Health grant HL 63,178 and the Mayo Foundation.

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This article has an online supplement, which is accessible from this issue's table of contents at www.atsjournals.org

Am J Respir Cell Mol Biol Vol 44, pp 692–699, 2011

Originally Published in Press as DOI: 10.1165/rcmb.2009-0359OC on July 8, 2010

Internet address: www.atsjournals.org

second batches were “released” after 15 and 60 minutes, respectively. The remaining batches were collected in 8–12 hourly increments.

ATI Cell Liquid Culture

Freshly isolated ATIs were seeded at a density of 2×10^5 cells on glass coverslips in 35-mm cell culture dishes in high-glucose Dulbecco's modified Eagles medium supplemented with 10% FBS, 200 units (μg)/ml of penicillin and streptomycin, and maintained in a 5% CO_2 humidified 37°C incubator.

ATI Identification and Purity, Yield, and Viability Assessment

ATI cells were identified and distinguished from contaminating cells by morphologic criteria and their immunostaining characteristics for T1- α , caveolin-1, intercellular adhesion molecule-1 (ICAM-1), pro-surfactant protein C (proSP-C), cytokeratin, vimentin, vascular endothelial (VE)-cadherin, fetal liver kinase (Flk)-1, fms-like tyrosine kinase (Flt)-4, and aquaporin-5 (13, 15, 28).

Immunocytochemistry

A cytospin was prepared immediately after isolation, and the cells fixed in 4% formaldehyde for 15 minutes. Normal rabbit IgG and mouse IgG1 served as controls for nonspecific binding of the antibodies. After fixation, cells were blocked with 5% normal goat serum and 5% glycerol for 45 minutes at room temperature before adding primary antibodies (see list of reagents in the online supplement). The specific immunoreactivity was quantified with fluorescence microscopy.

Cell Proliferation Assay and Cytogenetic Testing

Cell proliferation was determined using a Bromodeoxyuridine incorporation assay (29). Complimentary approaches included cell cycle FACS analysis and immunostaining for Ki67 (27). ATI cultures were incubated with colcemid overnight to screen for metaphase-arrested chromosomes. These were stained with Leishman's working stain solution and visualized with a Zeiss Axioplane 2 light microscope (Jena, Germany).

Cell Migration and Wound Healing Assay

Confluent monolayers of ATI were wounded with a 21-G needle in the presence of 0.5% FITC-dextran, while the other monolayer served as uninjured control. As previously described, FITC-dextran served to label wounded but resealed cells at the epithelial wound edge (30). After removal of FITC-containing media, BrdU-containing culture medium was applied to both wells, and the culture was incubated at 37°C in a humidified incubator for up to 16 hours. Cells were then fixed and stained for BrdU. Fluorescent and phase-contrast images of the wound were recorded intermittently for the analysis of cell migration and proliferation.

Statistical Analysis

All group data are presented as means and SD. Comparisons between groups were performed using Student *t* tests for unpaired observations on a Microsoft Excel 2003 platform (Microsoft Corp., Redmond, WA).

RESULTS

Yield and Purity of ATI Harvest and Culture Model

The isolation and immunoselection process yielded between four and five increasingly pure batches of ATIs (Figure 1). The first batch was routinely discarded. The number of cells typically ranged between 0.75 and 1.0×10^6 cells/lung for batches 2 and 3, and tended to decrease to 0.3 – 0.5×10^6 cells/lung for batches 4 and 5. Many times, the number of remaining cells after the fourth immunobead release was insufficient to generate a fifth batch. The viability of ATIs, as estimated by Trypan blue exclusion varied from $94.0 (\pm 0.86)\%$ to $92.60 (\pm 1.31)\%$ to $88.2 (\pm 3.42)$ between releases 2, 3, and 4/5, respectively. The number of T1- α -positive cells increased from $82 (\pm 5)\%$ in batch

2 to $97 (\pm 1)\%$ in batch 4. Corresponding immunostaining rates for caveolin-1 and ICAM-1 were concordant with T1- α -derived estimates of ATI purity (Figure 1). Based on proSP-C immunostaining patterns, the vast majority of contaminating cells were ATII. The fraction of proSP-C-positive cells decreased from $16 (\pm 7)\%$ in batch 2 to $1 (\pm 0.6)\%$ in batches 4 or 5.

Upon seeding on glass coverslips in 10% Dulbecco's modified Eagles medium growth media, cells adhered, grew as colonies, and formed confluent monolayers by Days 5–7. The cells in the center of the monolayer tended to be smaller ($1,413 \pm 71 \mu\text{m}^2$) than those in the periphery ($6,342 \pm 639 \mu\text{m}^2$). Over 95% of confluent cells derived from batch 3 or higher expressed the ATI markers, T1- α , ICAM-1, and caveolin-1 (Figure 2). However, unlike freshly isolated ATIs, most cells in culture stained positive for proSP-C. The proSP-C staining pattern was perinuclear and very different from that seen in ATII, which is punctuate throughout the cytoplasm. Colabeling of proSP-C with the Golgi marker, GM-130, indicated that a large fraction of proSP-C resides in compartments other than the Golgi network.

Because ATII begin to express T1- α after several days in culture, we had to rely on morphology and caveolin-1 immunostaining intensity to distinguish between ATII-derived ATI-like cells and primary ATIs (Figure E1 in the online supplement). Although we know from Western blot analyses that ATII also begin to express caveolin-1 during the transition to an ATI-like phenotype (data not shown), the intensity of caveolin-1 immunofluorescence in contaminating cells tended to be much lower than that of primary ATI. Accordingly, the degree of contamination with ATII-derived ATI-like cells, and possibly other cell species, decreased from 5% in 5- to 7-day-old cultures from batch 2 to 2% from batch 3, and less than 1% from batches 4 or 5. We could not identify vimentin-positive cells in ATI cultures, suggesting that fibroblast contamination was not an issue. Moreover, 99% of cells stained positive for cytokeratin, identifying them as being of epithelial origin.

Whereas the plasma membranes of freshly isolated rat ATIs were coated with murine antigens, and therefore reacted with isotype control mouse IgG1, confluent ATIs in primary culture did not (Figure 2). We speculate that the murine antigens on the plasma membranes of freshly harvested rat ATIs were remnants of the murine anti-T1- α antibody with which the immunobeads had been coated. After several days in culture, ATIs had presumably internalized and degraded the murine antigens, and no longer stained positive for either isotype-matched normal mouse IgG or a secondary murine antibody. As to the specificity of the T1- α immunofluorescence reactions, we should note that freshly harvested rat ATII, which do not express T1- α , and therefore did not acquire murine antigens during the immunobead selection procedure, did not react with normal mouse IgG or a secondary murine antibody (Figure 1).

Because there is species-dependent variation in podoplanin expression in lymphatic endothelial cells (6), we compared the immunolabeling characteristics of rat ATIs with those of human lymphatic endothelial cells and human umbilical vein endothelial cells (Figures E2 and E3). It is of interest that rat ATIs expressed the endothelial markers vascular endothelial growth factor receptor 2 (labeled with FLK-1) and 3 (labeled with FLT-4). Both endothelial cell lines were T1- α positive, but the staining pattern was very different from that seen in rat ATIs.

ATIs Spread and Proliferate in Primary Culture

After initial adherence and spreading by Day 3, highly refractive mitotic figures, ranging from prophase to telophase and cytokinesis, were first noted (Figure 3). Together with the appearance of metaphase-arrested chromosomes, these observations suggest that ATIs are capable of cell division in primary

Figure 1. Phenotypic characteristics of alveolar epithelial type (AT) I and ATII cell harvest. (A) Immunostaining of freshly harvested ATIs and ATIIs for T1- α , caveolin-1 (cav-1), intercellular adhesion molecule-1 (ICAM-1), pro-surfactant protein C (proSP-C), mouse IgG (MlgG), and rabbit IgG (RlgG) isotype negative controls. (B) Relative marker expressions in successive batches (successive DNase-mediated bead releases) of ATIs.

culture (Figure 3F). This interpretation was subsequently affirmed by cell cycle FACS analyses (Figure 3M), as well as the avid BrdU uptake and Ki67 labeling of T1- α - and caveolin-1-positive cells in culture (Figure 4A and Figure E4). The number of cells with BrdU-positive nuclei increased from 34% on Day 3 to 45% on Day 5 and 49% on Day 7. Corresponding rates of Ki67 labeling were 41% on Day 2 increasing to 63, 66, and 62% on Days 5, 7, and 9, respectively. Because BrdU is only incorporated during DNA synthesis, whereas Ki67 labels nuclei throughout all phases of the cell cycle with the exception of Go, the two estimates of cell division are intrinsically consistent.

On average, the cell number tripled between Days 1 and 6 with an estimated doubling time of 36 hour between Days 3 and 6. In contrast to ATIs, ATIIs in primary liquid culture were incapable of cell division even after their transition to an ATI-like phenotype (Figure 4B). When ATIs were passaged on Days 8–10, they formed monolayers within 3–4 days, and maintained their phenotype with respect to the specified ATI surface markers (data not shown).

We looked for topographical differences in Ki67 staining patterns across low-power image fields, knowing that larger cells tended to be in the periphery of the culture, but did not detect

such differences (Figure E4). Moreover, there was no correlation between direct estimates of projected cell surface area and markers of proliferation. For example, Figures E4G and E4H show very large ATIs undergoing cell division.

ATIs Repair Culture Wounds by Spreading and Migration

The wound repair response of a confluent ATI monolayer grown on glass slides was assessed with a scratch assay; 73 (± 13)% of ATIs at the scratch margin retained FITC-dextran, indicating a robust plasma membrane wound repair capacity (Figure 5). Moreover, FITC-dextran-labeled “injured and resealed” ATIs participated in the repair process and retained the capacity to divide (Figure E5). However, the intensity of cell proliferation had no bearing on the rate of wound closure. Scratch-injured monolayers of ATII-derived ATI-like cells, which do not divide in culture, closed wounds at a similar rate (6.44 $\mu\text{m/hr}$), as did monolayers of primary ATIs (8.98 $\pm$ 1.28 $\mu\text{m/hr}$). Moreover, inhibition of cell division with mimosine did not retard the rate of ATI wound closure (9.18 $\pm$ 0.9 $\mu\text{m/hr}$). This is in contrast to monolayer wounds of 2-culture-day old ATIIs, which were much slower to heal (4.09 $\mu\text{m/hr}$) (Figure 5 and videos in the online supplement).

Figure 2. Phenotypic characteristics of ATI in primary culture. ATIs maintain surface expressions of T-1 α , caveolin-1, ICAM-1, and aquaporin (AQP)-5 in primary culture. ATIs stain positive for the epithelial lineage marker, cytokeratin, but do not express vimentin. ATIs in culture express proSP-C, in a perinuclear distribution in a pattern that is very different from the punctate cytoplasmic patterns seen in ATII. Although proSP-C did colocalize with the Golgi marker, GM-130, a large fraction of proSP-C resided outside the Golgi network. Mouse and rabbit IgG served as negative controls. HSF, human skin fibroblast.

DISCUSSION

The main findings of this study are as follows: (1) rat ATIs can be harvested and maintained with a high degree of purity in primary culture; (2) *in vitro* growth characteristics of primary ATIs differ from those of ATII-derived ATI-like cells; ATI, but not *ex vivo* ATII-derived ATI-like cells, are capable of cell division, confirming recent reports by Gonzales and colleagues (25) and Dobbs and colleagues (26); (3) ATIs readily repair plasma membrane wounds without the subsequent loss of their ability to divide; and (4) ATI monolayers heal scratch wounds primarily by cell spreading and migration.

ATIs Can Be Harvested and Maintained with a High Degree of Purity in Primary Culture

Our ATI isolation procedures were based on the descriptions of Dobbs and Chen (13, 27) with some modifications. These include the use of magnetic immunobeads for selection instead of FACS, the use of a purified, highly concentrated mouse monoclonal antibody (1.5–2.5 mg/ml) instead of a polyclonal antibody or culture supernatant, and the use of a slightly larger pore size filter (150 versus 100 μ m). The immunoselection with T1- α antibody-coated magnetic beads allows the investigator to balance yield against purity. We generally discarded the first batch of release buffer-treated cells, because of their high ATII contamination rate. As a result, our yield decreased from four million cells per rat to, typically, 1.5 million cells per rat. The number of release buffer exposures had no significant effect on cell viability.

As summarized in a comprehensive review (2), ATIs could be easily distinguished from contaminant ATII and mesenchymal cells by their expressions of T1- α , caveolin-1, ICAM-1, and

the epithelial lineage marker, cytokeratin (14, 17, 28). The latter provided a clear distinction from lymphatic endothelial cells, which also express T1- α , albeit in a very different distribution. As expected, freshly isolated ATIs lacked proSP-C, but, interestingly, after 6 days in culture acquired a faint perinuclear signal, which is very different from the typical cytoplasmic, punctate proSP-C staining pattern of ATII. As previously pointed out by Borok and Crandall (24), as well as Dobbs (25), the latter speaks to the plasticity of ATIs, at least in culture, and raises uncertainty about the conventional view, that ATIs are terminally differentiated.

In Vitro Growth Characteristics of Primary ATIs Differ from Those of ATII-Derived ATI-Like cells

As distinct from ATII and ATII-derived ATI-like cells, rat ATIs in primary culture formed confluent monolayers in liquid culture, developed network structures on matrigel (data not shown), and, by Day 3, exhibited clear evidence of cell proliferation. The number of ATIs increased with an estimated doubling time of 36 hours, many mitotic forms could be seen in metaphase-arrested specimens, and BrdU incorporation reflected new DNA synthesis. None of these features were observed in ATII-derived ATI-like cells. Because the number of ATII, which are the main contaminants in early release batches, remains more or less static, the purity of ATI cultures increased with time. Alerted by the recent publication by Gonzalez and colleagues (26), in which the rate of ATI proliferation was assessed by Ki67 labeling, we compared the rates of BrdU incorporation, a marker of DNA synthesis, with the rates of nuclear localization of Ki67. Ki67 is a 360-kD nuclear protein, which is induced when quiescent arrested cells enter the G₁- to S-phase transition. It continues to be

Figure 3. Cell cycle FACS analysis of primary ATIs in culture. (A–E) highly reflective mitotic figures. (F) Metaphase-arrested chromosomal staining pattern. (G–L) Phase-contrast and 4',6-diamidino-2-phenylindole-stained nuclear images at different stages of cell division: Bromodeoxyuridine-positive cell (G) and corresponding late prophase/prometaphase (H); metaphase (J); and telophase/cytokinesis (L). (M) Cell cycle FACS analysis of 5-culture-day-old ATI indicates proliferation rates comparable to those seen in A549 cells (a human ATII-derived transformed cell line). This is in contrast to the very low proliferation rate seen in 5-culture-day-old ATIs. Dip, diploid; FL, flow.

expressed throughout G₂ and M phases. Its expression is undetectable in cells in G₀. Despite its widespread use in cancer research, its function remains poorly understood (31). We identified a significant number of Ki67-positive cells as early as Day 2, and confirm that, by Day 5, more than 50% of ATIs are positive for this proliferation-associated antigen. Not unexpectedly, the rates of BrdU incorporation are slightly lower, because DNA synthesis does not extend into the cell cycle M phase.

The ability of ATIs to proliferate *in vitro* is of interest insofar as ATIs are considered terminally differentiated cells. As summarized in a comprehensive review of the alveolar epithelial kinetics (5), old autoradiography studies using tritiated thymidine suggested that ATII are the primary, if not the sole source of ATIs that repopulate alveolar wounds (4, 32–34). Advances in stem cell biology, the birth of regenerative medicine, and the emergence of new molecular tools to conduct cell lineage studies have generated renewed interest in this topic (35, 36). For example, subsets of Clara cells and ATIs, such as bronchioalveolar duct junction cells, are thought to function as stem/progenitor cells in bronchioles and alveoli. However, bone marrow-derived stem cells seem to play little if any role in repopulating injured alveoli with epithelial cells of either

phenotype (37). The complex interactions between soluble growth factors, cell shape, cytoskeletal prestress, and the biophysical, as well as biochemical, matrix properties have been investigated to define the limits of alveolar epithelial plasticity (38–40), and there is now evidence that, *in vitro*, the transition between ATII and ATI is, at least in part, reversible (41).

Injured ATIs Readily Repair Plasma Membrane Wounds

The observation that the injured ATI monolayers “heal” scratch wounds, primarily by spreading and migration of the cells at the wound margins, is in keeping with data from other lung epithelial injury models (42). However, the model also informed about fate of injured ATIs at the wound margin. Accordingly, more than 70% of wounded ATIs at the scratch margin revealed their plasma membranes and apparently survived the insult at least in the short term. We infer membrane repair from the cytoplasmic retention of FITC-labeled dextran (Figure 5). This rate of repair is comparable to that of ATI-like cells (43), but, at least in our hands, tends to be higher than that seen in freshly isolated ATIs when injured by micropuncture (L. Godin, personal communication). It is well established that wounded mesenchymal cells must repair defects within minutes if they are to survive the insult (44). Less is known about the long-term effects of wounding and

Figure 4. (A) BrdU labeling (red nuclei) of ATIs maintained between 1 and 6 days in primary culture. Columns 1 and 3 show corresponding phase-contrast images (merged) and nuclear DAPI stains. Column 4 shows corresponding caveolin-1 expression as phenotypic control. (B) Corresponding images of ATIs after 3 and 4 days in primary liquid culture. Note the absence of BrdU incorporation.

repair on epithelial cell kinetics, let alone on those of ATIs. We now know that *in vitro* wounded and repaired ATIs retain the ability to divide (Figure 5 and Figure E5). This is in keeping with the seminal observations by Steinhardt and colleagues (45) on wounded sea urchin eggs. Although, to date, our investigation of cell repair in injured lungs has not specifically focused on ATIs, our data strongly suggest that ATIs not only repair *in vitro*, but also in the intact lung (46).

Although the evidence that ATI divide after Day 3 in culture is inescapable, we note that cell proliferation was not restricted to the scratch wound site, nor did wounding seem to enhance the proliferative response. How can our observations on cell

culture models be reconciled with the notion that ATIs are incapable of dividing *in situ* (4, 47–49)? Although the evidence from cell lineage studies of the 1970s and 1980s is strong, it is, nevertheless, not ironclad. In several of the original thymidine-labeling experiments, the origin of up to 12% of labeled cells was undetermined. In addition, the analyses did not account for unlabeled cells, and might have been further limited by a much slower turnover of ATIs compared with ATIs, with the former estimated to range between 120 and 140 days in normal lungs (2). An alternative explanation for the apparent differences between the *in vivo* and *in vitro* proliferative potential of ATIs is the important shape constraints imposed by the connective

Figure 5. Upper panel (T0): phase-contrast and green fluorescence image (FITC-dextran) of a scratch wound at time 0 hours. The cytosolic retention of dextran (green fluorescence) in ATIs at the injury margin is consistent with cell wounding followed by plasma membrane repair. Middle panel (T16): phase-contrast and fluorescence images of FITC dextran (green), Alexa 594 BrdU (red), and DAPI (blue) of scratch wound after 16 hours. Note the uniform BrdU uptake pattern (lack of spatial organization of the proliferative response) and the participation of wounded and healed ATI (green) in the repair response. Lower panel: rates of wound closure in 5-day-old monolayers of ATIs (dark blue), ATIs treated with mimosine (0.2 mM, pink), and 5-day-old ATII-derived ATI-like cells (yellow), compared with 2-day-old ATIs (light blue). CTL, untreated control.

tissue of the intact lung. A single ATI can extend over as many as four airspaces (1), and it has been argued that this mechanical constraint may prevent them from assuming the kind of shape that is imperative for cell division (50). However, at least in primary culture, one finds numerous large, spread out ATIs in the process of cell division.

Conclusions

ATIs are the targets of many lung insults, including ventilator-associated lung injury. Given our interest in the topic, we sought to refine currently available ATI harvest and culture techniques. In doing so, we made a chance observation—namely, that ATIs proliferate *ex vivo*, and that they are capable of plasma membrane wound repair. The former is in keeping with recent reports from the Dobbs laboratory (26). Although the ability of ATIs to divide may be limited to the *in vitro* environment, we concur that their role in alveolar wound repair

deserves to be revisited, and the molecular control of ATI–ATII plasticity further explored (24).

Author Disclosure: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

Acknowledgments: The authors thank Dr. Ianko Lankov (Mayo Clinic) for his assistance in T1- α antibody preparation and purification, and are grateful to Dr. Ramirez (Boston University School of Medicine) and Dr. Wetterwald (University Bern, Switzerland) for their generous gift of hybridoma E11.

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