Rho-Associated Protein Kinase Inhibition Enhances Airway Epithelial Basal-Cell Proliferation and Lentivirus Transduction

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The identification of factors that regulate airway epithelial cell proliferation and differentiation are essential for understanding the pathophysiology of airway diseases. Rho-associated protein kinases (ROCKs) are downstream effector proteins of RhoA GTPase that direct the functions of cell cytoskeletal proteins. ROCK inhibition with Y27632 has been shown to enhance the survival and cloning of human embryonic stem cells and pluripotent cells in other tissues. We hypothesized that Y27632 treatment exerts a similar effect on airway epithelial basal cells, which function as airway epithelial progenitor cells. Treatment with Y27632 enhanced basal-cell proliferation in cultured human tracheobronchial and mouse tracheal epithelial cells. ROCK inhibition accelerated the maturation of basal cells, characterized by a diminution of the cell size associated with cell compaction and the expression of E-cadherin at cell-cell junctions. Transient treatment of cultured basal cells with Y27632 did not affect subsequent ciliated or mucous cell differentiation under air-liquid interface conditions, and allowed for the initial use of lower numbers of human or mouse primary airway epithelial cells than otherwise possible. Moreover, the use of Y27632 during lentivirus-mediated transduction significantly improved posttransduction efficiency and the selection of a transduced cell population, as determined by reporter gene expression. These findings suggest an important role for ROCKs in the regulation of proliferation and maturation of epithelial basal cells, and demonstrate that the inhibition of ROCK pathways using Y27632 provides an adjunctive tool for the *in vitro* genetic manipulation of airway epithelial cells by lentivirus vectors.

Keywords: airway epithelial cells; lentivirus; apoptosis; proliferation; ciliogenesis

The epithelial basal-cell population holds a central role in the maintenance of the airway, owing to its function as a progenitor of differentiated cell types during development, injury, and pathological states (1–4). Basal cells are activated by the epidermal growth

CLINICAL RELEVANCE

Mechanisms for basal-cell proliferation are central to airway epithelial cell homeostasis and repair. We have ascertained that Rho kinase inhibition induces basal-cell proliferation, providing a tool for experimental manipulation and a potential pathway for therapeutic application.

factor and other regulatory molecules that facilitate survival and proliferation (4–7). Moreover, *in vitro* exposure to an air-liquid interface (ALI) in the presence of specific growth factors induces basal-cell differentiation (5, 6, 8, 9). We and others previously described the isolation and *in vitro* culture and differentiation of the basal-cell population from human and mouse airways (5, 6, 8–11). Despite these advances, the isolation and culture of airway epithelial cells may be unsuccessful in cases of human biopsies that are very small or in transgenic mice that are difficult to breed, yielding few basal cells. This lack of success is in part attributable to the need for high basal-cell densities in the successful culturing of primary airway epithelial cells, to facilitate their survival, proliferation, and subsequent differentiation (5, 9, 12).

Recent reports suggest that Rho/Rho-associated protein kinase (ROCK) proteins play an important role in the survival of embryonic stem cells during *in vitro* manipulation (13–16). The Rho family of GTPases is composed of small, signaling G proteins that regulate the actin cytoskeleton and cell migration and proliferation (17, 18). Downstream effectors of Rho include Rho-associated coiled-coil kinases including the isoforms ROCK1 and ROCK2 (Rho-associated coiled-coil-containing protein kinases 1 and 2). The roles of ROCK proteins in cell-cell adhesion and cell migration, differentiation, apoptosis, proliferation, and other functions have been extensively studied in epithelial cells from many tissues (19, 20). The association of ROCK with cell apoptosis initially promoted the use of ROCK inhibition as a tool to enhance embryonic stem-cell (ESC) survival *in vitro* (13, 16, 21). Toward this end, Y27632, a specific ROCK1 and ROCK2 inhibitor, is now routinely used in the culture and manipulation of human ESCs, induced pluripotent stem (iPS) cells, and some tissue-related stem-cell populations for its effects on the inhibition of dissociation-induced apoptosis (13, 16, 21, 22). Y27632 also promotes the proliferation of keratinocytes when cocultured with fibroblasts that function as feeder cells (23, 24). This method has similarly been used to expand very small samples of normal and malignant cells obtained from clinical samples (21).

We hypothesized that ROCK inhibition exerts similar effects on the *in vitro* survival and proliferation of the airway epithelial stem cell-like population of basal cells. Both ROCK1 and ROCK2 are expressed in airway epithelial cells, and are active

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in directing cell morphology (25). Because ROCK activation and inhibition regulate the cell cytoskeleton and tight-junction organization (17, 18, 26), we explored the effects of ROCK inhibition on basal-cell maturation during compaction, as cells achieve contact (27). In addition, the genetic modification of airway epithelial cells (gene overexpression or silencing) by lentivirus transduction is desirable but often inefficient because of low transduction efficiency and the inherent toxicity of the virus itself (28). To address this, we explored the use of Y27632 during transduction to allow for improved transduction.

MATERIALS AND METHODS

Cell Culture

See the online supplement for additional details. Primary human airway epithelial cells (hTECs) were isolated from the tracheas and proximal bronchi of lungs donated for transplantation, expanded on collagen-coated plastic dishes, and then studied as Passage 1 cells or cryopreserved (29). Cells from more than 20 donors were used for experiments. Mouse airway epithelial cells (mTECs) were isolated from the tracheas of 8- to 12-week-old C57BL/6J mice, and then studied as Passage 0 cells (10). Cells from either source were cultured on plastic dishes or semipermeable supported membranes (Transwell; Costar, Corning, NY), as described elsewhere (30). IL-13 at 10 ng/ml was added to the medium on ALI Days 10–17 to induce mucous cells. These experiments were approved by our Institutional Review Committees for Human and Animal Research.

Rho Kinase Inhibitor Treatment

Y27632 (Sigma-Aldrich, St. Louis, MO) was solubilized in PBS to create a 10-mM stock solution that was diluted in culture media. Fresh Y27632 and media were provided every 2 days.

Cell Quantification, Proliferation, and Apoptosis Assays

Cultured cells were lifted from substrates, using minicell scrapers (LEAP Biosciences, Palo Alto, CA) after treatment with 0.05% trypsin and 0.53 mM EDTA in Hanks' balanced salt solution for 20–30 minutes at 37°C. Live cells were counted in triplicate in a hemocytometer after trypan blue exclusion. Cell apoptosis was evaluated using flow cytometry to detect annexin V binding (Annexin-V FITC kit; Sigma-Aldrich), according to the manufacturer's protocol. Cell proliferation was assessed after a 6-hour incubation with 5-ethynyl-2'-deoxyuridine (EdU; Click-iT EdU Alexa Fluor 555 HCS Assay; Life Technologies, Carlsbad, CA), and analyzed using flow cytometry or immunohistochemistry, according to the manufacturer's protocol.

Flow Cytometry

Airway epithelial cells were incubated in 0.25% trypsin and 2.7 mM EDTA for 15 minutes at 37°C, and then suspended in PBS containing 2% BSA. Cells were collected by centrifugation ($300 \times g$ for 10 min at 4°C), and then analyzed by flow cytometry (FACSCalibur; BD BioSciences, San Diego, CA) using CellQuest (BD BioSciences) and FlowJo (Ashland, OR) software. Airway epithelial cells were gated, based on size and granularity.

Immunofluorescent Staining and Microscopy

Cells on Transwell membranes were fixed, immunostained, and imaged as previously described (29). Methods and antibodies are detailed in Table E1 in the online supplement.

Lentivirus Infection

See the online supplement for further details. A recombinant lentivirus to convey the expression of the yellow fluorescent protein (YFP) and puromycin resistance was generated using a three-plasmid cotransfection system in Human Embryonic Kidney 293T (HEK293T) cells, as previously described (31). Transgenes included the YFP reporter gene driven by a human ubiquitin C promoter sequence, and the puromycin resistance gene, under the

control of a SV40 promoter (32) (see the online supplement for further details). Virus titers determined by p24 levels, using a QuickTiter Lentivirus Titer Kit (Cell Biolabs, San Diego, CA), were approximately $7 \times 10^4 \pm 2.5 \times 10^4$ Transfection Units/ml (mean $\pm$ SD) in 16 preparations. Transduction efficiency was determined using flow cytometry, and calculated as the percentages of YFP-expressing cells.

Statistical Analysis

An unpaired Student *t* test and ANOVA with the Bonferroni test were used to compare the effects of multiple conditions. The significance level was defined as $P < 0.05$.

RESULTS

Rho Kinase Inhibitor Y27632 Enhances the Proliferation of Airway Epithelial Cells

Rho kinase inhibition has been shown to enhance the survival and improve the yield of ESCs in culture (13, 16, 21, 22). To determine whether airway epithelial basal cells were similarly affected, cells isolated from human or mouse airways were treated with Rho kinase inhibitor Y27632, and then assayed for cell proliferation and survival (Figure 1). hTECs (Passage 1) and freshly isolated mTECs (Passage 0), were seeded on plastic dishes at a low density of 1×10^4 cells/cm², and continuously treated with 0, 2.5, 5, or 10 μ M of Y27632. Treatment with Y27632 resulted in a significant increase in the cell numbers of both hTECs and mTECs when evaluated 96 hours after the initiation of culture (Figures 1A and 1B). All cells were P63-positive according to immunostaining. To determine whether the increase in cell numbers was attributable to the proliferation of P63-expressing basal cells, the thymidine analogue EdU was added to media during the final 6 hours of treatment with Y27632. EdU incorporation was enhanced in hTECs and mTECs treated with Y27632 (Figures 1D and 1E). In hTECs, the effect of Y27632 on cell proliferation was most pronounced at 5 μ M, whereas in mTECs, 10 μ M was most favorable. A marked difference (approximately one log) was evident between the numbers of mTECs and hTECs that responded to Y27632. However, hTECs were initially expanded in culture after isolation from lung tissue and before seeding. On the other hand, mTECs were isolated from the tracheas and directly seeded for assay. hTECs retrieved from culture plates were close to 100% p63-positive according to immunostaining, whereas mTEC isolated from tracheas contained approximately 1–5% P63⁺ cells.

We further asked whether a specific population of basal cells was activated by Y27632, based on immunostaining for keratin 5 (K5) and keratin 14 (K14), because these keratins may exhibit variable expression under different conditions (11, 33, 34). Treatment with Y27632 did not differentially affect the proportions of K5-stained or K14-stained cells, as determined by flow cytometry (Figures 1F and 1G).

Y27632 Does Not Significantly Ameliorate Airway Epithelial Cell Apoptosis in Early Culture

We next tested whether the Y27632 treatment of airway epithelial cells also enhanced cultures by inhibiting apoptosis, as shown for ESCs (13, 16). For these experiments, hTECs were treated with Y27632, immunostained with apoptosis marker annexin V after 2, 24, and 72 hours, and compared with a media-only control group, using flow cytometry. Although apoptosis was detected in a considerable percentage of cells during the initiation of culture, no significant differences were evident in the percentages of hTECs immunostained with annexin V among conditions (Figure E1A). Similarly, no significant differences in annexin V staining

Figure 1. Rho kinase inhibitor Y27632 enhances the proliferation of airway epithelial basal cells. (A and B) Effects of Y27632 on the number of human tracheal epithelial cells (hTECs) and mouse tracheal epithelial cells (mTECs), seeded at $1.0 \times 10^4/\text{cm}^2$ and evaluated at 96 hours. (C) Effects of Y27632 on the proliferation of P63-expressing hTECs ($n = 3$ donors). Cells were treated with Y27632 (10 μM) for 72 hours, pulsed with 5-ethynyl-2'-deoxyuridine (EdU) for 6 hours, and then immunostained with P63 (green), EdU (red), and 4',6-diamidino-2-phenylindole (DAPI; blue). (D and E) The effects of Y27632 on EdU labeling were determined, using flow cytometry. (F) The effects of Y27632 treatment on the proliferation of epithelial basal-cell subpopulations were identified via keratin 5 and keratin 14 (K5 and K14, respectively; green) and EdU (red) incorporation. hTECs were cultured and assayed as in C. (G) Quantification of K5 and K14 expression in EdU-labeled cells after 72 hours of Y27632 treatment. For quantitative data, values represent means $\pm$ SDs (hTECs, $n = 3$ donors; mTECs, $n = 3$ preparations). A significant difference compared with control samples is indicated. $*P < 0.05$.

were observed in mTECs, up to 24 hours after seeding (Figure E1B).

In ESCs, Y27632 improved postcryopreservation recovery in culture (22, 35). To test whether the survival of hTECs was similarly enhanced, cells were cultured for 72 hours in media alone or supplemented with 10 μM Y27632. Cells were then collected from plates and suspended in a freezing medium supplemented with or without Y27632 to continue the same treatment. After storage in liquid nitrogen vapor, cells were thawed, resuspended in hTECs plus media, and counted. The mean cell survival amounted to $89.7\% \pm 5.6\%$ and $77.3\% \pm 7.0\%$ in the Y27632-treated and untreated groups, respectively ($n = 4$ donors, $P < 0.005$). The absolute difference in cell survival between the treated and untreated groups amounted to $11.6\% \pm 4.6\%$ (range, 7.3–16.9%), indicating a slight but statistically significant improvement in recovery after cryopreservation. Taken together, these data indicate

that Y27632 enhances the number of cells in culture, primarily through increasing cell proliferation.

Y27632 Accelerates Basal-Cell Compaction, but Not Airway Epithelial Cell Differentiation

Rho kinase pathways contribute to cytoskeletal function and cell-junction formation (17, 18). Therefore, to determine the effects of Y27632 on these parameters was important. We first evaluated the morphology of Y27632-treated cells seeded at very low density (6.25×10^3 cells/ cm^2) on collagen-coated plastic plates and treated with media only, using 5 or 10 μM of Y27632 (Figure 2A). Treatment with Y27632 caused cells to round as cell confluence increased. A significant decrease in cell size was evident by Day 2, compared with nontreated cells (Figure 2B). The differences in cell shape and size became less prominent among treatment groups as proliferation continued. Previous studies demonstrated that during this process of compaction, proliferation is inhibited as cells form cell junctions by mobilizing E-cadherin along the plasma membrane (27, 36). Therefore, we next evaluated the formation of cell-cell junctions by monitoring E-cadherin expression and actin organization during basal-cell proliferation and before differentiation. For these experiments, hTECs were seeded on Transwell membranes using a seeding density typically used for ALI differentiation (1×10^5 cells/ cm^2) (5, 10). Compared with the media-treated control samples, Rho kinase inhibition accelerated the localization of both E-cadherin and actin at the cell junction (Figure 2C). However, a difference in the distribution of both E-cadherin and actin among treatment groups was not apparent when cells formed a confluent monolayer, that is, the point at which an ALI condition is established to induce differentiation (ALI Day 0). These findings further support our observation that Y27632 promotes the proliferation and maturation of basal airway epithelial cells. In practical terms, the Y27632 treatment of cells seeded on supported membranes achieved confluence, to allow for the establishment of an ALI condition at an earlier time compared with nontreated cells, even when seeded at a lower density (Figures 2D and 2E).

Y27632 Treatment Does Not Alter Ciliated or Mucous Cell Differentiation

To determine whether the treatment of basal cells with Y27632 affected airway epithelial cell differentiation, hTECs and mTECs were seeded at 1×10^5 cells/ cm^2 , and then incubated with media only (5 or 10 μM Y27632) until cells reached confluence. Subsequently, Y27632 was discontinued, and an ALI was established. In these preparations, Y27632 treatment did not affect subsequent ciliated cell differentiation, as determined by the extent of ciliation in preparations (Figures 2F and 2G). Furthermore, no significant effect of Y27632 on ciliogenesis was observed, as determined by the percentages of FOXJ1⁺ cells assessed on ALI Days 7 and 14, in hTEC cultures seeded at 1×10^5 cells/ cm^2 or $0.125 \times 10^5/\text{cm}^2$, respectively (Figures E2A and E2B). We also evaluated the effects of Y27632 on the numbers of mucous cells (MUC5AC⁺). MUC5AC⁺ cells were not detected in mTECs cultured in the absence or presence of Y27632. Few MUC5AC⁺ cells were evident in well-differentiated hTECs, independent of Y27632 (range, 0–19 MUC5AC⁺ cells per $\times 100$ -power field; $n = 3$ independent preparations). In additional experiments, Y27632 did not increase the percent of IL-13-induced mucus cells in hTEC preparations (Figure E2C).

Y27632 Enhances Lentivirus Transduction Efficiency

We next asked whether Y27632-enhanced proliferation would improve efficiency in the lentiviral transduction of primary airway epithelial cells. We used an approach adapted from our previous

Figure 2. Y27632 treatment accelerates changes in basal-cell morphology, without altering airway epithelial cell differentiation. (A) The effects of Y27632 treatment on basal-cell morphology are illustrated by representative photomicrographs of hTECs treated with Y27632. hTECs were cultured on collagen-coated plastic at 6.25×10^3 cells/cm² with the indicated concentrations of Y27632, for the specified times. (B) Quantification of cell size in A. (C) The effects of Y27632 treatment on the development of cell-cell junctions. hTECs cultured on Transwell membranes (Costar, Corning, NY) were stained for actin (phalloidin, red) and E-cadherin (green) on Day 3 and Day 5 (air-liquid interface Day 1). (D and E) The effects of Y27632 on the development of a confluent cell layer are indicated as time to creation of the air-liquid interface (ALI) condition. Cells

were cultured on Transwell membranes at the indicated seeding densities. Untreated cells seeded at 1.25×10^4 cells/cm² did not reach confluence (NC). (F) The effects of Y27632 treatment on differentiation. Representative images of hTECs and mTECs were immunostained for the cilia marker acetylated α -tubulin (α -tub; green) and for nuclei (DAPI; blue). Cells were seeded at 1.0×10^5 cells/cm². (G) Percent ciliated cells treated with Y27632 seeded at 1.0×10^5 cells/cm². Cells were evaluated on ALI Days 10–14 (mTECs) or ALI Days 18–28 (hTECs). Ranges under all conditions for hTECs were 25–28%, and for mTECs, 38–45%. $P > 0.05$. For quantitative data, values represent means $\pm$ SDs (hTECs, $n = 3$ donors; mTECs, $n = 3$ preparations). A significant difference compared with control samples is indicated. $*P < 0.05$.

experience and that of others for airway epithelial cell transduction (29, 37–39) (Figure 3A). A lentivirus mediating the expression of YFP reporter and puromycin resistance genes was used to quantify transduction. Cells were resuspended in freshly produced lentivirus-containing media, supplemented with or without Y27632 immediately after removal from tissue-culture plates (hTECs, Passage 1), or purified from mouse tracheas (mTECs, Passage 0). At 72 hours, a significant increase was evident in both the percentages and absolute numbers of YFP⁺ hTEC cells, compared with media treatment (Figures 3B and 3C, Day 3). After puromycin selection, the percentages of YFP⁺ hTEC cells were not different among treatment conditions. However, the absolute number of YFP⁺ hTECs was considerably higher in the Y27632-treated group (Figure 3C, Day 7). Y27632 did not enhance the transduction efficiency of bulk mTECs (i.e., basal and differentiated cell types freshly purified from mouse tracheas) (Figure 3D, Day 3). However, after puromycin selection (on Day 7), both the percentages and absolute numbers of YFP⁺ mTECs were significantly higher (Figures 3D and 3E, $P < 0.05$). These data suggest that Y27632 improved the transduction efficiency of human epithelial basal cells. In both hTECs and mTECs, Y27632 increased the total number of transduced cells. Furthermore, transduced cells retained their differentiation potential, and were able to achieve ALI even when seeded at lower densities. Moreover, YFP expression was retained in fully differentiated cells in culture up to several months (Figures 3F and E3). This retained expression can be attributed to the use of the ubiquitin C promoter to drive the YFP gene (see the online supplement for further details) (32, 40). The percentage of ciliated cells was similar in transduced hTEC preparations treated with Y27632 compared with untreated cells (Figure E3). Based on these observations, we developed a protocol for the use of Y27632 to enhance the transduction and selection of primary airway epithelial cells in culture (Figures 3A and 4; also see the online supplement).

DISCUSSION

Advances in stem-cell biology and cell culture during the past decade have improved our understanding of lung-cell biology

and the development of *in vitro* models (2, 3). In this study, we determined that Y27632, an inhibitor of ROCK previously shown to enhance the culture of ESCs and iPS cells, promoted the proliferation of P63⁺ airway epithelial basal cells. We found that Y27632 accelerated basal-cell compaction during proliferation, as indicated by a smaller cell size and the rapid development of well-organized E-cadherin at cell-cell junctions. In contrast to ESCs, supplementation with Y27632 did not decrease basal-cell apoptosis. However, the use of Y27632 slightly improved survival after cryopreservation. Furthermore, like ESC culture, Y27632 treatment maintained the cell's ability to differentiate. Our observation that cell proliferation was enhanced led us to test the capacity of Y27632 to improve genetic modifications of basal cells, using lentivirus transduction. We found that Y27632 improved transduction efficiency and especially the ability to obtain pure transduced populations by antibiotic resistance gene transduction and drug selection. These methodologies will provide an additional, simple tool for the culture of primary airway epithelial cells, particularly for the study of samples with limited availability.

ROCK inhibitor Y27632 is but one factor found to induce airway epithelial cell proliferation. Components included in specialized growth media, such as epidermal growth factor, insulin, and cholera toxin (5, 8, 9, 30), in addition to fibroblast growth factor 7 (also known as keratinocyte growth factor) and hepatocyte growth factor (41, 42), also share this property. Moreover, seeding density is a critical factor contributing to the proliferation of cultured cells (8, 43). hTECs or mTECs cultured at densities of 3×10^4 /cm² or lower proliferate at a slow pace, and in many instances fail to survive, suggesting an important role for cell-cell interactions during the early phases of culture (8, 44). Y27632 has been shown to enhance the proliferative capacity of ESCs as well as of epithelial cells that are concomitantly cultured with fibroblasts, acting as feeder cells that immortalize cells (13, 16, 21, 22).

The favorable effect of Y27632 in enhancing cell numbers was independent of the effect on cell apoptosis. This is in contrast to the reported effect of Y27632 in ESCs and iPS cells, where ROCK kinase inhibition improves survival through the suppression of

Figure 3. Y27632 treatment enhances the lentivirus transduction efficiency of epithelial basal cells. (A) Scheme for lentivirus transduction, selection, and differentiation of hTECs and mTECs. (B–E) The effects of Y27632 treatment on transduction efficiency ($n = 6$ hTECs, $n = 3$ mTEC preparations). Shown are yellow fluorescent protein-positive (YFP⁺) cells before puromycin (Puro) selection (Day 3) and after puromycin selection (Day 7). Cells were transduced with an YFP-expressing lentivirus, and seeded on Transwell membranes. (B and D) YFP expression was assayed using flow cytometry. (C and E) Total numbers of cells assayed at each time point in B and D, respectively. (F) hTECs

transduced with a YFP-expressing lentivirus were treated with 10 μ M Y27632 before differentiation. Cells were retained in culture for approximately 3 months. A significant difference compared with control samples is indicated. * $P < 0.05$.

dissociation-induced cell apoptosis (13, 21, 45). Apoptosis was significant when airway epithelial cells were initially placed in culture. However, we did not observe increased rates of cell apoptosis with Y27632 treatment, as reported by others (46). Furthermore, the increased proliferation of basal cells treated with Y27632 is in conflict with the effect in corneal epithelial cells, where treatment with Y27632 did not alter proliferation (47). These findings might be attributable to differences in cell-specific targets of ROCK.

We found that treatment with Rho kinase inhibition speeds changes in basal-cell morphology during proliferation. However, differences between treated and nontreated cells were minimal as the cells became confluent and progressed through early ALI conditions. In this regard, our experiments make clear that the basal cells themselves undergo a process of maturation, marked by cytoskeletal arrangement. The changes are consistent with ROCK activation and inhibition, including the reorganization of the cellular tight junctions (17, 18, 26, 47). The balance between cell proliferation and the formation of cell junctions is governed by an intricate relationship between RhoA and focal adhesion kinase activities (48). Furthermore, E-cadherin function also influences proliferation, in a manner directly related to cell density (43). Our findings are in line with these previous findings and with observations that constitutive Rho activity releases cells from proliferation arrest when confluent, and that Rho inhibition with Y27632 retains that control (48).

Figure 4. Flow chart for lentivirus-mediated transduction protocol, using Y27632. The transduction of human or mouse tracheal epithelial cells (hTECs and mTECs, respectively) is depicted. See the protocol in the online supplement for details.

Although Y27632 altered the rate of cytoskeletal reorganization, we did not observe an effect on the capacity for subsequent cell differentiation under ALI conditions. Airway epithelial cells treated with Y27632 achieved ALI earlier than untreated control cells, but retained their differentiation potential. Moreover, in additional experiments not included in this report, the continued Y27632 treatment of mTEC preparations throughout the ALI phase did not promote the development of additional ciliated cells or incur a differentiation advantage. We therefore limited the use of Y27632 during cell culture to the point of development of ALI.

We also found that supplementing viral transduction media with Y27632 improved the efficiency of lentiviral transduction for genetic modifications *in vitro*. Several methods have been used to increase lentivirus transduction efficiency, including the use of a high virus titer, serial transduction, centrifugation to enhance the contact of lentivirus with the cell (spin-transduction), the use of different polycations for cotransduction, and altering the coat protein of the virus (31, 49). We have not directly compared the use of Y27632 with these methods. However, in preliminary experiments using serial transduction (two treatments with fresh virus-containing

media 24 hours apart), concentrating the virus or using the cationic polymer hexadimethrine bromide, rather than protamine, did not increase the transduction efficiency of mTECs to greater than 60% (T. Huang and S. L. Brody, unpublished observations). Our findings that Y27632 improves transduction efficiency are consistent with a recent report on the enhanced transduction efficiency observed in keratinocytes previously maintained in culture in the presence of 10 μ M Y27632 and feeder cells (23). The use of Y27632 not only improved transduction efficiency, but also increased cell proliferation and facilitated selecting a purified population of transduced cells using antibiotic resistance. This facilitated selection is important for the culture of more demanding cells such as mTECs that poorly tolerate flow cytometry-based sorting and reculturing, and that exhibit decreased differentiation with each passage (10).

In conclusion, Y27632 can be used to enhance the proliferation of primary cultured airway epithelial cells without affecting their differentiation potential. Moreover, we present a protocol to improve the efficiency of lentiviral transduction in primary airway epithelial cells, which may ultimately permit the use of fewer cells for the transduction process. Although we have used Y27632 *in vitro* to enhance basal-cell proliferation, the potential use of Rho kinase inhibition *in vivo* to improve recovery from lung injury has been reported, suggesting that effects on lung stem cells could be at play (50). Thus, as stem-cell biology advances, insights from *in vitro* systems may be extended to lung-cell regeneration and repair or *in vivo* gene delivery.

Author disclosures are available with the text of this article at www.atsjournals.org.

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