Reuse of Cell Culture Inserts for *In Vitro* Human Primary Airway Epithelial Cell Studies

To the Editor:

Human primary bronchial epithelial (HBE) cells have a long history in respiratory research. With the establishment of protocols for expansion of primary airway epithelial cells with conditionally reprogrammed cell methods (1, 2), cellular redifferentiation using air–liquid interface (ALI) conditions on porous membranes, and lentiviral infection and CRISPR/Cas9-mediated transduction and gene knockdown technologies (3, 4), the use of HBE cell cultures for respiratory research has greatly expanded.

Because of the worldwide coronavirus disease (COVID-19) pandemic in 2020, a shortage of clinical supplies—for example, masks and hand sanitizers—emerged as a serious issue (5). Likewise, supplies for basic research have been limited. In the case of cell culture, Transwell (3460; Corning) and Millicell (PIH P01250; Millipore) inserts have been in exceptionally short supply. Owing to their limited supply and high cost, insert reuse is a rational approach to maintain timely and cost-efficient research.

The only studies reporting insert reuse focused on reuse for cell line applications (6). In this study, we investigated the reuse of Transwell and Millicell inserts for primary HBE cell culture research. All experiments were conducted using cells from three to five different donor lung specimens. Detailed protocols are found in the data supplement.

Protocols to remove primary airway cells from inserts were developed first. We tested four reagents (0.25% Trypsin-EDTA [25200056; Gibco], Accutase [A6964; Sigma], 6M urea in PBS, and RIPA buffer) to dissociate/solubilize and remove well-differentiated HBE cells from Transwell inserts. Dissociation solutions were applied to the apical surface for between 5 and 20 minutes, followed by pipetting or scraping with a cell lifter. Trypsin-EDTA, 6M urea, and RIPA buffer effectively removed cells. Accutase was less effective. Following cell dissociation, an overnight incubation with 0.25% Trypsin-EDTA at 37°C completed removal of residual cells and debris from inserts. Apical and basolateral surfaces of the inserts were then washed twice with PBS and twice with pure water. After air-drying in a hood, the inserts were coated with collagen (5005; Advanced BioMatrix), UV irradiated, and stored at 4°C until reuse.

The reused inserts were tested in a series of HBE cell culture studies. HBE cells from new donors were seeded and cell differentiation was monitored over 4 weeks in ALI culture (7). None

of the 110 second-use (U2) Transwell inserts exhibited contamination or a loss of integrity. Preservation of cellular differentiation in U1 (first-use) versus U2 cultures was tested by three approaches. First, mRNA expression of cell type markers was measured by qRT-PCR and was similar for U1 versus U2 cultures (Figure 1A) (8). Second, MUC5B protein in apical washes was measured by Western blotting and was found to be similar between U1 versus U2 cultures (Figure 1B). Third, hematoxylin and eosin and whole mount immunofluorescent staining for ciliated (tubulin), secretory (CCSP), and goblet (MUC5B) cell markers confirmed similar differentiation between U1 and U2 inserts (Figures 1C and 1D).

Measurements of HBE cell function on U1 versus U2 inserts were made for ion transport in Ussing chambers (Figure 1E) and cilia beat frequency with video microscopy (Figure 1F) (9–11). Both assays revealed virtually identical cellular functional activities of HBE cells on U1 versus U2 inserts. Importantly, donor-to-donor variability in all parameters was reproduced in assays comparing HBE cells on U1 versus U2 inserts, suggesting that insert reuse did not affect donor-specific cellular function.

We then tested whether a low number of residual cells escaped the reuse protocol and caused DNA contamination of subsequent cultures. ACE2 knockout cells (GenBank MT505392, generated by introduction of gRNA/Cas9 complexes with electroporation [3]), were cultured on U2 inserts and, after 28 days in ALI, the HBE gene-modified cells were removed from the insert and the insert reconditioned for third use (U3). Wild-type (WT) donor cells were plated on U3 inserts. After attachment, expansion, and 7-day ALI culture, DNA was extracted from the U3 culture and the gRNA target region was amplified by PCR and sequenced. Gene-editing efficiency analyses (Inference of CRISPR Edits [ICE] analysis; Synthego) (12) demonstrated that the gene-edited U2 insert cells did not contaminate U3 cultured WT cells (Figure 1G).

HBE cells exposed to inflammatory mediators exhibit changes in epithelial structure/function that may modify the produced basement membrane matrix and affect subsequent culture properties. Accordingly, U2 HBE cells were treated with IL-1 β (1 ng/ml, 201-LB; R&D Systems) for 7 days. IL-1 β treatment resulted in induction of *MUC5B* and reduction of *SCGB1A1* mRNA (Figure 1H). Apical washes confirmed MUC5B protein induction by IL-1 β (Figure 1I) (8). During IL-1 β treatment, 8 of 27 U2 inserts exhibited leakage of media onto the apical surface. U2 inserts containing IL-1 β -treated versus vehicle-treated HBE cells were prepared for reuse and plated with naive HBE cells (U3). HBE cells on U3 inserts from IL-1 β -treated versus vehicle-treated HBE cells exhibited similar *MUC5B* and *SCGB1A1* gene expression, similar MUC5B protein in apical washes, and absence of media leakage, suggesting residual IL-1 β effects were not transmitted to subsequent HBE cultures with insert reuse (Figures 1J and 1K).

We directly investigated whether the porous membrane structure of inserts was affected by reuse using scanning EM. Significant damage to or obstruction of the pores was not observed after any of the cell dissociation/solubilization protocols (Figure 1L).

Finally, multiple reuses of Transwell and Millicell inserts were tested. HBE cells were grown and differentiated on U2 to U4 (fourth-use) Transwell and U2 to U3 Millicell inserts (Figure 1M). Insert reuse four times with a cumulative longevity of use for 185 days was feasible. No bacterial contamination was observed at any time in these studies.

In these experiments, insert reuse was limited to inserts maintained under sterile conditions at all times to minimize bacterial and/or endotoxin contamination. Therefore, it remains unknown

Author Contributions: T.K. conceived and designed the study, performed experiments, analyzed data, and wrote the initial and the revised manuscript. Y.M., L.S., T.D.R., B.R.G., and C.B.M. performed experiments and analyzed data. P.R.S. and L.E.O. supervised cilia beat frequency assays. C.E. supervised scanning microscopy imaging. S.H.R. provided human airway epithelial cells and guidance. R.C.B. supervised the study, analyzed data, and revised the manuscript. All authors reviewed and approved the final version of the manuscript.

This letter has a data supplement, which is accessible from this issue's table of contents at www.atsjournals.org.

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Figure 1. Cell culture insert reuse. (A) mRNA expression from five human primary bronchial epithelial (HBE) cell donors cultured on U1 versus U2 Transwell inserts. Cell type-specific markers (*MUC5B* [goblet cell], *SCGB1A1* [club/secretory cell], *FOXJ1* and *DNAH5* [ciliated cell], and *FOXI1* [ionocyte]) and ion channel markers (*CFTR*, *SCNN1B*, and *ANO1*) were measured. Except for *FOXJ1*, no significant differences between U1 versus U2 were observed ($n=5$, paired t test). Dot colors in U1 versus U2 represent matched donors. (B) Western blotting for MUC5B in U1 versus U2 apical washes. (C) Hematoxylin and eosin staining of U1 versus U2 HBE cell cultures. (D) Whole mount immunofluorescent staining of HBE differentiated cell cultures

Figure 1. (continued) on U1 versus U2 Transwells (nuclei [DAPI, blue], cilia [tubulin, green], secretory cells [CCSP, red], goblet cells [MUC5B, white], and F-actin [phalloidin, purple]). Scale bars, 20 μm . The character colors for each donor in *D* correspond to the dot colors in *A*, *E*, and *F*. (*E*) Ussing chamber studies for three donor HBE cell preparations cultured on U1 versus U2 Transwells. (*F*) Cilia beat frequency measurements of HBE cells from five donors cultured on U1 versus U2 Transwells. (*G*) DNA sequencing of the gRNA target region in truncated ACE2 gene from U2 (gene-edited) and U3 (wild-type) cells cultured on reused inserts. The images were captured from Inference of CRISPR Edits (ICE) analysis by Synthego (12). The U2 and U3 cells exhibited gene-edited efficiencies of 40% and 0%, respectively. (*H*) *MUC5B* and *SCGB1A1* mRNA expression of HBE cells on U2 inserts after 7-day treatment with IL-1 β ($n=5$, paired t test). (*I*) *MUC5B* Western blotting in HBE cell apical washes and cell lysates on U2 inserts after 7-day IL-1 β administration. (*J*) *MUC5B* and *SCGB1A1* mRNA expression in HBE cells on U3 inserts previously used for IL-1 β exposure in U2 cultures. (*K*) *MUC5B* Western blotting for HBE cell apical washes on U3 inserts previously used for IL-1 β administration in U2 cultures. (*L*) Scanning microscopy images of new Transwell and U3 Transwell inserts after cell removal with 0.25% trypsin-EDTA, 6M urea, or RIPA. Scale bars, 1 μm . (*M*) Whole mount staining of the HBE cells cultured on U4 Transwell and U3 Millicell (staining for the same proteins as in *D*). CBF = cilia beat frequency; PAM = protospacer adjacent motif.

Figure 1. (continued)

whether reuse of inserts after sterilization by γ irradiation or other sterilization procedures after nonsterile experiments—for example, Ussing chamber assays—is feasible.

In summary, Transwell inserts can be reused after cell removal and membrane washing when inserts are maintained in sterile conditions. Multiple measurements/assays suggest that no significant negative impact is incurred by insert reuse. Insert reuse provides a buffer for long backorder periods and may reduce experimental costs.

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COVID-19 and Coagulopathy

To the Editor:

We read with interest the recent review article by Rodriguez and colleagues regarding endothelial dysfunction and consequent thrombotic complication in patients with coronavirus disease (COVID-19) (1). Here, we would like to add a point. We will provide supplementary explanation on a molecular group (e.g., IL-1, IL-6, and TNF- α) that has been implicated in coagulation activation in the paper.

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We wish to explain the complex role of IL-6 in coagulopathy accompanied by severe inflammation. Using IL-6 knockout (IL-6^{-/-}) mice, we have previously demonstrated that IL-6 serves as a protector in coagulopathy disturbance and thrombocytopenia during devastating systemic inflammation induced by intraperitoneal administration of bacterial endotoxins (LPS) (2), which was evidenced by prothrombin time and activated partial thromboplastin time. Furthermore, we have confirmed that in the presence of LPS, α_2 -plasmin inhibitor activity was significantly lower in IL-6^{-/-} mice than in wild-type mice, indicating that IL-6 can partially inhibit LPS-provoked fibrinolysis by enhancing α_2 -plasmin inhibitor activity (3). Interestingly, fibrin degradation product was significantly lower in the IL-6^{-/-} mice administered with LPS than in the wild-type mice, whereas D-dimer was comparable in both groups (unpublished observation; these complicated results require future research for further clarification). Regardless, IL-6 may not be introduced in the same way as other proinflammatory cytokines, such as IL-1 and TNF- α , in coagulopathy disturbance with persistent inflammation.

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Reply to: COVID-19 and Coagulopathy

From the Authors:

We read the letter from Dr. Inoue and colleagues with interest and recognize the importance of pointing out that IL-6

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