Culturing Human Alveolar Organoids with Corning® Matrigel® Matrix and Transwell® Permeable Supports for Emerging Viruses Infectivity Assessment

Application Note

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Introduction

The lung is comprised of at least 40 to 60 resident cell types, each with specific physiologic functions[1-2]. Within the alveolus, alveolar epithelium comprises two cell types: type II alveolar epithelial cells that secrete surfactant proteins and are considered as tissue stem cells and type I alveolar epithelial cells that form a thin wall for gas exchange[3]. Alveolar epithelial type II cells are stem cells of the alveoli and play crucial roles in maintaining lung homeostasis and the pathogenesis of lung diseases.

Coronavirus disease 2019 (COVID-19) is a disease that causes fatal disorders including severe pneumonia. It has been reported that severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), the causative virus of COVID-19, infect and entry host cells via binding to the human angiotensin-converting enzyme II (ACE2)[4-5]. A variety of animal and cell models have been used for SARS-CoV-2 research, but a suitable *in vitro* lung model, such as alveolar organoid, is essential for investigating the mechanism underlying the viral infection in alveoli and provides a platform to evaluate candidate therapeutic agents.

Here we describe a method for generating alveolar organoids derived from human alveolar epithelial cells in Corning's Transwell permeable supports in combination with Corning Matrigel matrix. In the alveolar organoids, most of the alveolar type II cells are preserved, as well as the cell that express coronavirus entry receptor ACE2.

Materials and Methods

Alveolar Organoid Culture Medium Preparation and Corning Matrigel Matrix Handling

One day prior to experiment, Corning Matrigel matrix for organoid culture (Corning Cat. No. 356255) was thawed by submerging the vial in ice in a 4°C refrigerator overnight. Aliquots of Corning Matrigel matrix for organoid culture were always stored at -20°C and thawed as needed the night before use. Pre-chilled tips and tubes were always used when working with Matrigel matrix. Prepare alveolar organoid culture medium according to the formulation in Table 1.

Human Alveolar Organoid Culture and Passaging

After thawing the human pulmonary alveolar epithelial cells at 37°C, the cell density was adjusted to 1 x 10^5/mL with SAGM. Cell suspension was mixed at a 1:1 ratio with Matrigel matrix containing Jagged-1 peptide (1 µM; AnaSpec Cat. No. AS-61298). Aliquots of the mixture (90 µL/well) were placed in the center of the 24-well Transwell inserts (Corning Cat. No. 3470). After placing the plate at 37°C for 20 min. to allow Matrigel matrix to polymerize, 500 µL alveolar culture medium were added to the lower chamber. The medium was replaced every three days. Y-27632 (10 µM; MCE Cat. No. HY-10071) was added to the alveolar culture medium for the first three days.

For magnetic-activated cell sorting, alveolar organoids were dissociated from the Matrigel matrix using cell recovery solution (Corning Cat. No. 354253) and further incubated with Accutase® (Corning Cat. No. 25-058-CI) for 10 min. at 37°C to obtain single-cell suspensions. The cells were then incubated with anti-HT2-280 antibody (mouse IgM; Terrace Biotech Cat. No. TB-27AHT2-280; 1:100) for 20 min. at 4°C, followed by incubation with anti-mouse IgM microbeads (Miltenyi Biotec Cat. No. 130-047-302) for 20 min. at 4°C. The labeled cells were magnetically sorted twice with LS columns (Miltenyi Biotec Cat. No. 130-122-729) according to manufacturer's instructions. After sorting, 3 x 10^3 to 1 x 10^4 cells were seeded into each Transwell insert of a 24-well plate.

NOTE: Generally the cells after sorting is enough to reach the mentioned density 1x10^5. If, for some reason, the sorted cells is not enough, it is better to reduce the well numbers during the passaging process.

Table 1. Alveolar Organoid Culture Medium Formulations

Item	Vendor	Cat. No.	Final Concentration
FGF7	Novoprotein	CH73	100 ng/mL
Noggin	Novoprotein	CB89	100 ng/mL
SB431542	MCE	HY-10431	10 µM
CHIR99021	MCE	HY-10182	3 µM
Small airway epithelial cell growth medium (SAGM)	Lonza	CC-3118	Remaining

NOTE: Small airway epithelial cell growth medium (SAGM, Lonza Cat. No. CC-3118) was prepared by adding all additives except epinephrine to SABM medium.

Gene Expression Analysis

After organoid collection from the Transwell® membrane, alveolar organoids were washed several times with cold PBS (Corning Cat. No. 21-040-CV). RNA was extracted with the Magnetic Tissue/Cell/Blood Total RNA kit (Tiangen Cat. No. DP761). One-Step TB Green® PrimeScript™ RT-PCR kit (Takara Bio Cat. No. RR066A) with primers synthesized by GENEWIZ (Table 2) was used with the LightCycler® (Roche) for real-time PCR.

Table 2. Primers used for Alveolar Organoid Gene Expression Analysis

Gene	Target	Primer Sequence (5' to 3')
SPTPB	Alveolar type II cells	CCCCATTCCTCTCCCCTAT
		ACAGCTAGCGCACCCTTG
SFTPC	Alveolar type II cells	ATATAAGACCCTGGTCACACCTG
		GGGGAGCTGCGGAGTAGT
HOPX	Alveolar type I cells	ACCACGCTGTGCCTCATC
		GCGCTGCTTAAACCATTTCT
FOXJ1	Ciliated cells	GTGCTTCATCAAAGTGCCTCG
		GCCTCGGTATTCACCGTCA
KRT5	Basal cells	AGCAGATCAAGACCCTCAACA
		GGTCCACTTGGTGTCCAGAA
ACE2	Human CoV receptors	GGATACCTACCCTTCCTACATCAGC
		CTACCCCACATATCACCAAGCA

Organoid Immunohistochemistry

Alveolar organoids were harvested and washed several times with cold PBS, and then fixed using 4% paraformaldehyde (PFA) at 4°C overnight. Alveolar organoids were washed with PBST (PBS + 0.05% Tween® 20) and permeabilized with 0.2% Triton X™-100 for 30 min., followed by PBST washing several times prior to staining. For immunostaining, organoids were incubated with primary antibodies in 1:100 dilution at 4°C overnight. Prosurfactant Protein C antibody (SFTPC; Abcam Cat. No. ab90716), was used to label alveolar type II cells. ACE2 (Abcam Cat. No. ab108252) was used for SARS-CoV-2 entry receptor staining. The next day, organoids were washed with PBST, then incubated with fluorescent secondary antibodies, and nuclei stained with 2 µg/mL of DAPI. Images were captured with the Yokogawa CQ1 Image Cytometer.

Results and Discussion

It has been reported by Shiraishi K, et al. that endogenous human alveolar epithelial type II cells could be cultured and passaged with GSK-3β, Tgf-β, and Bmp4 inhibitors and Notch and Fgf7 ligands instead of co-culturing with fibroblast[7-8]. In this study, such a fibroblast-free method was used to generate alveolar organoid on Transwell permeable supports with Corning Matrigel matrix (Figure 1). The monoculture generated organoids with empty cavities in 14 days of culturing (Figure 2A; Figure 4A and 4C). For cell passaging, after magnetic-activated cell sorting, HT2-280+ cells were seeded on Transwell permeable supports and could grow into alveolar organoid after 15 days in culture (Figure 2B).

Expression data was utilized to compare expression of alveolar and non-alveolar epithelial cell markers, as well as coronavirus entry receptor, ACE2, in alveolar organoids compared to expression in the source human pulmonary alveolar epithelial cells. The qPCR analysis in Figure 3 revealed upregulation of alveolar epithelial type II cells markers, SFTPB and SFTPC, but not type I cell marker, HOPX (p=0.256). No significant change was observed in expression of ciliated cell marker, FOXJ1 (p=0.185). The transcriptional level of basal cell marker, KRT5, was decreased in alveolar organoids. The presence of alveolar type II cells in alveolar organoids was confirmed with positive staining for SFTPC (Figure 4B and 4C). These data revealed that alveolar organoid was mainly composed of alveolar epithelial type II cells.

As reported by Zhao Y, et al., the majority of the ACE2-expressing cells (83% in average) in lung are alveolar type II cells[8]. Immunofluorescence staining indicated the expression of ACE2 in alveolar organoids (Figure 4D), which was consistent with the upregulated transcriptional level of ACE2 (Figure 3). These data indicate that the expression of corona virus receptor ACE2 in alveolar cells in human can be well maintained in alveolar organoid, which provide an *in vitro* 3D model for rapid assessment of respiratory virus such as SARS-CoV-2 infection.

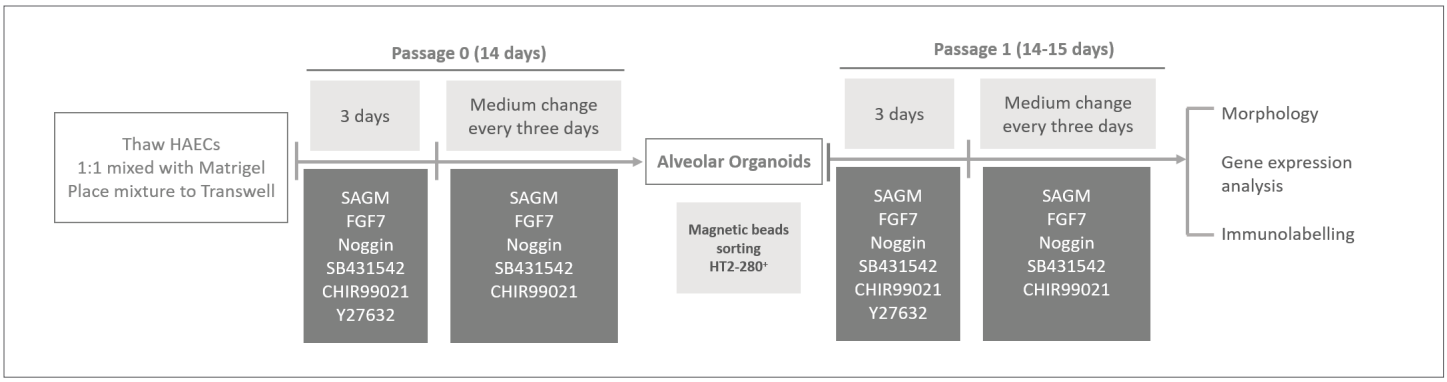

Figure 1. Schematic of the alveolar organoid generation workflow.

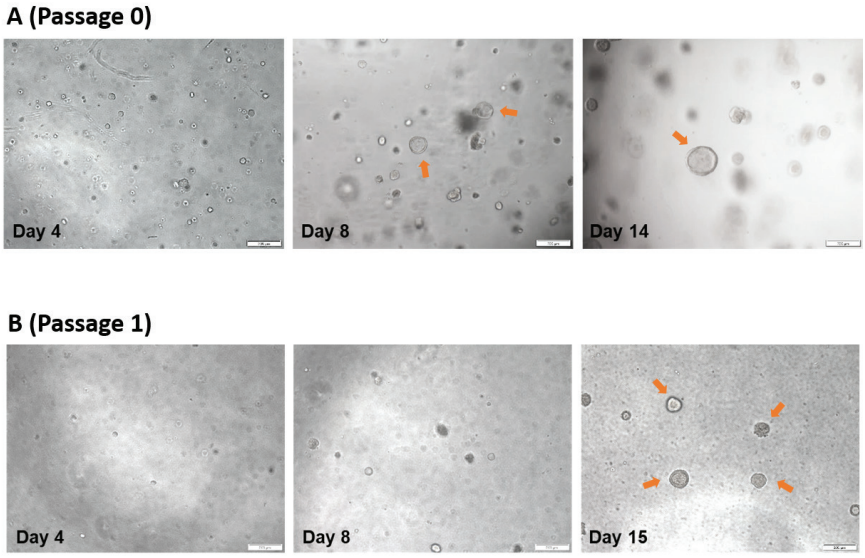

Figure 2. Bright-field images of representative wells of passaged alveolar organoids. Representative photomicrographs of alveolar organoids (passage 0, passage 1) in culture. Images were taken using 10X objective from the Olympus IX53 microscope. Scale bars are 200 µm.

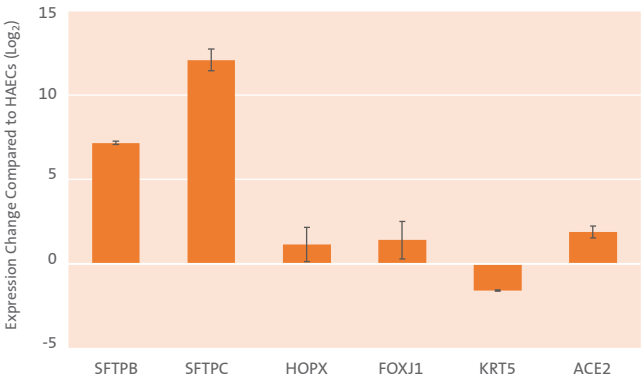

Figure 3. Gene expression in alveolar organoids. qPCR analysis of alveolar type II cell markers, SFTPB and SFTPC, alveolar type I cell marker, HOPX, non-alveolar epithelial markers, FOXJI and KRT5, and SARS-CoV entry receptor, ACE2, using primary human alveolar epithelial cells (pHAECs) and cells obtained from organoids. mRNA levels were normalized to that of β-actin. Data represent mean ± SD.

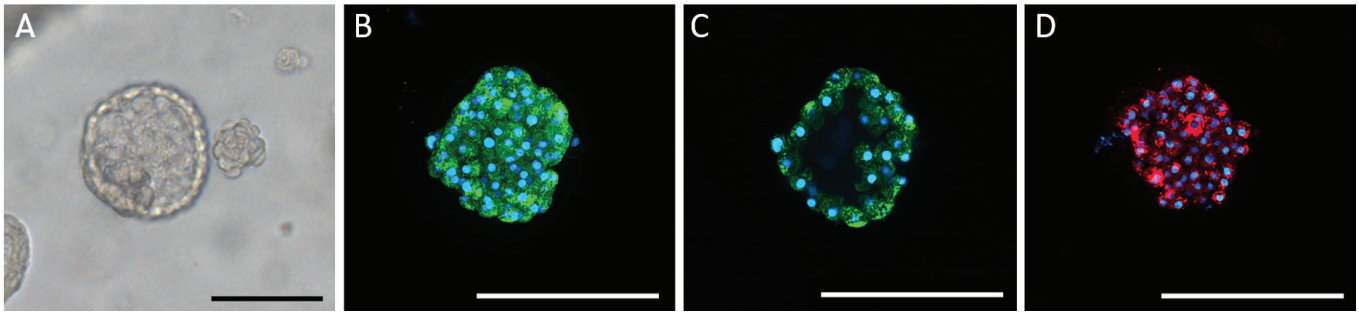

Figure 4. Representative photomicrographs of alveolar organoids. Brightfield image taken with 10X objective (A) and composite of confocal Z-stacked images of SFTPC (B) and ACE2 (D) expression with DAPI nuclei counterstain. (C) cross-sectional view of alveolar organoid in (B). Brightfield image was obtained with Olympus IX53 microscope and fluorescent images with YOKOGAWA CQ1. Scale bars are 200 µm.

Conclusions

- Alveolar organoids can be generated and passaged on Transwell® permeable supports using fibroblast-free method.
- According to the detection from both mRNA and protein levels, alveolar type II cell markers showed a high expression level in the organoids.
- Elevated expression level from both mRNA and protein of ACE2 indicate the appearance of SARS-CoV-2 entry receptor in alveolar organoids.

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