Development of alveolar and airway cells from human iPS cells: toward SARS-CoV-2 research and drug toxicity testing

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ABSTRACT — Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causes coronavirus disease 2019 (COVID-19). SARS-CoV-2 enters host cells by binding with the receptor angiotensin-converting enzyme 2 (ACE2). While ACE2 is expressed in multiple cell types, it has been implicated in the clinical progression of COVID-19 as an entry point for SARS-CoV-2 into respiratory cells. Human respiratory cells, such as airway and alveolar epithelial type II (ATII) cells, are considered essential for COVID-19 research; however, primary human respiratory cells are difficult to obtain. In the present study, we generated ATII and club cells from human induced pluripotent stem cells (hiPSCs) for SARS-CoV-2 infection and drug testing. The differentiated cells expressed ATII markers (SFTPB, SFTPC, ABCA3, SLC34A2) or club cell markers (SCGB1A1 and SCGB3A2). Differentiated cells, which express ACE2 and TMPRSS2, were infected with SARS-CoV-2. Remdesivir treatment decreased intracellular SARS-CoV-2 viral replication and, furthermore, treatment with bleomycin showed cytotoxicity in a concentration-dependent manner. These data suggest that hiPSC-derived AT2 and club cells provide a useful in vitro model for drug development.

Key words: Human iPS cell, Alveolar epithelial cell, Club cell, SARS-CoV-2, Bleomycin

INTRODUCTION

The coronavirus disease 2019 (COVID-19) pandemic started in November 2019 in Wuhan, China (Wang et al., 2020; Zhou et al., 2020). Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a pathological virus of COVID-19, and its symptoms include fever, cough and fatigue, while complications of the virus include pneumonia, pulmonary fibrosis and respiratory failure (Xu et al., 2020; Yin et al., 2021; Huang et al., 2020a). SARS-CoV-2 is known to infect cells via angiotensin-converting enzyme 2 (ACE2) and transmembrane serine protease 2 (TMPRSS2), which primes to SARS-CoV-2 spike protein to facilitate entry of the virus into mammalian cells (Hoffmann et al., 2020). Various types of cells, such as those of the cornea, esophagus, ileum, colon, liver, and heart, are known to express ACE2 in humans.

In the context of COVID-19, respiratory cells, such as airway basal, club, ciliated cells and alveolar epithelial type II cells (ATII), are strongly implicated in the pathogenesis and clinical progression of the disease (Sungnak et al., 2020). While pulmonary fibrosis is a major symptom of COVID-19 (John et al., 2021), some drugs can also induce pulmonary fibrosis as side effects, such as bleomycin, antibiotics, and anti-tumor drugs (Rossi et al., 2000; Froudarakis et al., 2013). Although bleomycin-induced pulmonary fibrosis is primarily evaluated in animal models, human relevant lung cells would be valuable for modelling pulmonary fibrosis in COVID-19 research and drug development (Wu et al., 2008; Moore and Hogaboam, 2008).

Human induced pluripotent stem cells (hiPSCs) are an attractive source for obtaining lung cells. Several groups have successfully developed respiratory cells from human induced pluripotent stem cells (hiPSCs) using two-dimen-
sional (2D) or three-dimensional (3D) organoid culture methods (Huang et al., 2014; Jacob et al., 2017; McCauley et al., 2017; Yamamoto et al., 2017). Organoid culture methods provide human ATII cells from human embryonic stem cells (hESCs) and hiPSCs. However, the apical side of epithelial cells localize inside organoids (Korogi et al., 2019; Co et al., 2019). Apical-out organoid and airway-liquid interface cultures have been examined for SARS-CoV-2 (Salahudeen et al., 2020). However, a simple and easy 2D culture method has not been fully understood.

In this study, we obtained ATII and club cells from hiPSCs using a 2D culture method. We found that the differentiated cells were able to be infected with SARS-CoV-2. In addition, treatment with remdesivir inhibited SARS-CoV-2 replication in a concentration-dependent manner. Furthermore, bleomycin induced cell death in hiPSC-derived ATII and club cells; thus, hiPSC-derived ATII and club cells in 2D culture can facilitate further research into treatments for COVID-19.

**MATERIALS AND METHODS**

**Cells**

253G1 hiPSCs (RIKEN BioResource Research Center, Tsukuba, Ibaraki, Japan) were maintained as described previously (Yamada and Kanda, 2019). VeroE6/TMPRSS2 (JCRB Cell Bank, Osaka, Japan) were maintained in DMEM supplemented with 5% fetal bovine serum (FBS; Biological Industries, Ashrat, Israel) and penicillin-streptomycin (Thermo Fisher Scientific, Waltham, MA, USA).

**Differentiation into lung progenitor cells from hiPSCs**

Lung progenitor cells were differentiated from hiPSCs using a previously described protocol (Yamamoto et al., 2017) with some modifications. Briefly, hiPSCs were dissociated with Accumax at 37°C for 5 min, and then dissociated into single cells using Accumax at 37°C for 20 min. The cells were incubated with anti-CPM antibody (Fujifilm Wako) for 30 min on ice. After washing with PBS containing 1% BSA, cells were incubated with Alexa 488 conjugated anti-mouse IgG and 7-amino-actinomycin-D (BioLegend, San Diego, CA, USA), washed with PBS containing 1% BSA, and analyzed and sorted by flow cytometry (FACS Aria II Cell Sorter, BD Biosciences, San Diego, CA, USA). Dead cells were discriminated against using 7-AAD staining. The sorted CPM+ were seeded at a density of 5 × 10^4 cells/cm^2 in Ham’s F-12 medium supplemented with 3 μM CHIR99021, 10 ng/mL human KGF, 50 nM dexamethasone (Sigma-Aldrich), 100 μM 8-Bromoadenosine 3′,5′-cyclic monophosphate sodium salt (8-Br-cAMP, Biolog Life Science, Bremen, Germany), 100 μM 3-Isobutyl-1-methylxanthine (Fujifilm Wako), 10 μM SB431542, and 1 μM LDN193189 (Fujifilm Wako) on cell culture plate pre-coated with Matrigel Growth Factor Reduced (GFR) Basement Membrane Matrix (Corning, Acton, MA, USA). The culture medium was changed every two days.

**Quantitative real-time reverse transcription PCR (qPCR)**

Total RNA was extracted from differentiated cells using TRIzol reagent (Thermo Fisher Scientific) as previously described (Yanagida et al., 2021). qPCR was performed using a QuantiFect SYBR Green RT-PCR kit (QIAGEN, Valencia, CA, USA) using an ABI PRISM 7900HT sequence detection system (Applied Biosystems, Foster City, CA, USA). Gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase GAPDH expression. Total RNA from human adult lungs

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Immunocytochemistry

Cell staining was performed as previously described (Hirata et al., 2014). Briefly, cells were fixed, permeabilized, blocked, and incubated with primary antibodies against SOX17 (1:500; R&D Systems), FOXA2 (1:1000; Cell Signaling Technology), TTF1/NKX2-1 (1:400; Abcam, Cambridge, UK), mature-SFTPB (1:500; Seven Hills Bioreagents, Cincinnati, OH, USA), pro-SFTPC (1:200; Abcam), SCGB3A2 (1:500; Abcam), and ACE2 (1:100; R&D systems), SARS-CoV-2 nucleocapsid (1:100; GeneTex, Irvine, CA, USA), or SARS spike glycoprotein (1:100; Abcam) in 5% FBS in PBS overnight at 4°C. After washing with PBS, cells were incubated with secondary antibodies, Alexa 488-conjugated anti-mouse IgG, Alexa 488-conjugated anti-goat IgG, and Alexa 594-conjugated anti-rabbit IgG (1:200; Thermo Fisher Scientific) in 5% FBS for 1 hr at room temperature. Nuclei were counterstained with 4,6-Diamidino-2-phenylindole dihydrochloride (DAPI; Nacalai Tesque, Kyoto, Japan). Cells were mounted with VECTASHIELD mounting medium (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were captured using a BIOREVO BZ-9000 fluorescence microscope (Keyence, Osaka, Japan) or a Nikon A1 confocal microscope (Nikon, Tokyo, Japan).

SARS-CoV-2 infection

The SARS-CoV-2 strain (JPN/TY/WK-521) was obtained from the National Institute of Infectious Diseases in Japan. The differentiated cells on day 35 were infected with SARS-CoV-2 at a multiplicity of infection (MOI) of 1. Remdesivir was administered 1 hr post-infection. After 48 hr post infection, the supernatant of the cell culture was collected and stored at −80°C until use in the plaque assay. After washing with PBS, RNA was extracted using the CellAmp direct RNA prep kit (TAHARA) and then qPCR was performed with TaqMan Fast Virus 1-Step Master Mix (Thermo Fisher Scientific), 2019-nCoV RUO Kit, and 2019-nCoV_N_positive control (Integrated DNA Technologies, Coralville, IA, USA) using the ABI PRISM 7900HT sequence detection system (Applied Biosystems).

Plaque assay

The plaque assay was performed as previously described (Baer and Kehn-Hall, 2014). Briefly, VeroE6/TMPRSS2 cells were seeded on 12-well plates and incubated with serially diluted cell culture supernatant stock at 48 hr post-infection. After 1 hr of incubation, the medium was replaced with 1% methylcellulose-containing medium and cultured for 72 hr. The cells were fixed with 4% paraformaldehyde and stained with methylene blue. Plaques were counted to determine the virus titers.

Cell viability assay

The differentiated cells were treated with bleomycin sulfate (Selleck Chemicals, Houston, TX, USA). After 72 hr, cell viability was measured using the CellTiter 96 AQueous One Solution Cell Proliferation Assay (Promega, Madison, WI, USA). Absorbance was measured at 490 nm using an iMark microplate reader (Bio-Rad, Hercules, CA, USA). The 50% inhibitory concentration (IC50) value was calculated by nonlinear regression using GraphPad Prism (GraphPad Software, La Jolla, CA, USA).

Table 1. PCR Primers for qPCR.

<table>
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<th>Target gene</th>
<th>Forward primer sequence</th>
<th>Reverse primer sequence</th>
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<td>GAPDH</td>
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<td>ACCACCCCTTGTCTGTAGCACA</td>
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<td>NKX2-1</td>
<td>AGCCACAGTCCTCGTCTCCT</td>
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<tr>
<td>CPM</td>
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<td>ACE2</td>
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<td>TMPRSS2</td>
<td>CAGGAGTGACACGGGAACTGTGGT</td>
<td>GATTAGCGTCTGGCCCTTCTTT</td>
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RESULTS

Differentiation into lung progenitor cells from hiPSCs

Previous reports suggest that lung progenitor cells are developed by NKX2-1+ cells (Kimura et al., 1996). Because NKX2-1 is a transcription factor, the surface marker carboxypeptidase M (CPM) has been identified to characterize lung progenitor cells (Gotoh et al., 2014). First, we differentiated hiPSCs into CPM+ lung progenitor cells (Fig. 1). qPCR revealed that the differentiated cells expressed definitive endoderm markers SOX17 and FOXA2 on day 6 (Fig. 1B, C). In addition, FOXA2 was expressed as an anterior foregut endoderm marker on day 10 (Fig. 1B). Immunocytochemical analysis confirmed the PCR data. On day 21, the cells expressed the lung progenitor markers CPM and NKX2-1 (Fig. 1D, E), and then CPM+ cells were sorted by flow cytometry (Fig. 1F).

Differentiation of ATII and club cells from hiPSCs-derived lung progenitor cells

The resultant CPM+ cells were cultured to induce the formation of lung cells (Fig. 2A). As shown in Fig. 2B and C, qPCR analysis revealed that the differentiated cells expressed ATII markers (SFTPB, SFTPC, ABCA3, SLC34A2) and club cell markers (SCGB3A2, SCGB1A1) on day 35. Furthermore, immunocytochemical analysis showed that the differentiated cells expressed both ATII markers (SFTPB and SFTPC) and club cell markers (SCGB3A2). In contrast, other cell markers, such as basal cell marker KRT5, goblet cell marker MUC5AC, and ciliated cell marker SNTN, were not detected by qPCR (data not shown). These data suggest that human iPSC-derived lung progenitor cells differentiate into both ATII and club cells.

Infection of SARS-CoV-2 in hiPSCs-derived ATII and club cells

We next examined whether hiPSC-derived ATII and club cells were able to be utilized for SARS-CoV-2 research. The entry of SARS-CoV-2 into target cells is initiated by the binding of the spike (S) protein to ACE2 (Hoffmann et al., 2020). The S-protein is cleaved by the TMPRSS2 serine protease and triggers virus entry into the target cell. We investigated ACE2 expression levels in differentiated cells. qPCR analysis revealed that both AT2 and club cells from hiPSCs expressed ACE2 and TMPRSS2 on day 35 (Fig. 3A). Immunocytochemistry analysis showed that ACE2 was co-expressed with SFTPB or SCGB3A2 (Fig. 3B). We then examined whether SARS-CoV-2 was infected with hiPSC-derived ATII and club cells. As shown in Fig. 4A, RNA copies of SARS-CoV-2 were detected in the differentiated cells (Fig. 4A). Spike proteins were co-stained with SFTPB or SCGB3A2 (Fig. 4B), suggesting that SARS-CoV-2 was infected into ATII and club cells.

To confirm whether the cells could be used for testing antiviral drugs, we investigated the effect of remdesivir in hiPSC-derived ATII and club cells. Treatment with remdesivir at 1 hr post-infection decreased the RNA copies of SARS-CoV-2 in a concentration-dependent manner (Fig. 4C). The plaque assay confirmed the RNA copies of SARS-CoV-2 replication in the cells (Fig. 4D). These data indicate that cells differentiated from hiPSCs could be used to evaluate drugs for COVID-19.

Effect of bleomycin treatment

We further examined whether hiPSC-derived ATII and club cells could be utilized for drug testing. Progressive fibrosis is a potential consequence of pulmonary pneumonia induced by SARS-CoV-2 (John et al., 2021). Because bleomycin is known to induce pulmonary fibrosis and cytotoxicity (Yue, 2017; Rossi et al., 2000), we investigated the effect of bleomycin in differentiated cells. After treatment with BLM for 72 hr, cell viability decreased in a concentration-dependent manner. The IC50 of bleomycin was approximately 13.1 μM (Fig. 5). These data suggest that differentiated cells from hiPSCs can be used to assess toxicity in fibrosis.

DISCUSSION

In the present study, we demonstrated that ATII and club cells differentiated from hiPSCs can be used as in vitro models for COVID-19 research and drug testing. Treatment with remdesivir inhibited viral replication in hiPSC-derived ATII and club cells. We further demonstrated that bleomycin induced cytotoxicity in hiPSC-derived cells.

We differentiated hiPSCs into ATII and club cells by confirming ATII markers (SFTPB, SFTPC, ABCA3, SLC34A2) and club cell markers (SCGB3A2, SCGB1A1) (Fig. 2). Similar to previous studies, we found that SFTPC expression levels in 2D culture were lower than those in SFTPB expression. This may be due to the immature property of hiPSC-derived differentiated cells. Indeed, other studies have also reported the immature properties of differentiated cells (Yamamoto et al., 2017; Huang et al., 2014). To date, many efforts have been made to find compounds that improve immaturity or differentiation propensity. For example, temporal withdrawal of the WNT agonist CHIR99021 after sorting NKX2-1+/SFTPC+ cells increased ATII maturation but not ATII
Fig. 1. Differentiation from hiPSCs to lung progenitor cells. (A) Scheme of differentiation protocol from hiPSCs to lung progenitor cells. (B) Expression levels of SOX17 and FOXA2 were examined in the differentiated cells on day 6 and 10 by qPCR. Data were normalized by day 0 and represented as mean ± standard deviation (S.D.; n = 3). (C) Immunocytochemical analysis in the differentiated cells on day 6. Cells were stained with antibodies against SOX17 and FOXA2. Nuclei were stained with DAPI. Scale bars, 20 μm. (D) Expression levels of CPM and NKX2-1 on day 21 were examined by qPCR. Data were normalized by day 0 and represented as mean ± SD (n = 3). (E) Immunocytochemical analysis in the differentiated cells on day 21. Cells were stained with antibodies against CPM and NKX2-1. Nuclei were stained with DAPI. Scale bars, 20 μm. (F) Representative flow cytometry plots of the differentiated cells on day 21. Cells were stained with antibody against CPM. CPM⁺ cells were sorted by flow cytometry for further differentiation.
Fig. 2. Generation of ATII and club cells from hiPSC-derived lung progenitor cells. (A) Scheme of generation of ATII and club cells from hiPSC-derived lung progenitor cells. (B) Gene expression levels of SFTPB, SFTPC, ABCA3, SLC34A2, SCG-B3A2, and SCGB1A1 were examined in the differentiated cells on day 21 by qPCR. Data were normalized by day 0 and represented as mean ± S.D. (n = 3). (C) Immunocytochemical analysis in the differentiated cells on day 35. Cells were stained with antibodies against SFTPB, SFTPC, and SCGB3A2. Nuclei were stained with DAPI. Scale bars, 20 μm.
cell proliferation in human ES-derived ATII cells (Jacob et al., 2017). CHIR99021 was also shown to enhance airway fate in lung progenitor cells (McCauley et al., 2017). Further studies should be conducted to obtain more mature ATII and club cells using a customized protocol.

We found that hiPSC-derived ATII and club cells expressed ACE2 were infected with SARS-CoV-2 (Fig. 3, 4). AT2 cells have been studied for SARS-CoV-2 infection. Several studies have reported SARS-CoV-2 infection in club cells (Salahudeen et al., 2020; Hui et al., 2020). Single-cell RNA sequencing analysis revealed that ACE2 expression in club cells is higher than AT2 in human lung tissue (Sungnak et al., 2020), suggesting that both cells are infected by SARS-CoV-2. Remdesivir reduced viral replication in hiPSC-derived ATII and club cells (Fig. 4 C and D). This is consistent with the results of our study. Pretreatment with 1 μM remdesivir decreased SARS-CoV-2 gene expression from approximately 10^8 to 10^5 using an air-liquid interface culture of ATII differentiated from hiPSCs (Huang et al., 2020b). Remdesivir at 1 μM SARS-CoV-2 infection decreased RNA copies from 10^8 to 10^5 at 2 days post-infection using ATII from primary human lung tissue (Ebisudani et al., 2021). The sensitivity of remdesivir was very similar among these in vitro models. Our culture system is expected to screen and confirm the effectiveness of drugs against SARS-CoV-2.

In addition to our 2D model, 3D organoids from hESCs have been reported to be infected with SARS-CoV-2 (Han et al., 2021; Pei et al., 2020). These hESC-derived lung organoids have been used to evaluate antiviral compounds. The 3D models are expected to be superior to the 2D models in terms of maturity; however, the apical side is known to localize inside organoids (Han et al., 2021). Furthermore, it is not fully understood
whether maturity enhances drug safety evaluation using hiPSC-derived cells. Future studies should determine whether apical-out mature organoids are suitable for drug development. Our culture system focuses only on alveolar and airway cells. However, serial immunological reactions post-infection with SARS-CoV-2 have not yet been studied. The cell types most relevant for understanding the pathophysiological significance of SARS-CoV-2 need to be determined. In addition, immunological reactions should be considered in the model for virus research.

**Fig. 4.** SARS-CoV-2 infection and effect of remdesivir in hiPSC-derived ATII and club cells. (A) Immunocytochemical analysis of the differentiated cells on day 35, which were infected with SARS-CoV-2 (MOI = 1, 48 hr post infection). Cells were stained with antibodies against SARS-CoV-2 nucleocapsid and SARS spike glycoprotein. (B) Immunocytochemical analysis of the differentiated cells on day 35, which were infected with SARS-CoV-2 (MOI = 1, 48 hr post infection). Cells were stained with antibodies against SFTPB, SCGB3A2, and SARS spike glycoprotein. Nuclei were stained with DAPI. Scale bars, 20 μm. (C) SARS-CoV-2 nucleocapsid protein RNA copies in differentiated cells on day 35 infected with SARS-CoV-2. Cells were treated with remdesivir or vehicle 1 hr after SARS-CoV-2 infection (MOI = 1). After 48 hr post infection, RNA was extracted for qPCR. (D) The differentiated cells on day 35 were treated with remdesivir (1 μM) or vehicle 1 hr after SARS-CoV-2 infection (MOI = 1). After 48 hr post infection, viral titers in the culture supernatants were determined by plaque assay in Vero E6 cells. Data are represented as mean ± S.D. (n = 3).
We demonstrated that hiPSC-derived ATII and club cells are useful in drug-induced pulmonary fibrosis tests (Fig. 5). The anti-cancer drug bleomycin is known to induce lung fibrosis. Animal models and A549 cell lines have been widely used to investigate bleomycin-induced lung damage (Aoshiba et al., 2003; Tian et al., 2017; Moore and Hogaboam, 2008). IC50 value for cell viability of bleomycin in our differentiated cells (13.1 μM) is lower than that in A549 cells (93.9 ± 8.7 mg/L = 65 μM; Wu et al., 2008). Because the maximum plasma concentration (Cmax) of bleomycin is 4.32 μg/mL, which is equivalent to approximately 3 μM, in Hodgkin’s lymphoma patients (Malik et al., 2016), A549 cells do not have sufficient sensitivity to detect fibrosis. Thus, hiPSC-derived models are considered suitable for drug safety evaluation. A recent study showed that TGF-β1 induced fibrosis in the organoid from hiPSCs provided alveolar epithelial cell type 1 cells (AT1), ATII, and mesenchymal cells (Kim et al., 2021). Future studies should examine suitable endpoints to detect drug-induced fibrosis using in vitro models.

In summary, our results demonstrate that hiPSC-derived ATII and club cells are permissive to SARS-CoV-2 infection. Human hiPSC-derived cells can be used to evaluate the effectiveness of remdesivir and bleomycin toxicity. Thus, the in vitro models developed here are expected to be useful for drug development.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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