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## FULL-LENGTH ARTICLE

## Basic Research

## Differentiation of immortalized human multi-lineage progenitor to alveolar type 2-like cells: angiotensin-converting enzyme 2 expression and binding of severe acute respiratory syndrome coronavirus 2 spike and spike 1 proteins

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## ABSTRACT

Along with the nasal epithelium, the lung epithelium is a portal of entry for sudden acute respiratory syndrome coronavirus 2 (SARS-CoV-2) and many other respiratory viruses. In the case of SARS-CoV-2, the virus surface spike proteins bind to the angiotensin-converting enzyme 2 (ACE-2) receptor to facilitate entry into the respiratory epithelium. Alveolar type 2 (AT2) cells are committed respiratory progenitor cells responsible for the integrity and regeneration of the respiratory epithelium and production of respiratory surfactant proteins. AT2 cells express high levels of surface ACE-2 and thus are a leading target for primary infection by SARS-CoV-2. This study describes a method for directly differentiating telomerase reverse transcriptase-immortalized human cord blood-derived multi-lineage progenitor cells (MLPCs) to AT2-like cells for the purpose of generating an *in vitro* cellular platform for viral studies. Differentiation was confirmed with the acquisition of AT2 and absence of alveolar type 1 (AT1) specific markers by confocal microscopy. Expression of the ACE-2 receptor was confirmed by immunofluorescence antibody staining, quantitative reverse transcription polymerase chain reaction and binding of biotinylated SARS-CoV-2 spike and spike 1 proteins. The binding of biotinylated spike proteins was specifically blocked by unlabeled spike proteins and neutralizing antibodies. Additionally, it was demonstrated that the spike protein was internalized after binding to the surface membrane of the cells. The authors defined the culture conditions that enabled AT2-like cells to be repeatedly passaged and cryopreserved without further differentiation to AT1. The authors' method provides a stable and renewable source of AT2 cells for respiratory viral binding, blocking and uptake studies.

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## Introduction

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is a highly transmissible virus that is responsible for the current coronavirus disease 2019 pandemic, negatively impacting global health and economies [1–3]. High-level transmission and mortality rates distinguish the SARS-CoV-2 virus from related coronaviruses severe acute respiratory syndrome-associated coronavirus (SARS-CoV) and Middle East respiratory syndrome. However, pneumonia-related symptoms,

including cough, fever, shortness of breath and fatigue, are similar in all three infections [2,4]. Differences in transmissibility and variable individual immune responses to SARS-CoV-2 account for dramatically increased numbers of infections, prolonged periods of morbidity and higher mortality compared with SARS-CoV and Middle East respiratory syndrome [5–8].

Viral entry into cells is the essential first step in the infectivity and pathogenesis of SARS-CoV-2 [5,9–11]. Studies have determined that a major portal of entry for the SARS-CoV-2 virus is the cell surface expressed protein angiotensin-converting enzyme 2 (ACE-2). Attachment and entry of the virus via the ACE-2 receptor are mediated by spike proteins located on the surface of the virus. The spike protein is expressed as a trimer, with each monomer consisting of two distinct

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domains: S1 and S2. The S1 domain contains the receptor-binding domain (RBD) responsible for the initial binding of the virus to ACE-2. The presence of TMPRSS2 proteases and lysosomal cathepsin proteins on the target cells are responsible for cleavage of the S1 and S2 domains, which allows the S2 domain to mediate viral entry via membrane fusion [5]. The SARS-CoV virus also relies on the ACE-2 receptor for cellular invasion, but differences in the RBD of the two viruses may contribute to the higher affinity of the SARS-CoV-2 spike protein in binding to ACE-2 and, potentially, the severity of infection [5,12–14]. Inhibition of binding of the spike protein to the ACE-2 receptor has been identified as a target for the development of therapeutics against SARS-CoV-2 [10,15]. Antibodies present in convalescent serum and monoclonal antibodies directed against the spike protein have been tested for therapeutic potential, but their potential effectiveness has yet to be fully determined.

The lung epithelium is involved in many, if not most, SARS-CoV-2 infections, even those that are asymptomatic. Epithelial cells that line the alveoli and bronchi of the lungs express high levels of ACE-2 and as such are highly susceptible to viral attachment and entry [4]. Severe cases of pulmonary SARS-CoV-2 infection are associated with cell death and fibrosis of lung tissue. Alveolar type 2 (AT2) cells play multiple roles in proper alveolar functionality [16]. They act as stem cells for the regeneration of the lung epithelium by differentiating to alveolar type 1 (AT1) cells that line the alveoli and mediate gas exchange. They also produce the surfactants (lipids and proteins) that maintain the osmotic integrity of the alveoli. The regenerative properties of AT2 cells and their potential loss due to pathogenic processes such as SARS-CoV-2 infection make them a desirable cell type for study. Commercial sources exist for lung epithelial cells; however, the utility of these cells is limited by their low *in vitro* longevity, limited expansion potential and high cost. Additionally, donor-to-donor differences in primary small airway epithelial cells (SAEpi) could affect functionality. The authors sought to define the process by which immortalized AT2 cells could be generated and propagated as a tool to study viral infection.

A previous study using human umbilical cord blood-derived multilineage progenitor cells (MLPCs) demonstrated a simple one-step method for the differentiation of MLPCs to cells resembling AT2 cells in morphology and expression of surfactant protein C (SPC) [17]. The method was confirmed using polyclonal and clonal non-immortalized MLPCs. MLPCs are distinguished from mesenchymal stem cells (MSCs) by their gene expression, ability to be differentiated to non-mesodermal lineages and greatly enhanced capacity to be expanded, enabling the development of clonal cell lines with uniform characteristics [18–22].

Recently, the authors demonstrated the ability of telomerase reverse transcriptase (TERT)-transfected MLPCs to become functionally immortalized cells while retaining the ability for further differentiation [23]. The methodology to differentiate non-immortalized MLPCs to AT2-like cells [17] was utilized with the TERT-immortalized MLPCs. This resulted in the development of long-lived and expandable human AT2-like cells that overcame the myriad limitations observed with commercially available primary human SAEpis.

The TERT-MLPC-derived AT2-like cells were characterized by their expression of surfactant protein, AT2- and AT1-specific markers, ACE-2 and ability to bind and internalize SARS-CoV-2 spike proteins. The ability of the AT2-like cells to specifically bind spike proteins makes them useful for the study of SARS-CoV-2 binding and agents that could inhibit that binding. Ultimately, with additional characterization, these novel cells could provide an *in vitro* model to study the direct effects of SARS-CoV-2 and other respiratory pathogens on AT2 cells and their role in cell injury.

## Methods

### Development of MLPCs

MLPCs are multi-potent stem cells isolated from human umbilical cord blood. Umbilical cord blood was collected as part of a Food and

Drug Administration submission to market PrepaCyte-CB (Cryo-Cell International, Oldsmar, FL, USA), a product to debulk cord blood for cryobanking and transplantation. Institutional review board approval of the study was conducted March 3, 2005, by the University of Minnesota (Minneapolis, MN, USA) and St Louis Cord Blood Bank (St Louis, MO, USA) using quorum review protocol #800. The cord blood samples were collected by the American Red Cross Cord Blood Program (Saint Paul, MN, USA) and Ridgeview Medical Center (Waconia, MN, USA). Donations were collected with donor consent for research use only.

MLPCs were isolated as previously described [21–24]. Briefly, after mixing and sedimentation with PrepaCyte-MS (CytoMedical Design Group, LLC, Saint Paul, MN, USA), cord blood mononuclear cells isolated from the supernatant were plated at a concentration of  $1.33 \times 10^6$  cells/cm<sup>2</sup> in MSCGM medium (PT-4105; Lonza, Walkersville, MD, USA). After 24 h, non-adherent cells were removed, leaving adherent cells with a mostly fibroblastic morphology. Cells were cultured in MSCGM until 80–90% of the cells had a fibroblastic morphology. These cells were used to develop MLPC clonal cell lines, TERT-transfected polyclonal cells and clonal TERT cell lines.

### Immortalization of MLPCs

MLPCs were immortalized by insertion of the gene for human TERT as previously described [23]. The pRRlsin.hCMV human TERT lentiviral expression plasmid was the kind gift of Dr Noriyuki Kasahara, Department of Medicine, University of California, Los Angeles (Los Angeles, CA, USA). Polyclonal TERT-transduced MLPCs were the kind gift of Dr Eve Kelland, Department of Neurology, Keck School of Medicine, University of Southern California (Los Angeles, CA, USA).

### Development of E12 cell line

Clonal MLPC-TERT cell lines were developed by limited dilution cloning as previously described [23]. One clone, E12 TERT (E12), exhibited the desired characteristics of immortality and differentiation beyond mesodermal outcomes. E12 cells have been repeatedly cryopreserved, thawed and expanded for over 14 years and were used in this study.

### Primary SAEpis

Primary SAEpis were obtained from ScienCell Research Laboratories (3230; Carlsbad, CA, USA). A total of  $5 \times 10^5$  cells were plated in a 75-cm culture vessel coated with poly-L-lysine. Cells were grown and expanded using 13 mL of small airway growth medium (SAGM) (3118; Lonza), with three medium exchanges per week.

### Differentiation of E12 cells to AT2-like cells

E12 cells ( $3 \times 10^5$  cells/mL) in MSCGM were added to non-coated tissue culture vessels and allowed to attach overnight. Medium was then exchanged with SAGM and allowed to culture for 8–14 days, with three medium changes per week. Upon achieving 70% confluence, cells were harvested by treatment with TrypLE (12605-028; Life Technologies, Grand Island, NY, USA) to dissociate them from the culture vessel, and cells were used for confocal analysis, further expansion in SAGM or cryopreservation.

### Confocal immunofluorescence analysis

An aliquot of cells ( $2 \times 10^4$  cells/200  $\mu$ L) was plated per well in 16-well chamber slides (Nalge Nunc International, Rochester, NY, USA). E12 cells were plated in MSCGM in non-coated wells, E12 AT2 cells were plated in SAGM in non-coated wells and SAEpis were plated in SAGM in poly-L-lysine-coated wells. Cells were cultured for 1–2 days to allow attachment prior to processing and analysis. To

prepare the cells for staining, cells were fixed for 1 h in 1% formalin and permeabilized using PermaCyte medium (WBP-1000; CytoMedical Design Group, LLC). This procedure allowed for visualization of both cytoplasmic and surface markers.

Cells were incubated with unlabeled primary antibody (100 ng) for 30 min at room temperature. Antibodies specific for cytokeratin 19 (CK19) (MAB3608), epithelial cell adhesion molecule (EpCAM) (MAB9601), SOX17 (MAB1924), CD26 (MAB1180) and ACE-2 labeled with Alexa Fluor 594 (FAB9332T) were obtained from R&D Systems (Minneapolis, MN, USA). Antibodies specific for TERT (NB100-317) and caveolin-1 (CAV1) (NBP2-80617) were obtained from Novus Biologicals (Littleton, CO, USA). Antibodies specific for surfactant protein C (PIPA579988), TM4SF1 (PA5-21119) and aquaporin (MA5-32593) were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Antibody specific for HT2-280 (TB-27A-HT2-280) was obtained from Terrace Biotech (San Francisco, CA, USA). Marker expression was confirmed by positive staining compared with cells stained with mouse, rat or rabbit antibody isotype controls (CytoMedical Design Group, LLC).

Unbound antibody was removed by washing each well twice with 200  $\mu$ L of PermaCyte. Cells were incubated with 200 ng of secondary antibodies labeled with Alexa Fluor 594 specific for mouse (A-11005), rabbit (A-11072) or rat (A-11007) primary antibodies (Life Technologies) for analysis on the Olympus FluoView 1000 confocal microscope (Olympus, Center Valley, PA). Nuclei were visualized by staining with 4',6-diamidino-2-phenylindole (DAPI) in which the nuclei appeared blue. This procedure is outlined in [Figure 1A](#). All images were observed by separation of blue and red fluorescence and enumerated for expression of each marker as a percentage of blue nuclei, also staining for the red fluorescence of each marker.

#### Quantitative reverse transcription polymerase chain reaction analysis

Total RNA was isolated using the RNeasy mini kit (Qiagen, Hilden, Germany) and reverse-transcribed using SuperScript IV VIL0 (Thermo Fisher Scientific). TaqMan gene expression was performed using ACE-2 ([Hs01085331\\_m1](#)), DPP4 (CD26) ([Hs00897405\\_m1](#)), EpCAM ([Hs00158980\\_m1](#)), SPC ([Hs00951326\\_g1](#)), CK19 ([Hs00761767\\_s1](#)), TM4SF1 ([Hs05418027\\_s1](#)) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) ([Hs02786624\\_g1](#)) probes (Thermo Fisher Scientific). Data were normalized to GAPDH.

#### Analysis of SARS-CoV-2 spike and spike 1 binding

The ability of cells to bind SARS-CoV-2 spike and spike 1 proteins was analyzed by confocal microscopy using biotinylated spike proteins. Cells were prepared as described earlier and labeled with 250 ng of either biotinylated spike protein (RBD) (SPD-C82E9; ACRO-Biosystems, Newark, DE, USA) or biotinylated spike 1 protein (SIN-C82E8; ACROBiosystems) for 30 min. Unbound spike proteins were removed by washing cells twice with 200  $\mu$ L of PermaCyte medium. Bound spike proteins were visualized by staining with streptavidin-Alexa Fluor 594 (S11227; Life Technologies). Cells were counterstained with DAPI to visualize the nuclei. This procedure allowed visualization of binding to surface and cytoplasmic ACE-2 and is outlined in [Figure 1B](#).

#### Internalization of spike protein

The ability of the biotinylated spike protein to be internalized was examined with E12 MLPCs, E12 AT2 cells and primary SAEpis. Briefly, cells were plated in 16-well chamber slides as described earlier. A total of 250 ng of biotinylated spike protein was added to each well, and the cells were incubated for 2 h at 37°C. Unbound spike protein was removed by washing three times with phosphate-buffered saline. Cells were then fixed with 1% formaldehyde for 1 h at room

temperature. The cells were then permeabilized by PermaCyte and stained with streptavidin-Alexa Fluor 594. The nuclei of the cells were visualized with DAPI. This procedure allowed visualization of the spike protein binding to surface receptors, but cytoplasmic staining was due only to internalization of the spike protein. This procedure is outlined in [Figure 1E](#).

#### Specific blockade of spike and spike 1 binding

The specificity of biotinylated spike proteins binding to E12 AT2 cells was confirmed by blocking with a 5-M excess of unlabeled spike protein. Cells were prepared as described for confocal analysis. Cells were incubated with 1.25  $\mu$ g of unlabeled spike protein (SPD-S52H6; ACROBiosystems) for 1 h. Without washing the unbound unlabeled spike protein, biotinylated spike and spike 1 proteins were added to the cells and incubated for 30 min. Cells were washed twice with PermaCyte medium to remove any unbound proteins. Visualization of bound biotinylated spike proteins was accomplished by secondary labeling with streptavidin-Alexa Fluor 594. Cells were counterstained with DAPI to visualize nuclei. This procedure is outlined in [Figure 1C](#).

To examine the effects of neutralizing antibody on binding of the spike proteins, commercially available neutralizing antibodies were obtained from ACROBiosystems (SAD-S35) and Novatein Biosciences (PR-nCOV-mABS1; Woburn, MA, USA). A total of 1  $\mu$ g of either antibody was pre-incubated with the spike protein for 1 h prior to addition of the mixture to the cells prepared as previously described for binding of spike proteins. Visualization of the binding of biotinylated spike protein was accomplished by secondary staining with streptavidin-Alexa Fluor 594. The nuclei of the cells were visualized with DAPI. This procedure is outlined in [Figure 1D](#).

## Results

#### Cellular morphology typified the differentiated AT2 cells

The morphology of undifferentiated E12 MLPCs, differentiated E12 AT2 cells and primary SAEpis is shown in [Figure 2](#). E12 MLPCs were characterized as mononuclear with a spindle-shaped morphology. Differentiated E12 AT2 cells were round or cuboidal in morphology with either one or two nuclei per cell. Primary SAEpis also appeared round to cuboidal in morphology with one to two nuclei. Under less than confluent conditions, some of the differentiated E12 AT2 cells and SAEpis expressed a more elongated and spindle-shaped morphology.

#### Confocal microscopy indicated specificity of binding to AT2 cells

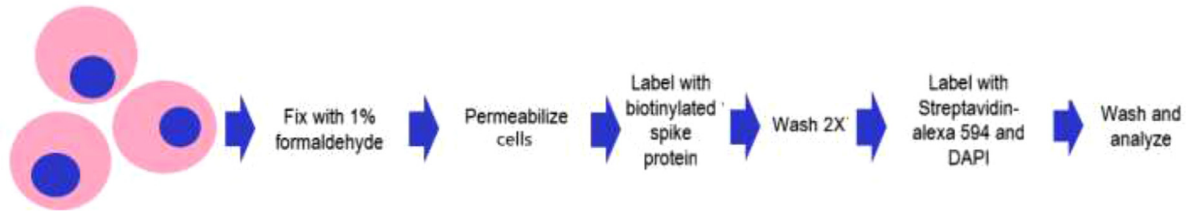
Confocal microscopy results are summarized in [Table 1](#) and shown in [Figures 3–5](#). Results with isotypic antibodies to determine baseline non-specific binding of antibodies to the different cell types are shown in supplementary Figure 1. E12 MLPC control cells, E12 AT2 differentiated cells and primary SAEpis were labeled with antibodies specific for markers associated with commitment to endoderm (SOX17), AT2-associated markers (SPC, ACE-2, TM4SF1 and HT2-280), AT1-associated markers (advanced glycosylation end product-specific receptor [AGER], CAV1 and aquaporin) and other markers not specific to AT2 cells but known to be expressed by AT2 cells (CK19, CD26 and EpCAM). Additionally, cells were tested for expression of TERT, a marker associated with the ability to repair telomeres and extend the lifetime of cells. In a previous study, TERT expression in E12 MLPCs was confirmed by fluorescence in situ hybridization analysis [24]. Additional analysis of SPC expression in SAEpis and E12 AT2-like cells is presented in supplementary Figure 2. Similarly, a study of ACE-2 expression in SAEpis and E12 AT2-like cells is presented in supplementary Figure 3.



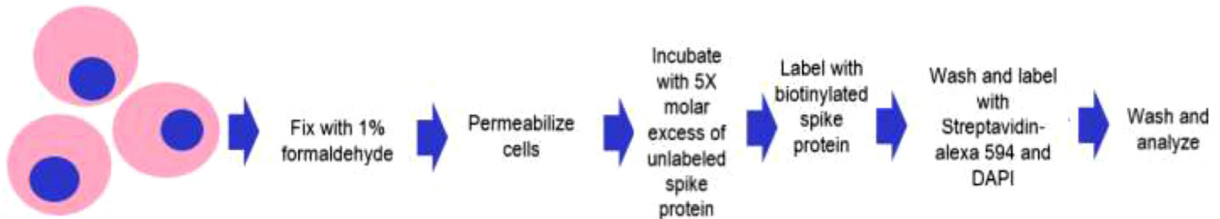
### A. Staining with Antibody



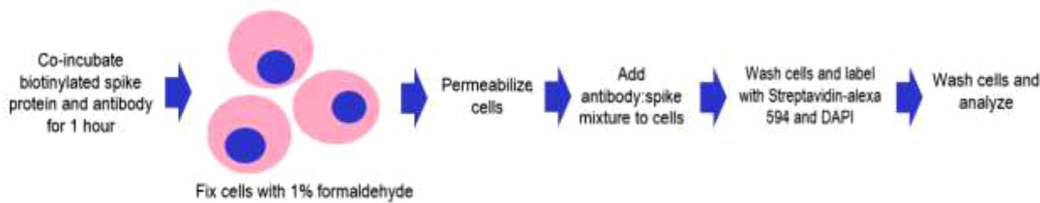
### B. Staining with Spike Protein



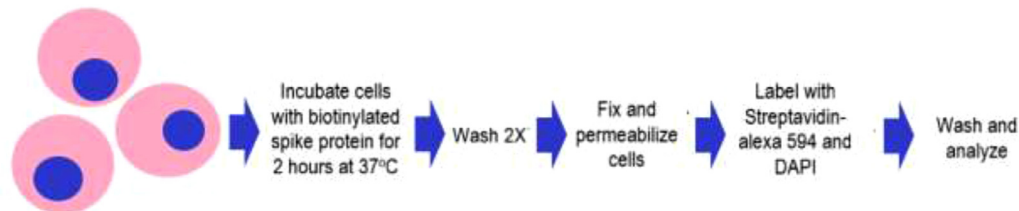
### C. Blocking by Spike Protein



### D. Blocking by Antibody



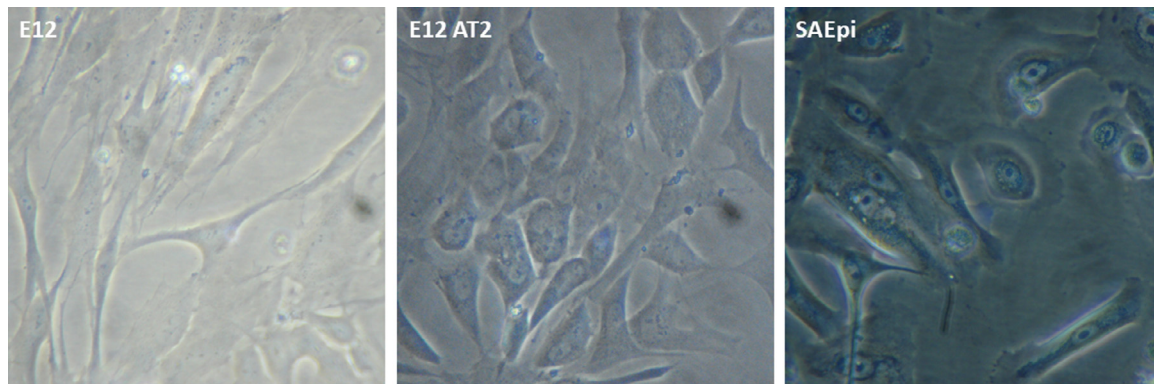
### E. Internalization of Spike



**Figure 1.** Cartoon depiction of staining procedures used in this study for confocal analysis. (A) Staining with antibody. The procedure allowed for staining of membrane and cytoplasmic proteins. (B) Staining with spike protein. The procedure enables binding of biotinylated spike protein to both membrane and cytoplasmic ACE-2. (C) Blocking by spike. A 5X molar excess of unlabeled spike protein was pre-incubated with the cells. Biotinylated spike protein was added without washing the unlabeled spike protein. This procedure enables blocking of binding of biotinylated spike protein to both membrane and cytoplasmic ACE-2. (D) Blocking by antibody. Antibody was pre-incubated with spike protein prior to addition of mixture to permeabilized cells. Antibodies that were effective at preventing the binding of spike to ACE-2 blocked the binding of biotinylated spike protein to membrane and cytoplasmic ACE-2. (E) Internalization of spike protein. Spike protein was incubated with biotinylated spike protein for 2 h at 37°C. Unbound biotinylated spike protein was washed away before fixation and permeabilization of cells. After permeabilization, bound spike protein was visualized with streptavidin-Alexa Fluor 594. This procedure allowed for detection of both membrane-bound and internalized spike proteins. AB, antibody.

Undifferentiated E12 MLPCs were positive for CK19, CD26 and EpCAM and negative for all other markers. E12 AT2 cells were positive for markers associated with AT2 cells (SPC, ACE-2, TM4SF1 and HT2-280), negative for markers associated with AT1 cells (AGER,

CAV1 and aquaporin) and positive for markers not unique to AT2 cells but known to be expressed on AT2 cells (CK19, CD26 and EpCAM). Primary SAEpis expressed markers consistent with AT2 cells and were negative for markers associated with AT1 cells. E12 AT2 cells



**Figure 2.** Phase contrast of undifferentiated control E12 clonal MLPCs. E12 clonal MLPCs differentiated to AT2-like cells and primary SAEpis. Results shown are representative of at least four separate cell culture experiments and determinations done on different days.

**Table 1**

Summary of confocal analysis of control E12 MLPCs, E12 AT2-like cells and SAEpis.

Marker	E12 MLPCs	E12 AT2-like cells	SAEpi
SOX17	Negative	Positive	Positive
CK19	Positive (weak)	Positive	Positive
Spike	Negative	Positive	Positive
Spike 1	Negative	Positive	Positive
ACE-2	Negative	Positive	Positive
SPC	Negative	Positive	Positive
TERT	Positive	Positive	Positive
EpCAM	Positive	Positive	Positive
CD26	Positive	Positive	Positive
TM4SF1	Negative	Positive	Positive
HT2-280	Negative	Positive	Positive
AGER	Negative	Negative	Negative
CAV1	Negative	Negative	Negative
Aquaporin	Negative	Negative	Negative

Cells were tested for non-lineage-specific markers (SOX17, CK19, EpCAM and CD26), AT2-specific markers (SPC, TM4SF1 and HT2-280) and AT1-specific markers (AGER, CAV1 and aquaporin). ACE-2 and binding of biotinylated spike proteins are also included. Summarized results are of at least six different determinations performed on different days. In each determination, at least 100 cells were counted. By examining each image as separate red and blue fluorescence, it was determined that each marker was 100% positive or negative.

maintained the same marker profile after continuous culture for 60 days after differentiation without further differentiation to AT1 cells. CAV1 and aquaporin, while negative on the cells tested in this article, are markers expressed on hepatocytes and were shown to be positive on E12/hepatocyte fusion cells (Figure 5) [24], confirming their stated specificity. As expected, E12 MLPCs and E12 AT2 cells were positive for TERT, as they were derived from E12 TERT-transfected MLPCs. Unexpectedly, primary SAEpis were also variably positive for TERT. This may be due to the AT2 cells acting as committed lung-specific stem cells that differentiate to non-proliferative AT1 cells upon *in vitro* culture or upon natural differentiation to AT1 cells within the alveoli. Collectively, these data showed that AT2-like cells could be generated from MLPCs and propagated long term without further differentiation to AT1-like cells. By the separation of blue and red fluorescence and enumeration of nuclei with positive expression of specific markers, it was determined that the cells were 100% positive or negative for each specific marker.

#### Quantitative reverse transcription polymerase chain reaction confirmed expression of ACE-2 and other AT2 cell markers

The positive expression of ACE-2 on SAEpis and E12 AT2-like cells observed by confocal analysis (Figure 3; also see

supplementary Figure 3) was confirmed by quantitative reverse transcription polymerase chain reaction (qRT-PCR). Additionally, messenger RNA was detected for DPP4 (CD26), EpCAM, SPC, CK19 and TM4SF1 in E12 AT2-like cells and SAEpis. E12 control cells were negative for ACE-2 by confocal analysis and were also found to be minimally detectable for ACE-2-specific RNA by qRT-PCR. Combined with confocal analysis, this level of RNA expression was insufficient to produce detectible levels of ACE-2 protein. Primary SAEpis and E12 AT2-like cells both tested positive for ACE-2 expression and expression of AT2-specific markers by confocal analysis and RNA by qRT-PCR. The results shown in Figure 6 were normalized to GAPDH.

#### Spike proteins exhibited binding and internalization in AT2 cells

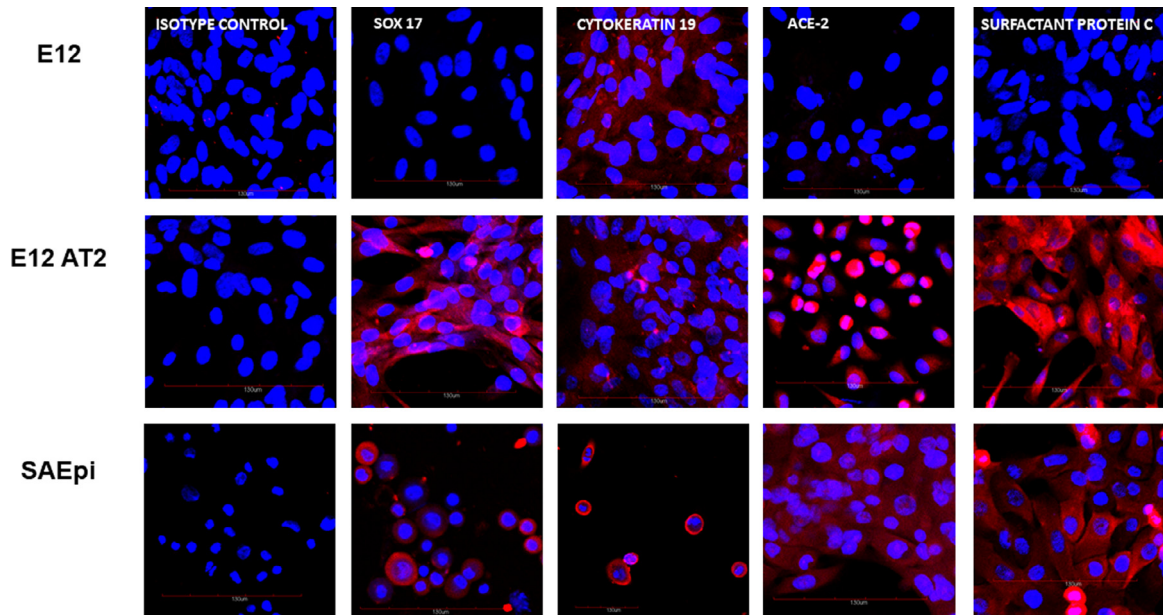
Expression of the ACE-2 surface protein suggested that AT2-like cells would be susceptible to binding by the SARS-CoV2 spike and spike 1 proteins. The authors tested this by binding biotinylated spike and spike 1 recombinant proteins (Figure 7). As shown, undifferentiated E12 MLPCs (negative for ACE-2) were negative for the binding of spike and spike 1 proteins. Both E12 AT2 cells and SAEpis (both positive for ACE-2 expression) were positive for the binding of spike and spike 1 proteins. Permeabilization prior to the addition of spike proteins measures the binding of spike by surface and internal ACE-2 proteins. Under the conditions described earlier, fixation and permeabilization of cells after binding and removal of spike proteins demonstrated surface-bound spike proteins and any spike proteins internalized by the cells. Internalization of spike proteins was observed in both E12 AT2 cells and SAEpis but not in control E12 cells.

#### Blocking of spike proteins confirmed the specificity of binding

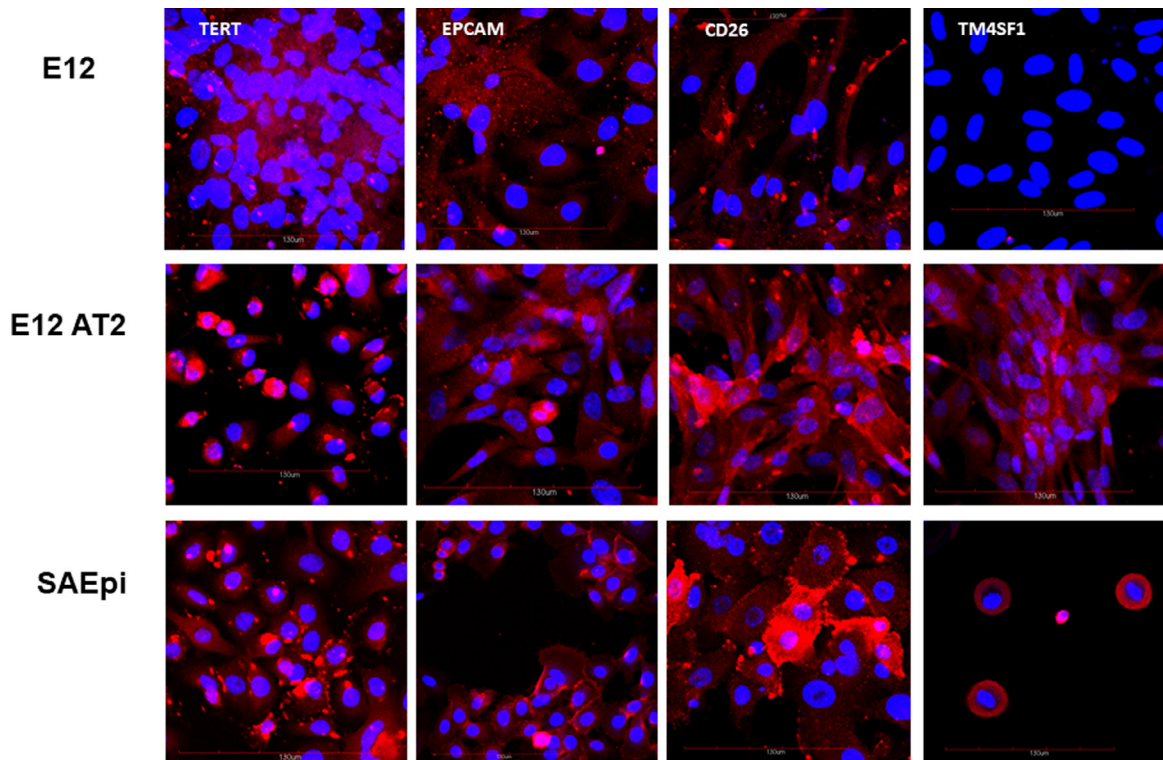
Specificity of the binding of spike proteins was confirmed by blockade of biotinylated spike protein with a 5-M excess of unlabeled spike protein and by neutralizing monoclonal human antibodies. As shown in Figure 8, binding of biotinylated spike protein was essentially inhibited by pre-incubation with unlabeled spike protein and by neutralizing antibody from ACROBiosystems but was not by the antibody from Novatein Biosciences. Without further investigation, the authors posited that the Novatein Biosciences antibody was specific for an epitope distinct from the RBD, as both antibodies were shown to bind to the spike protein attached to paramagnetic beads.

#### Discussion

The emergence of the novel SARS-CoV-2 virus has resulted in a pandemic that is severely affecting global health and the



**Figure 3.** Confocal analysis of undifferentiated E12 control MLPCs, differentiated E12 AT2-like cells and SAEpis. Cells were labeled with primary antibodies (isotype control, SOX17, CK19, ACE-2 and SPC) and secondary antibodies labeled with Alexa Fluor 594. Positive cells are stained red. DAPI staining shows the nuclei as blue. Negative cells are indicated only by blue nuclei. Results are representative of at least six different culture experiments done on separate days.

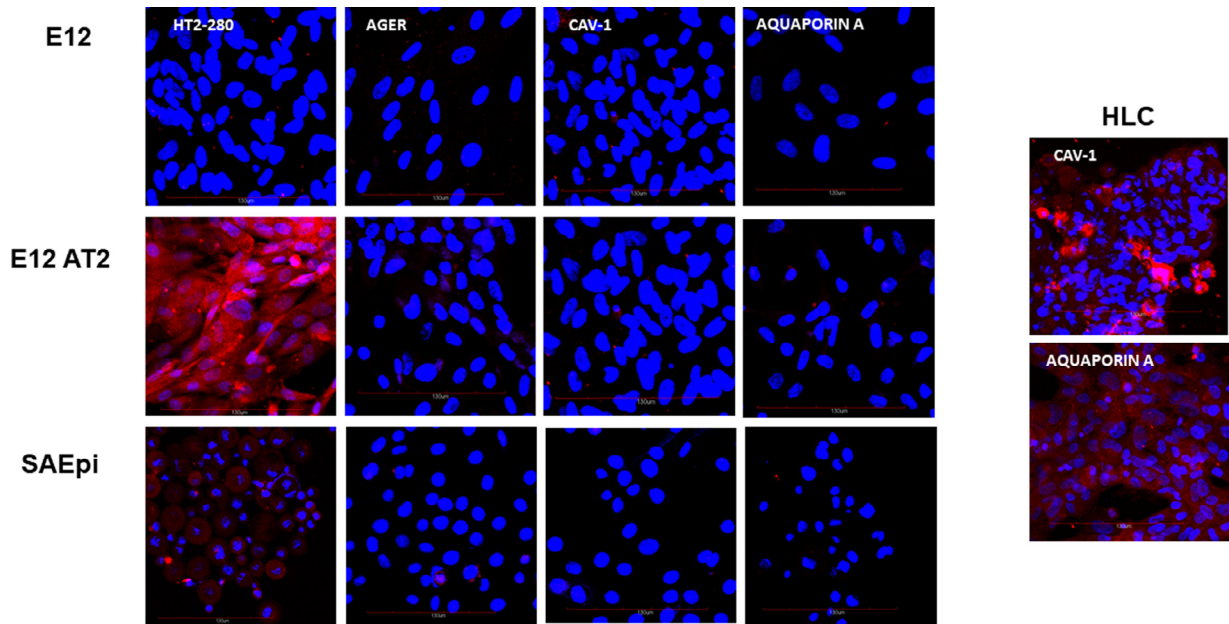


**Figure 4.** Confocal analysis of undifferentiated E12 control MLPCs, differentiated E12 AT2-like cells and SAEpis. Cells were labeled with primary antibodies (TERT, EpcAM, CD26 and TM4SF1) and secondary antibodies labeled with Alexa Fluor 594. Positive cells are stained red. DAPI staining shows the nuclei as blue. Negative cells are indicated only by blue nuclei. The results are representative of at least six different culture experiments and determinations done on different days.

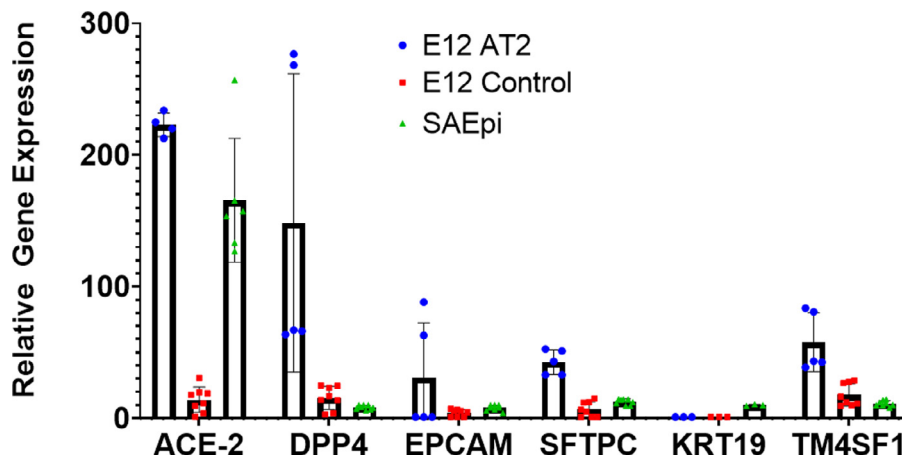
world economy [1–3]. Studies determining the nature of infection by the virus found that, much like the SARS-CoV virus, the ACE-2 cell surface molecule, expressed in a number of tissues, was the receptor for viral attachment and infection by the virus [5,9–11]. ACE-2 is expressed at high levels in the respiratory tract, making the lungs a target for infection and pathology [4].

AT2 cells exhibit a dual role in the lungs, acting as stem cells that can differentiate into AT1 cells to (i) maintain the structural integrity of alveoli and regenerate lung tissue after injury; and (ii) produce the surfactants that maintain osmotic integrity of the alveoli [16]. Long-term consequences of severe infection with SARS-CoV-2 include pulmonary fibrosis and reduced lung function. Indeed, reduced functionality of AT2 cells has been associated with increased fibrosis in





**Figure 5.** Confocal analysis of undifferentiated E12 control MLPCs, differentiated E12 AT2-like cells, SAEpis and HLC. Cells were labeled with primary antibodies (HT2-280, AGER, CAV1 and aquaporin) and secondary antibodies labeled with Alexa Fluor 594. Positive cells are stained red. DAPI staining shows the nuclei as blue. Negative cells are indicated only by blue nuclei. Results presented represent four different culture experiments and determinations carried out on separate days.



**Figure 6.** The qRT-PCR analysis of ACE-2, DPP4 (CD26), EpCAM, SPC, CK19 and TM4SF1 expression of control E12 MLPCs, E12 AT2-like cells and SAEpis. Data were normalized to GAPDH. The results represent at least four different culture experiments done on different days.

cases of chronic obstructive pulmonary disease and interstitial pulmonary fibrosis. In an unrelated pathology, augmented ACE-2 was associated with reduced fibrosis in dystrophic skeletal muscle [25], with AT2 cells playing a key role in recovery from the resultant lung injury. The expression of ACE-2 on AT2 cells and the ability of these cells to specifically bind SARS-CoV-2 spike proteins combined with their role in regeneration make AT2 cells crucial in the short- and long-term response of the lungs to SARS-CoV-2 infection.

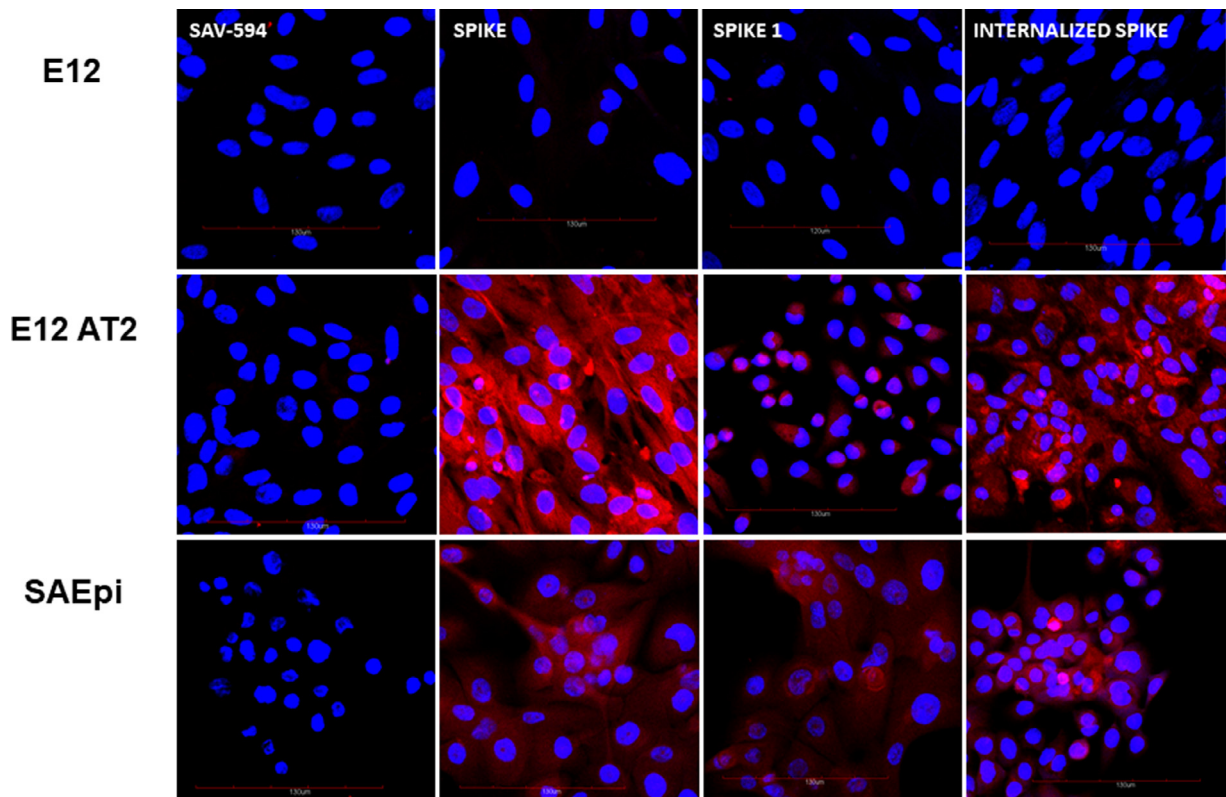
As a tool for studying respiratory pathogens, primary SAEpis appear to have characteristics associated with AT2 cells, including production of SPC, expression of AT2-specific markers and lack of AT1 markers. Additionally, they are shown to bind SARS-CoV-2 spike proteins, making them useful in the study of binding and infection. SAEpis are hampered by their limited viability *in vitro* and reduced proliferative capacity. The focus of this study was to develop a cell line with the desired characteristics of AT2 cells and the ability to be cultured for extended periods of time and expanded extensively. In previous studies with E12 MLPCs, differentiation of E12 cells into

hepatocyte-like cells (HLCs) resulted in cells that were shown to be functionally immortalized [23].

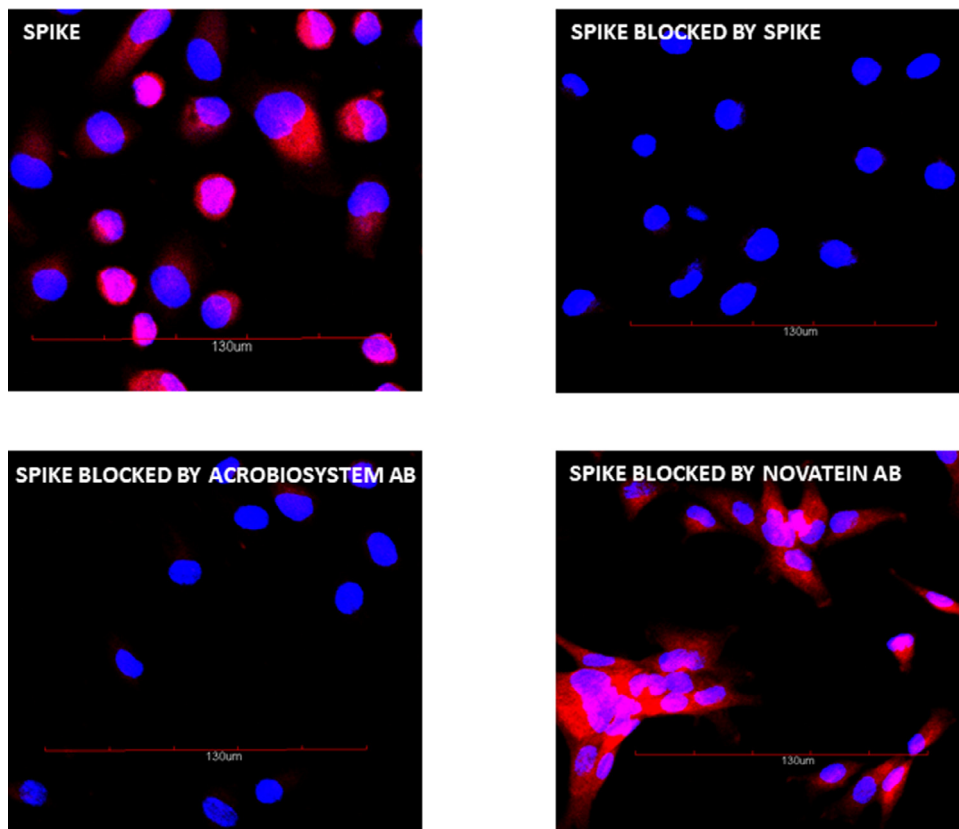
As an alternative to primary alveolar cells, there have been efforts to develop alveolar cells and organoids through the differentiation of human pluripotent stem cells, but these efforts have usually involved a multi-step process [26–28]. The first step involves commitment to anterior foregut endoderm, followed by differentiation to NKX2.1+ lung progenitor cells and final differentiation to SPC+ cells. Often, cell differentiation requires co-culture with fetal lung fibroblasts. These methodologies produce mixtures of AT2 and AT1 cells, as evidenced by cells expressing either SPC+ (AT2 cells) or AQP5+ (AT1 cells). This study describes the direct differentiation of TERT-immortalized MLPCs into AT2-like cells by a one-step culture method requiring 8 days for transition to cells expressing the AT2 phenotype.

MLPCs are a minor subset of cord blood-derived MSC-like cells that are distinguished from MSCs by gene expression, extended proliferative capacity and differentiation potential to tissues beyond mesodermal origin [18–22]. Earlier studies with non-immortalized





**Figure 7.** Confocal analysis of undifferentiated E12 control MLPCs, differentiated E12 AT2-like cells and SAEpis. Cells were labeled with biotinylated spike or spike 1 proteins and were visualized using streptavidin-Alexa Fluor 594. Positive cells are stained red. DAPI staining shows the nuclei as blue. Negative cells are indicated only by blue nuclei. Results are representative of at least four different culture experiments and determinations done separately.



**Figure 8.** Confocal analysis of differentiated E12 AT2-like cells that were tested for the blocking of biotinylated spike protein by unlabeled spike protein or neutralizing antibodies from ACROBiosystems or Novatein Biosciences. Bound spike was visualized using streptavidin-Alexa Fluor 594. Positive cells are stained red. DAPI staining shows the nuclei as blue. Negative cells are indicated only by blue nuclei. The results are representative of at least four different culture experiments carried out on separate days. AB, antibody.

MLPCs demonstrated differentiation to AT2 based on expression of SPC [17]. Transfection of MLPCs with the TERT gene resulted in functionally immortalized cells (cells have been cultured for >14 years) that retain their capacity for differentiation, as demonstrated in a recent study whereby TERT-MLPCs were differentiated to immortalized functional HLCs [23]. The authors' results showed that a simple procedure used to differentiate the non-immortalized MLPCs into AT2-like cells was confirmed by the expression of SPC by the E12 AT2-like cells. Analysis of additional specific markers confirmed the differentiation of E12 TERT-MLPCs to AT2-like cells (HT2-280+ and TM1SF4+) without further differentiation to AT1 cells (AGER<sup>-</sup>, CAV1<sup>-</sup> and AQP5<sup>-</sup>). Cells grown in the described medium continued to expand *in vitro* without further differentiation after 60 days of continuous culture. This enabled the ability to develop cell banks of AT2-like cells that could be cryopreserved and recultured and that retained the expandability of the E12 AT2-like cells.

E12 AT2-like cells were also shown to be positive for ACE-2, the known receptor for the SARS-CoV-2 virus, by both confocal analysis and qRT-PCR. The binding of the virus to the ACE-2 receptor and internalization of the virus are mediated by the viral surface spike protein. To establish that the ACE-2 expressed by the E12 AT2-like cells was capable of binding the spike protein, biotinylated spike (containing the RBD) and spike 1 recombinant proteins were incubated with the E12 AT2 cells and binding was visualized with streptavidin-Alexa Fluor 594 and confocal analysis. The authors' results suggested that the spike protein was internalized, although the precise mechanism by which that occurred is yet to be determined. One possibility is the internalization of the protein bound to the ACE-2 receptor via the clathrin-coated pit pathway [29]. Specificity of the observed binding of the spike and spike 1 proteins was confirmed by specific blockade by unlabeled spike protein.

Neutralizing antibodies have been a focal point in the development of therapeutics against the SARS-CoV-2 virus by blocking the binding of the spike protein RBD to the ACE-2 receptor. The majority of potentially therapeutic antibodies target the RBD of the spike protein, although it is likely that some neutralizing antibodies target other regions of the spike protein [30]. It has been recently shown that other cellular proteins (neuropilins) can act as alternative or co-factors for viral attachment to alveolar cells [31]. Using the E12 AT2-like cells, biotinylated spike proteins and neutralizing antibodies, the authors' spike blocking results suggested that one antibody was directed against the RBD of the spike protein, whereas the other antibody, unable to block the binding of the spike protein to the ACE-2 receptor, was likely directed against a separate epitope on the spike protein. The neutralizing activity of antibodies that are not directed to the RBD suggests that multiple epitopes on the spike protein are critical to the infection of cells by the virus. The identification of alternative binding or co-factors in the infectivity of the SARS-CoV-2 virus suggests that the development of combinations of monoclonal antibodies directed against the different binding epitopes on the spike protein should be investigated.

In a parallel study using confocal microscopy, the authors investigated the binding of SARS-CoV-2 spike proteins to human primary hepatocytes and immortalized human HLCs derived from the same E12 cell line used in this study [32]. It was shown that both primary human hepatocytes and HLCs could bind spike but not spike 1 protein. Although hepatocytes and HLCs do not express ACE-2, it was determined that the binding of spike to both hepatocytes and HLCs was through binding to the asialoglycoprotein receptor independent of the RBD of the spike 1 protein. Interestingly, unlike the results seen in this study, both neutralizing antibodies were able to inhibit the binding of the biotinylated spike protein to the hepatocytes and HLCs. The results observed with hepatocytes, E12 HLCs, E12 AT2 cells and SAEpis suggest that there are epitopes on the spike protein in addition to the RBD that affect attachment and infection by the SARS-CoV-2 virus. Variations in the non-RBD regions of the spike protein

could possibly confer advantages to newly emerging variants that appear to have advantages with regard to transmission and infectivity. Cellular studies could provide a platform for elucidation of the role of viral variants in infectivity.

The authors believe that E12 AT2-like cells can provide an important tool to study lung pathologies, specifically the binding of SARS-CoV-2 spike proteins to the ACE-2 receptor. E12 AT2 cells provide a highly proliferative, long-lived and reproducible source of cells for these studies, with significant potential for therapeutic agent discovery, testing and validation as well as mechanistic studies in respiratory pathologies.

## Conclusions

This study describes a methodology to differentiate MLPCs to AT2-like cells. The AT2 phenotype was confirmed by expression of AT2-specific markers, including SPC, TM4SF1 and HT2-280, and absence of the AT1-specific markers AGER, CAV1 and aquaporin. The AT2-like cells were demonstrated to express ACE-2 and were capable of specifically binding and internalizing SARS-CoV-2 spike proteins. The use of the AT2-like cells and biotinylated spike proteins enabled the discrimination of neutralizing antibodies that were RBD-specific or specific for other epitopes on the spike protein. In sum, AT2-like cells could provide a stable, reproducible and accurate source of cells to study spike binding and the development of antibodies or other agents that could inhibit the binding of SARS-CoV-2 and the entry of viral pathogens.

## Declaration of Competing Interest

DPC is the owner of CytoMedical Design Group, LLC, which does not alter the authors' adherence to *Cytotherapy* policies on sharing data and materials.

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## Author Contributions

Conception and design of the study: DPC, MJO and CJS. Acquisition of data: DPC and MJO. Analysis and interpretation of data: DPC, MJO and CJS. Drafting or revising the manuscript: DPC, MJO and CJS. All authors have approved the final article.

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## Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:[10.1016/j.jcyt.2021.07.017](https://doi.org/10.1016/j.jcyt.2021.07.017).

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