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Research article

Characterization of distal airway stem-like cells expressing N-terminally truncated p63 and thyroid transcription factor-1 in the human lung

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ABSTRACT

Distal airway stem cells (DASCs) in the mouse lung can differentiate into bronchioles and alveoli. However, it remains unclear whether the same stem cells exist in the human lung. Here, we found that human lung epithelial (HuL) cells, derived from normal, peripheral lung tissue, in monolayer, mostly express both the N-terminally truncated isoform of p63 (ΔNp63), a marker for airway basal cells, and thyroid transcription factor-1 (TTF-1), a marker for alveolar epithelial cells, even though these two molecules are usually expressed in a mutually exclusive way. Three-dimensionally cultured HuL cells differentiated to form bronchiole-like and alveolus-like organoids. We also uncovered a few bronchiolar epithelial cells expressing both ΔNp63 and TTF-1 in the human lung, suggesting that these cells are the cells of origin for HuL cells. Taken together, ΔNp63$^+$ TTF-1$^+$ peripheral airway epithelial cells are possibly the human counterpart of mouse DASCs and may offer potential for future regenerative medicine.

1. Introduction

There is no highly effective treatment for several chronic respiratory diseases, such as idiopathic pulmonary fibrosis (IPF) and chronic obstructive pulmonary disease (COPD). The growing awareness of stem cell biology has highlighted the potential utility of “cell therapy” as an efficacious strategy against chronic respiratory diseases [1]. Indeed, there is an ongoing clinical trial testing the efficacy of autologous bronchial stem cells as a treatment for IPF patients (clinicaltrials.gov: NCT02796781).

Normal epithelial stem cells have already been identified in the stomach, small intestine, and large intestine [2,3]. The adult lung is a static tissue, with a slow turnover rate (less than 1% per day) in airway epithelia (bronchi and bronchioles) and alveoli [4–6]. Different epithelial stem cells have been found at the various levels of lung tissue (bronchi, bronchioles, and alveoli), and these stem cells maintain epithelial integrity and play key roles in regenerating the lung. Alveolar epithelial type 2 (AT2) cells, for example, function as stem/progenitor cells in the alveoli, and are endowed with the potential for self-renewal and the ability to give rise to alveolar epithelial type 1 cells in the steady state [7,8]. AT2 cells express thyroid transcription factor-1 (TTF-1), an essential transcription factor for lung development. Club cells, formerly called Clara cells, are another type of regional progenitor cell, which functions to repair the bronchiolar epithelium in damaged tissues. Intriguingly, seminal articles in the field have recently identified and characterized a specific epithelial stem cell—distinct from AT2 cells and club cells—in the peripheral tissues of the mouse lung [9–11], referred to as distal airway stem cells (DASCs) or lineage-negative progenitors. Studies in mice show that these DASCs undergo active proliferation, and migrate and differentiate to regenerate the epithelia of bronchioles and alveoli ablated by influenza virus infection. Whereas DASCs can differentiate into AT2 cells, they express p63 and keratin 5 (KRT5), two representative markers for basal cells of the bronchi or bronchioles. It remains unclear whether the same cells exist in the human lung.

We recently demonstrated that human lung epithelial cells (HuL cells), derived from normal, peripheral lung tissues, undergo dynamic epithelial-to-mesenchymal transition or mesenchymal-to-epithelial

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transition in the presence or absence, respectively, of transforming growth factor-β (TGF-β) signaling in vitro [12,13]. We previously showed that HuL cells, especially in the absence of TGF-β signaling, express TTF-1, a representative marker for AT2 cells [13], and, more recently, noted that they are also positive for ΔNp63, the N-terminally truncated isoform of p63. In the human lung, ΔNp63$^+$ basal cells are present in the trachea, principal bronchus, and bronchi through to the respiratory bronchioles but not in the alveolus [14,15]. ΔNp63 expression is also observed in basal cells of the esophagus and prostate. In human fetal lung, TTF-1 is specifically expressed in the nuclei of epithelial cells localized to the tips of developing terminal airway buds and is sparse or absent in more proximal airways. As development advances, the number of TTF-1$^+$ bronchiolar cells decreases while the number of AT2 cells expressing the molecule increases [16]. Thus, in principle, the expression of ΔNp63 and TTF-1 seems to be mutually exclusive in the lung. HuL cells, however, express both ΔNp63 and TTF-1, and this led us to hypothesize that HuL cells have the capacity to differentiate into not only bronchiolar tissues but also alveolus. Here, we report that HuL cells are capable of forming bronchiole-like or alveolus-like organoids, which suggests that these ΔNp63$^+$ TTF-1$^+$ dual-positive cells are the human counterpart of mouse DASCs.

2. Materials and methods

2.1. Isolation of human lung epithelial cells

HuL5 cells and HuL6 cells were isolated as described previously [13]. In the present study, human bronchial epithelial cells were also isolated from a normal bronchial tissue (0.26 g in weight) obtained from a female Japanese patient (55-year-old) who was operated on for metastatic lung cancer at Sapporo Medical University Hospital. Written, informed consents were obtained from the patients.

Briefly, the bronchial tissue was rinsed with phosphate buffered saline (PBS) containing penicillin-streptomycin (1:100, Nacalai Tesque, Kyoto, Japan) and amphotericin B (2.5 μg/ml, Sigma-Aldrich, St Louis, MO, USA), and then incubated with Dulbecco's modified Eagle's medium (DMEM) containing dispase II (2.0 unit/ml, Sigma-Aldrich) at 37 °C for 30 min. The digested bronchial tissue was then minced and incubated for another 30 min, and the suspension was filtered through a 100 μm Nylon Cell Strainer (Thermo Fisher Scientific Japan, Yokohama, Japan) and centrifuged at 1500 rpm for 5 min. The cell pellet was subsequently resuspended and incubated in PBS containing PharmLyse (BD Biosciences, San Jose, CA, USA) at room temperature for 5 min and then centrifuged at 1500 rpm for 5 min. The cells (5.0×10^5) were seeded onto 100 mm cell culture dishes (#353003, Falcon, Corning, NY, USA) that had been preseeded with mitomycin-C-treated NIH3/T3 cells (2.88×10^5 cells/dish) as feeder cells. Cultures were maintained in bronchial epithelial cell growth medium (BEGM, Lonza Japan, Tokyo, Japan), a serum-free media, for 13 days. On reaching sub-confluence, cells were trypsinized and used for experimentation as described below. Cells derived from the bronchial tissue, selected in BEGM culture, were termed human bronchial (HuB) cells.

2.2. Cell culture

2.2.1. Two-dimensional (2D) cell culture

Subsequent cultures were grown on 100 mm cell culture dishes in BEGM containing EW-7197, the potent TGF-β receptor inhibitor (1 μM, Selleck Chemicals, Houston, TX, USA) [17], and Y-27632, the selective Rho-associated coiled-coil containing protein kinase 1 and 2 inhibitor (10 μM, Cayman Chemical, Ann Arbor, MI, USA) at 37 °C in a humidified incubator with 5% CO_2. The medium was changed every 3 days until reaching sub-confluence. To split the cell cultures, the cells were dissociated with trypsin and re-seeded at 1:10 ratio.

2.2.2. Three-dimensional (3D) cell culture for bronchiolar or alveolar differentiation

Five hundred μl of 100% Growth Factor-Reduced Matrigel (Corning, NY, USA) was spread with a cold pipette tip gently in 6-well plate (Corning) and allowed to solidify at 37 °C for 30 min. HuL cells (1.0×10^6 /well) were suspended in warm CnT-PR-AD medium (Cellntec, Bern, Switzerland) containing 1 mM $CaCl_2$ and 1% Matrigel, and seeded on top of the Matrigel. Of note, EW-7197 (1 μM) was also used in medium to promote alveolar differentiation (condition A). Cells were also treated with fibroblast growth factor (FGF)-10 (100 ng/ml, Peprotech, Rocky Hill, NJ, USA) and the γ-secretase inhibitor N-[(3,5-difluorophenyl)acetyl]-L-alanyl-2-phenylglycine-1,1-dimethylethyl ester (DAPT, 20 μM, Selleck Chemicals, Houston, TX, USA) to favor bronchiolar differentiation (condition B). The former (FGF-10) was present in the medium throughout the differentiation culture while the latter (DAPT) was added at day 6 of the experiment.

2.3. Flow cytometry

Flow cytometric analysis was performed on single cell suspensions by using NovoCyte Flow Cytometer (ACEA Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. The antibodies used were as follows: phycoerythrin (PE)- trophoblast cell surface antigen 2 (TROP2, BioLegend, San Diego, CA, USA), fluorescein isothiocyanate (FITC)-integrin alpha 6 (ITGA6), PE-cyanine 7 (CY7)-podoplanin (PDPN, BioLegend), allophycocyanin (APC)-CY7-CD24 (BioLegend), and APC-CD90 (BioLegend). The anti-ITGA6 monoclonal antibody was previously developed in our laboratory.

2.4. Western blotting

Cultured cells were lysed in NuPAGE LDS Sample Buffer (Thermo Fisher Scientific). Whole cell lysates were subjected to SDS-PAGE (5–20% SuperSep Ace, Wako Pure Chemical Industries, Osaka, Japan) followed by blotting with specific antibodies, and detection using the Supersignal West Pico Plus Chemiluminescent Substrate (Thermo Fisher Scientific). The primary antibodies used were anti-KRT5 (D4U8Q; 1:1000; Cell Signaling Technology Japan, Tokyo, Japan), anti-keratin 7 (KRT7) (D1E4; 1:1000; Cell Signaling), anti-TTF-1 (EPR8190; 1:1000; Abcam Japan, Tokyo, Japan), anti-p63 (ab53039; 1:2000; Abcam), and anti-β-actin (AC-15; 1;10000; Sigma-Aldrich).

2.5. RT-PCR

Total RNA extraction and quantitative RT-PCR were carried out as previously described [18,19]. The following PCR primers were all purchased from Qiagen (QuantiTect Primer Assay. Valencia, CA, USA): β-actin, encoded by the *ACTB* gene (Hs_ACTB_1_SG); FOXJ1 (Hs_FOXJ1_1_SG); SCGB1A1 (Hs_SCGB1A1_1_SG); surfactant protein A2 (SFTPA2) (Hs_SFTPA2_2_SG); surfactant protein D (SFTPD) (Hs_SFTPD_1_SG); and AQP5 (Hs_AQP5_1_SG). The amount of β-actin mRNA in each sample was used to standardize the quantity of target mRNAs. Relative mRNA expression levels were calculated using the comparative $\Delta\Delta C_T$ method and are presented as the averages of triplicate experiments. We also conducted conventional RT-PCR for the mRNA expression of *TP63* and *NKX2-1* using primers for TP63 (Hs_TP63_2_SG) and NKX2-1 (Hs_NKX2-1_1_SG), both of which were purchased from Qiagen.

2.6. Xenograft mouse studies

All animal experimentations were conducted in accordance with a protocol approved by the Animal Committee at Sapporo Medical University. Studies were performed in 6- to 8-week-old female NOD-scid IL2Rgamma^{null} (NSG) mice (Charles River Laboratories Japan, Yokohama, Japan). Intratracheal instillation for bleomycin (BLM)

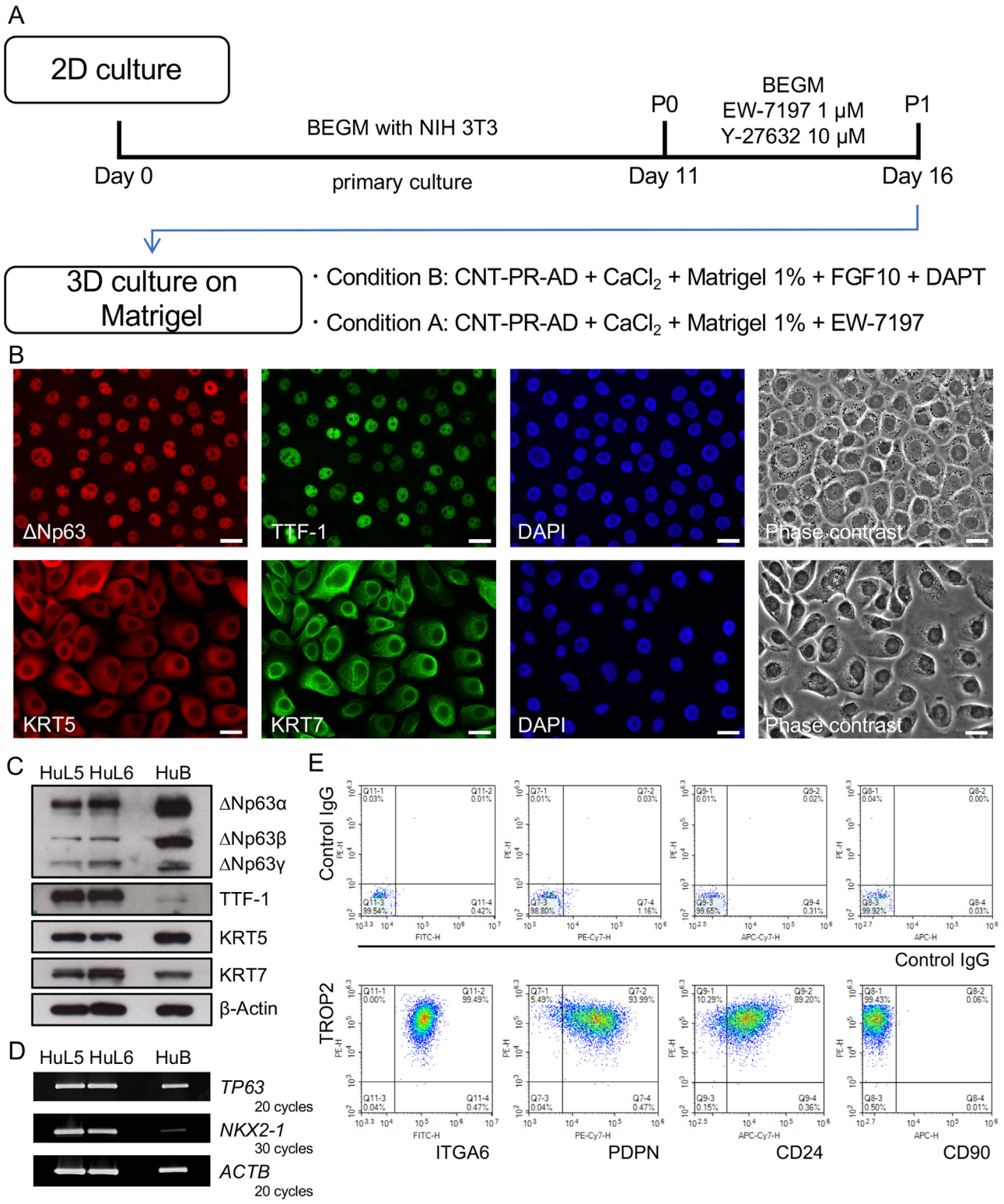

Fig. 1. Two-dimensionally cultured HuL cells are immature epithelial cells positive for ΔNp63, KRT5, TTF-1, and KRT7. (A) Experimental scheme showing the process of human lung (HuL) cell culture. Cells cultured in "condition A" were treated with 1 μM EW-7197 to promote alveolar differentiation. Cells grown in "condition B" were exposed to 100 ng/ml fibroblast growth factor (FGF)-10 in the medium throughout the differentiation culture, with 20 μM DAPT added on day 6. (B) Representative immunofluorescent and phase-contrast images of HuL5 cells in monolayer. Scale bars, 20 μm. (C) Western blots of HuL5 cells, HuL6 cells, and human bronchial epithelial (HuB) cells in monolayer. (D) Conventional RT-PCR for the expression of *TP63* and *NKX2-1* in HuL5, HuL6, and HuB cells in monolayer. (E) Flow cytometric analysis for TROP2, ITGA6, PDPN, CD24, and CD90 in HuL5 cells in monolayer.

treatment followed by HuL cells transfer experiments were performed according to the "oropharyngeal aspiration method" [20]. Briefly, mice were anesthetized and instilled with 50 μl of 5 mg/kg BLM sulfate (Nippon Kayaku, Tokyo, Japan) as per the method. On day 10 after BLM administration, HuL5 cells (5.0×10^6) in 50 μl suspension were instilled in the same way for intratracheal transfer experiment. Mice were sacrificed 60 days after the transplantation.

2.7. Immunofluorescence staining of cells

Cell culture, fixation, permeabilization, and blocking were performed as described previously [13]. Samples were then incubated overnight at 4 °C with the following primary antibodies: anti-TTF-1 (D2E8; 1:50; Cell Signaling), anti-ΔNp63 (BC28; 1:400; Biocare Medical, Pacheco, CA, USA), anti-KRT5 (2C2; 1:200; Sigma-Aldrich), and anti-KRT7 (D1E4; 1:400; Cell Signaling). Cells were subsequently incubated with two secondary antibodies, Alexa Fluor 488-conjugated goat anti-rabbit IgG and Alexia Fluor 594-conjugated donkey anti-mouse IgG (1:400 each; Thermo Fisher Scientific) at room temperature for 1 h. After mounting of cells and staining nuclei using SlowFade Diamond Antifade Mountant with DAPI (Thermo Fisher Scientific), cells were observed via fluorescent microscopy (IX-71; Olympus, Tokyo, Japan) and photographed (DP80; Olympus).

2.8. Immunofluorescence staining of tissues

We analyzed a normal bronchus and a peripheral lung tissue obtained from the same female patient already mentioned above. These tissues were processed by the AMeX (acetone, methyl benzoate, and xylene) method [21], which is well known to preserve antigens in better conditions than the conventional formalin fixation. The tissues were serially sectioned into 4 μm thick slides. A section was stained with hematoxylin-eosin (HE), and the other sections were used for immunohistochemical detection of TTF-1 and ΔNp63, or KRT7 and KRT5. The primary antibodies used were anti-TTF-1 (D2E8; 1:50; Cell Signaling), anti-ΔNp63 (BC28; 1:200; Biocare Medical), anti-KRT7 (OV-TL 12/30; prediluted; Nichirei Biosciences, Tokyo, Japan), and anti-KRT5 (D4U8Q; 1:400; Cell Signaling). As for the expression of TTF-1 and ΔNp63, the antigens were detected in exactly the same way as described in the paragraph before. As for the expression of KRT7 and KRT5, we used two secondary antibodies, Alexa Fluor 488-conjugated donkey anti-mouse IgG and Alexia Fluor 594-conjugated goat anti-rabbit IgG (1:400 each; Thermo Fisher Scientific).

2.9. Immunocytochemistry of 3D-cultured spheroids and immunohistochemistry of mouse lung tissues

Immunocytochemical or immunohistochemical staining was carried out on formalin-fixed, paraffin-embedded 3D-cultured spheroids and mouse lung tissues, respectively. Of note, cell blocks including organoids were prepared with the use of iPGell (Nippon Genetics, Tokyo, Japan) according to the manufacturer's recommendation. Cell or tissue sections were retrieved using Novocastra Epitope Retrieval Solution 1 for human mitochondria antigen or Epitope Retrieval Solution 2 (Leica Biosystems, Nubloch, Germany) for expression of TTF-1 and ΔNp63 at 100 °C for 20 min. As for SFTPA expression, antigen retrieval was unnecessary. The types and dilutions of primary antibodies used were anti-TTF-1 (SPT24; prediluted; Nichirei Biosciences), anti-ΔNp63 (BC28; prediluted; Biocare Medical), anti-SFTPA (PE10; 1.67 μg/ml; Immuno-Biological Laboratories, Gumma, Japan), and anti-human mitochondrial antigen (113-1; 1:100; BioGenex, Fremont, CA, USA) [22]. Immunocytochemistry or immunohistochemistry was conducted using a Lecia BOND-MAX.

2.10. Statistics

Differences in expression levels of each mRNA between 2D-cultured HuL cells and 3D-cultured cells in condition A or condition B were evaluated by unpaired *t*-tests. A P-value less than 0.05 was considered significant. All statistical calculations were performed with JMP software (JMP for Windows version 7; SAS Institute Japan; Tokyo, Japan).

3. Results

3.1. Two-dimensionally cultured HuL cells are immature epithelial cells positive for ΔNp63, KRT5, TTF-1, and KRT7

We isolated and analyzed normal human lung epithelial cells obtained from two individuals, referred to as HuL5 cells and HuL6 cells [12,13]. We found that both cells proliferated actively in monolayer cultures using BEGM supplemented with EW-7197, a TGF-β receptor inhibitor, and Y-27632, a Rho-kinase inhibitor (Fig. 1A). Both HuL cells were grown successfully through five passages (data not shown). Under these growth conditions, they exhibited a cobblestone-like appearance owing to the absence of TGF-β signaling, and were positive for TTF-1 and KRT7, markers for AT2 cells, as well as ΔNp63 and KRT5, markers for airway basal cells (Figs. 1B–1D; Supplementary Fig. S1A). Flow cytometry results indicated a typical expression pattern of immature epithelial cells: $TROP2^+$, $ITGA6^+$, $PDPN^+$, $CD24^{low}$, and $CD90^-$ (Fig. 1E; Supplementary Fig. S1B).

We also isolated and analyzed HuB cells from normal bronchial tissue. The flow cytometric expression pattern of HuB cells was similar to that of HuL cells (Fig. 1E; Supplementary Fig. S1B, S1D). However, HuB cells were positive for ΔNp63, KRT5, and KRT7 and showed very low expression of TTF-1 (Fig. 1C; Supplementary Fig. S1C). As shown in Fig. 1D, the results of conventional RT-PCR analyses confirmed this lower expression of TTF-1 (encoded by the *NKX2-1* gene) in HuB cells as compared with HuL cells, but comparable levels of p63 (encoded by the *TP63* gene).

3.2. Three-dimensionally cultured HuL cells form not only bronchiole-like organoids but also alveolus-like organoids

The phenotype of HuL cells expressing ΔNp63 and TTF-1 led us to hypothesize that HuL cells are a type of peripheral lung epithelial stem cell capable of differentiating into bronchioles or alveoli. Accordingly, we exploited a 3D on-top culture method to differentiate HuL cells into these distal tissue types (Fig. 1A). We found that HuL cells in 3D on-top culture rapidly aggregated to form spheroids within several hours. Over time, the spheroids gradually increased in size and then underwent spontaneous cavitation, hereafter referred to as "organoids" (Figs. 2A, 2B).

To induce differentiation, cultures were treated with 1 μM EW-7197 to promote alveolar differentiation ("condition A") or 100 ng/ml FGF-10 and 20 μM DAPT, a γ-secretase inhibitor, to favor bronchiolar differentiation ("condition B") (See Methods). Under "condition B," HuL5 and HuL6 organoids displayed a stratified wall with cilia on the apical sides (Fig. 2C; Supplementary Fig. S2A). Although most of the HuL cells in monolayer culture expressed ΔNp63 and TTF-1 (Fig. 1B; Supplementary Fig. S1A), most of the apical cells in the organoids in condition B were negative for ΔNp63, and a few basal cells were negative for TTF-1 (Fig. 2C; Supplementary Fig. S2A). More importantly, the organoids never expressed SFTPA at protein levels in condition B. These findings collectively suggest that condition B promotes bronchiolar differentiation of HuL cells.

In comparison, under "condition A," the organoids of HuL5 cells and HuL6 cells mostly displayed a thin wall of single- or double-layered cells (Fig. 2D; Supplementary Fig. S2B). Interestingly, there were a few apical cells positive for SFTPA in the cytoplasm, and the apical surface of the organoid was thinly covered with SFTPA, which was reminiscent

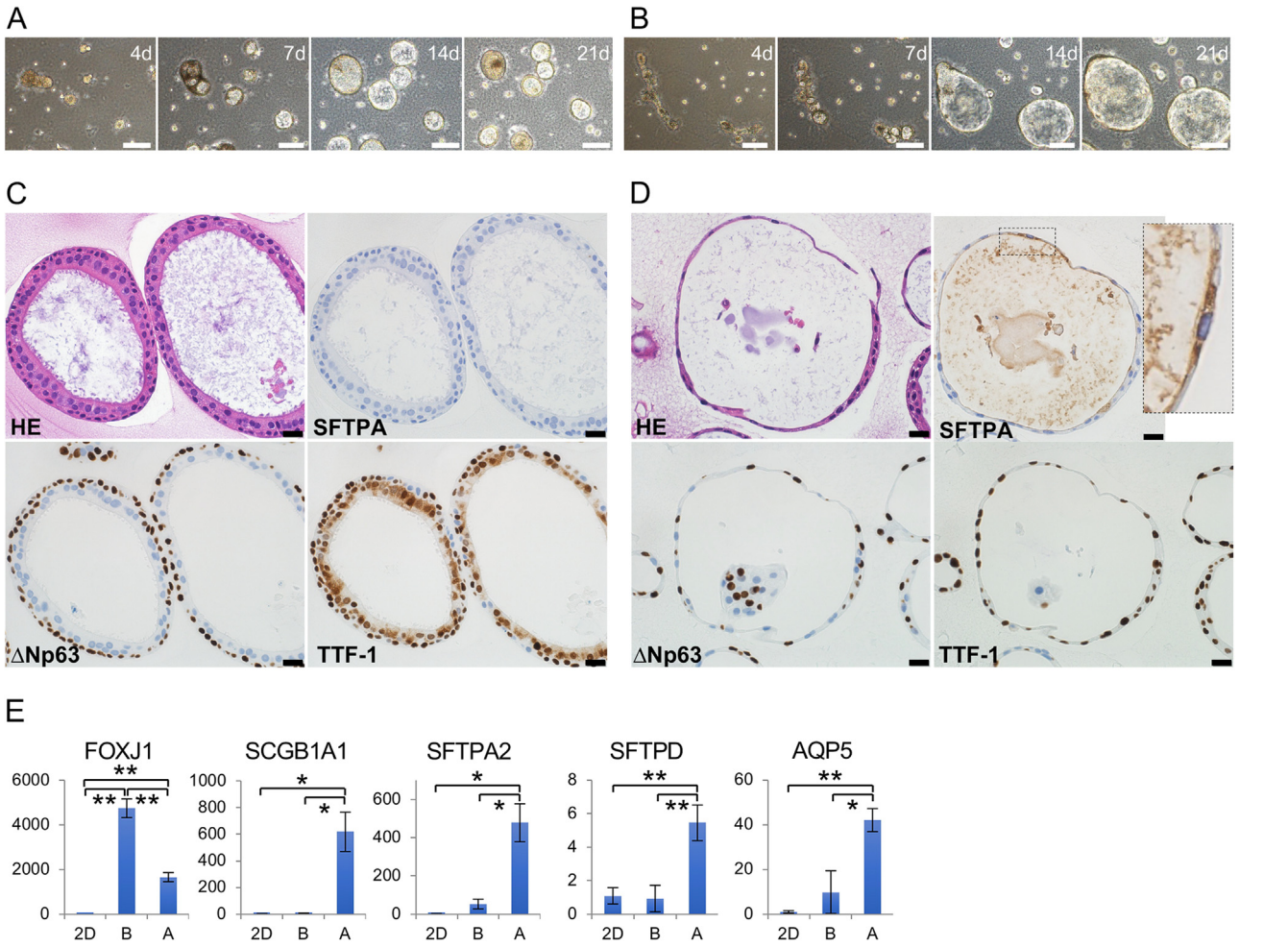

Fig. 2. Three-dimensionally cultured HuL5 cells differentiate to form not only bronchiole-like organoids but also alveolus-like organoids. (A and B) Time-lapse, phase-contrast images of HuL5 cells under three-dimensional (3D) on-top culture in "condition B" (100 ng/ml FGF10 and 20 μM DAPT) (A) and "condition A" (1 μM EW-7197) (B). Scale bars, 200 μm. (C and D) Hematoxylin-eosin (HE) staining and immunocytochemical images of HuL5 cells cultured in condition B (C) and condition A (D). Scale bars, 20 μm. (E) Quantitative RT-PCR for the expression of *FOXJ1*, *SCGB1A1*, *SFTPA2*, *SFTPD*, and *AQP5* in HuL5 cells in monolayer (2D) or under 3D on-top culture that includes condition B (B) and condition A (A). RT-PCR was performed in triplicate. Results are expressed as mean ± standard deviation (SD). $*P < 0.05$, $**P < 0.01$.

of the alveoli in the lung (Fig. 2D; Supplementary Fig. S2B). In the organoids in condition A, most of the HuL cells were positive for TTF-1, with some cells negative for ΔNp63. Taken together, condition A is likely to promote alveolar differentiation of HuL cells.

We next confirmed the morphological or immunocytochemical findings in organoids under 3D culture using RT-PCR (Fig. 2E; Supplementary Fig. S2C). For example, we found that *FOXJ1*, a marker for ciliated cells, markedly increased in condition B, whereas *SFTPA2*, *SFTPD*, and *AQP5*, all of which are mainly expressed in alveolar epithelia, were elevated in condition A. Intriguingly, although club cells are present in the distal airway or bronchioles, the expression levels of the club cell marker *SCGB1A1* remained low in condition B. We surmised that this was probably caused by the inhibition of Notch signaling by DAPT treated in condition B (Fig. 1A), which can turn club cells into ciliated cells [23].

HuB cells under 3D culture formed organoids in almost the same way as HuL cells, but HuB cell organoids consistently displayed a stratified wall with cilia on the apical side in condition A as well as in condition B (Supplementary Fig. S3A, S3B).

The findings above suggest that HuL cells can differentiate into alveoli capable of secreting SFTPA as well as bronchioles. In contrast, HuB cells are only able to differentiate into bronchial tissue. This raises the possibility that HuL cells are a type of peripheral lung epithelial stem cell and possibly the human counterpart of mouse DASCs. As for HuB cells, we surmise that the inability to differentiate into alveolar tissue depends, at least in part, on the very limited expression of TTF-1.

3.3. HuL cells transplanted into mice exhibit a bronchus-like structure

Next, we tested whether HuL cells could differentiate into bronchioles or alveoli in vivo. We used the oropharyngeal aspiration method [20] to instill 2D-cultured HuL5 cells (5.0×10^6 cells) into NSG mice whose bronchiolar and alveolar epithelia had been ablated with bleomycin. We harvested the mice lungs after 60 days and analyzed the tissue histologically (Figs. 3A–3G). We found that HuL5 cells—confirmed by positive immunohistochemical staining for human-specific mitochondria—replaced the damaged peripheral regions of the mouse lungs (Figs. 3A, 3B). Most of the HuL5 cells in vivo conspicuously differentiated into ciliated or goblet cells (Figs. 3C, 3D), and ΔNp63 expression was confined to basal cells (Fig. 3E). In other words, the structure that HuL5 cells formed in vivo histologically resembled the bronchus, and was quite different from normal alveoli. Importantly, however, a few HuL5 cells in the mice lungs probably differentiated into AT2 cells in terms of their SFTPA expression (Figs. 3F, 3G, arrows).

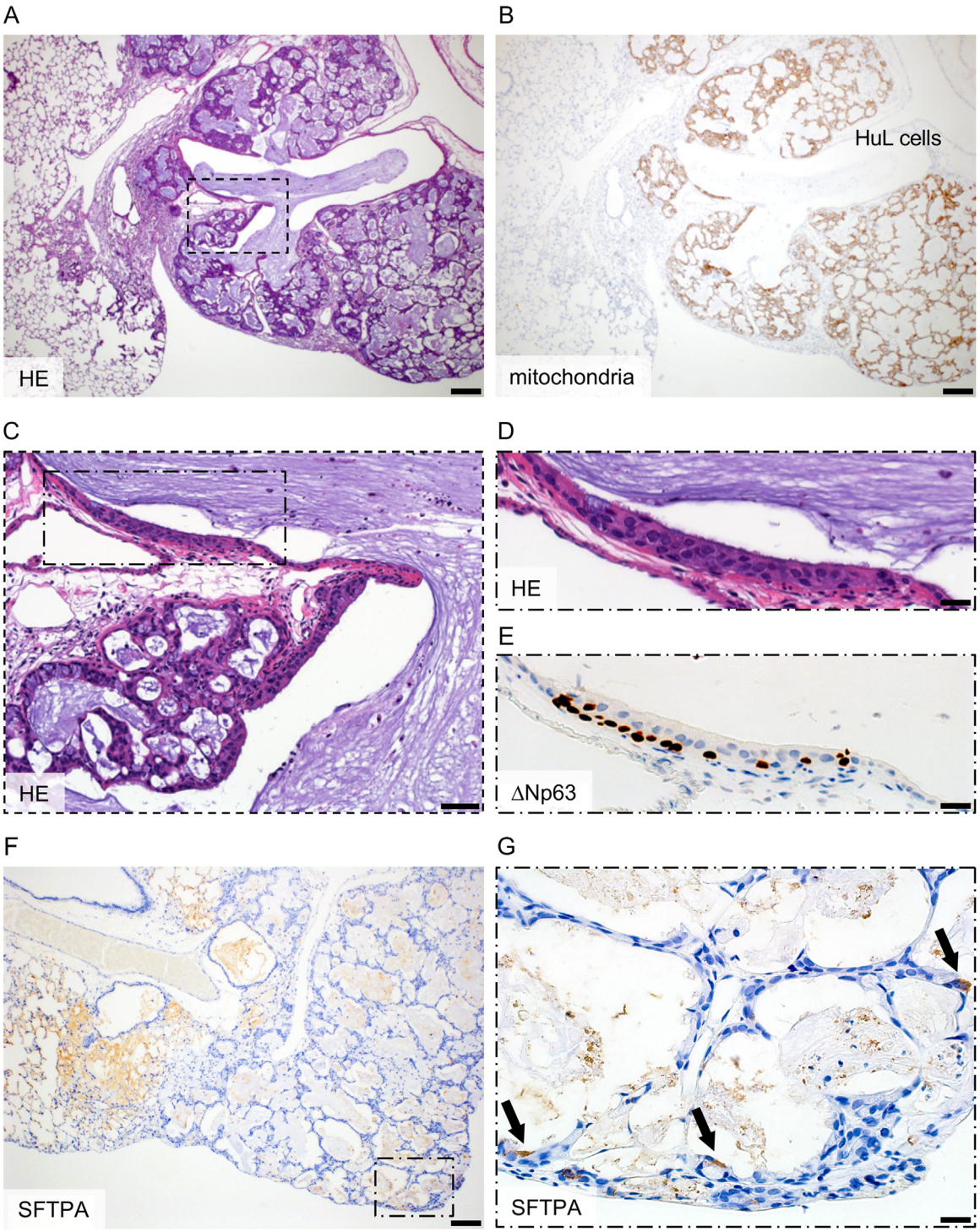

Fig. 3. HuL cells transplanted into mice exhibit a bronchus-like structure. (A–E) Representative images of a mouse lung in which transplanted HuL5 cells survived and differentiated into bronchial tissue. HE-stained images with low, middle, and high-power magnification demonstrating that most HuL5 cells differentiated into ciliated cells or goblet cells (A, C, and D). Immunohistochemistry for human-specific mitochondria clearly illustrating the localization of HuL5 cells in the mouse lung (B). Immunohistochemistry for ΔNp63 showing that the expression was confined to basal cells (E). (F and G) Immunohistochemistry for SFTPA showing the existence of a few positive HuL5 cells (arrows). Representative images of low- (F) and high- (G) power magnification are presented. Scale bars, 200 μm (A, B, F); 50 μm (C); and 20 μm (D, E, G).

3.4. ΔNp63$^+$ TTF-1$^+$ bronchiolar epithelial basal cells exist in the human lung

Considering that ΔNp63 and TTF-1 are expressed, in principle, in a mutually exclusive manner both in normal lung tissues [14–16] and in lung cancer [24–26], it seemed unusual that HuL cells would express both molecules. Accordingly, we sought to determine if human lung tissue contained such ΔNp63$^+$ TTF-1$^+$ dual-positive epithelial cells. As expected, basal cells in the bronchus were ΔNp63$^+$ TTF-1$^-$ (Supplementary Fig. S4). On the other hand, through immunofluorescence staining, we uncovered a few, specific epithelial cells in the distal airway that were positive for both ΔNp63 and TTF-1, as

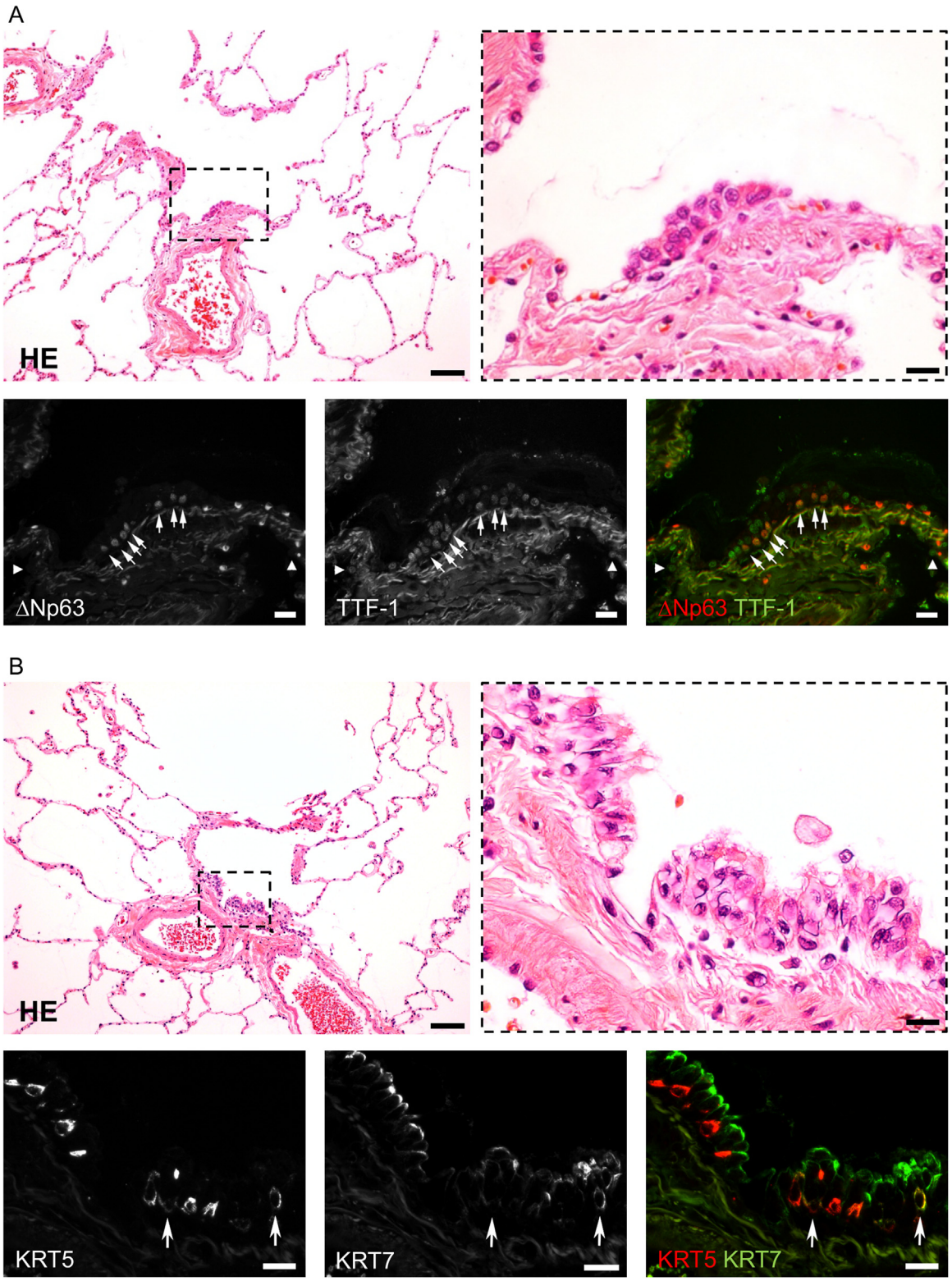

Fig. 4. ΔNp63$^+$ TTF-1$^+$ bronchiolar epithelial basal cells exist in the human lung. (A) Representative images of human peripheral lung tissue including a respiratory bronchiole. HE-stained images of low- and high-power magnification (top) and immunofluorescence for ΔNp63 and TTF-1 expression (bottom). Of note, HE staining (top) and immunofluorescence (bottom) were conducted on serial sections. The arrows indicate bronchiolar basal cells positive for both ΔNp63 and TTF-1, and the arrowheads indicate AT2 cells expressing TTF-1 alone. (B) Other representative images of human peripheral lung including a respiratory bronchiole. HE-stained images of low- and high-power magnification (top) and immunofluorescence for the expression of KRT5 and KRT7 (bottom). HE staining (top) and immunofluorescence (bottom) were conducted on serial sections. Arrows indicate bronchiolar basal cells expressing both KRT5 and KRT7. Scale bars, 100 μm (top left; A and B), 20 μm (top right and bottom; A and B).

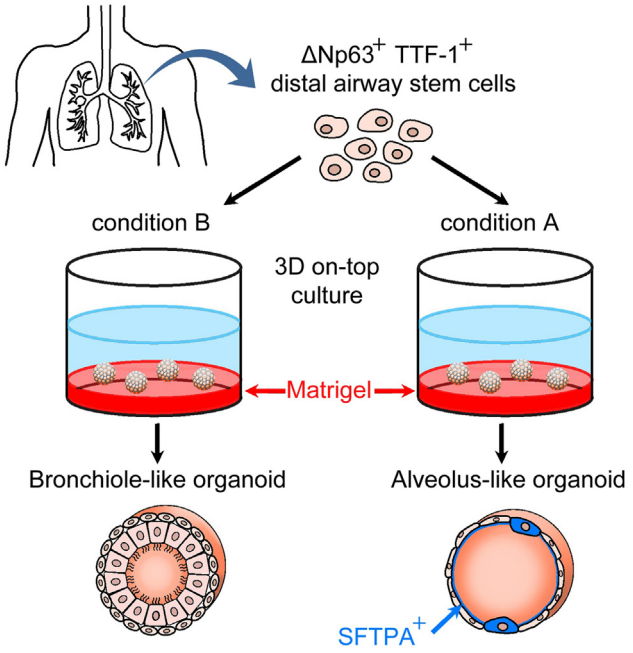

Fig. 5. ΔNp63$^+$ TTF-1$^+$ dual-positive distal airway cells are the human counterpart of mouse distal airway stem cells.

shown in Fig. 4A. Fig. 4B illustrates that KRT5$^+$ KRT7$^+$ dual-positive epithelial cells were also present in the respiratory bronchiole. These findings suggest that the HuL cells extensively analyzed in this study were derived from bronchiolar epithelial basal cells positive for ΔNp63 (or KRT5) and TTF-1 (or KRT7) in the human lung.

4. Discussion

Here we have demonstrated that HuL cells, derived from human peripheral lung tissues, in monolayer cultures, express both ΔNp63, a representative marker of airway basal cells and squamous cell carcinomas, and TTF-1, a well-known marker of AT2 cells and lung adenocarcinomas [24–26]. Furthermore, we show that HuL cells in 3D on-top culture can differentiate to form bronchiole-like or alveolus-like organoids. This suggests that HuL cells are the human counterpart of DASCs in the mouse lung (Fig. 5). On the other hand, HuB cells, derived from human bronchial epithelia, can only differentiate into bronchi in 3D on-top culture, even under "condition A." These findings are generally consistent with those of the seminal work reported by Kumar *et al.*; although, the group unexpectedly did not elucidate whether DASCs expressed TTF-1 [9].

One might doubt the existence of ΔNp63$^+$ TTF-1$^+$ HuL cells based on the prevailing notion that these two molecules are expressed in a mutually exclusive way in normal or neoplastic human lung tissues [14–16,24–26]. However, we present convincing evidence that a few epithelial basal cells in the human bronchiole express both markers, suggesting that HuL cells analyzed in this study were derived from these specific cells. Moreover, HuL cells are positive for both keratin markers (KRT5 and KRT7), which are also expressed, in principle, in a mutually exclusive manner [27,28]. Although several types of epithelial stem/progenitor cells in the peripheral lung have already been reported, including AT2 cells, club cells, and SCGB1A1$^+$ SFTPC$^+$ bronchioalveolar stem cells [7,29–31], these findings were mostly obtained by analyzing mice lungs. To the best of our knowledge, very few reports have extensively analyzed tissues or epithelial cells derived from human lung. It will be of critical importance to examine epithelial stem cells of the human lung, such as HuL cells, if we seek to apply "cell therapy" for the treatment of severe lung diseases.

To our knowledge, ΔNp63$^+$ TTF-1$^+$ dual-positive and KRT5$^+$ KRT7$^+$ dual-positive bronchiolar cells are a unique, previously unidentified, type of peripheral lung epithelial stem cell localized to the distal airway. An article has recently clarified that Barrett's esophagus—a condition in which the squamous mucosa in the distal esophagus is replaced by a columnar epithelium resembling that in the stomach or intestine—is formed by "transitional basal cells" expressing p63, KRT5, and KRT7 at the squamous–columnar junction [32]. The phenotype of the cells responsible for Barrett's esophagus resembles that of HuL cells. Considering that Barrett's esophagus is known as the precursor lesion of esophageal adenocarcinoma, HuL cells might also be the precursor cell for peripheral-type lung cancer, such as adenocarcinoma and squamous cell carcinoma. Further research on ΔNp63$^+$ TTF-1$^+$ dual-positive bronchiolar epithelial cells is required.

There are several limitations in the present study. Although HuL cells in 3D culture could form bronchiole-like or alveolus-like organoids, HuL cells transplanted in NSG mice survived and differentiated mostly into bronchial tissue, not bronchioles or alveoli. A few HuL cells were positive for SFTPA in the NSG mice, but we cannot confirm yet that HuL cells transplanted in the human lung can adequately regenerate the peripheral lung epithelia. McKeon and colleagues previously demonstrated that local DASCs in mice can grow and differentiate to restore the damaged peripheral lung epithelia caused by influenza virus infection [9,11], which suggests that various cytokines secreted from damaged lung tissue are crucial for DASCs to sufficiently regenerate the tissue. However, we transplanted HuL cells into bleomycin-pretreated lungs of NSG mice. Since the immunity of NSG mice is severely deficient, the tissues may fail to express several cytokines essential for promoting proliferation and/or differentiation of transplanted HuL cells [33]. This also may explain why HuL cells minimally differentiated into bronchiolar or alveolar epithelia in the NSG mice. More importantly, ΔNp63$^+$ TTF-1$^+$ dual-positive HuL5 or HuL6 cells extensively analyzed in this study were polyclonal cells derived from the peripheral lung tissues. Single cell cloning and subsequent differentiation study remains to be conducted in order to demonstrate that ΔNp63$^+$ TTF-1$^+$ dual-positive distal airway cells are the human counterpart of mouse DASCs. It has been increasingly appreciated that LGR5$^+$ epithelial stem cells in the gastrointestinal tract can be maintained in vitro for more than a year without losing pluripotency [2,3]. However, we have not established such an optimal method for the long-term culture of HuL cells and this could be a limitation in our study. We have found that treatment with a TGF-β receptor inhibitor and Y-27632 (a ROCK inhibitor) is effective, but not perfect, for the maintenance of the stemness of HuL cells (data not shown) [34,35]. An optimal long-term culture method for HuL cells is needed if we intend to exploit HuL cells for the treatment of chronic respiratory disease.

5. Conclusions

The present study shows that ΔNp63$^+$ TTF-1$^+$ HuL cells, derived from the peripheral lung tissue, can differentiate to form bronchiole-like and alveolus-like organoids. We also show that a few epithelial basal cells in the bronchiole express both ΔNp63 and TTF-1, suggesting that these cells are the precursor cell for HuL cells extensively analyzed. Thus, it seems that ΔNp63$^+$ TTF-1$^+$ bronchiolar cells are a type of peripheral lung epithelial stem cell that has the capacity to regenerate damaged bronchioles and/or alveoli. Cell therapy using ΔNp63$^+$ TTF-1$^+$ distal airway cells may offer a potential approach to treat chronic respiratory diseases, such as IPF and COPD, in the future.

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Authors' contributions

Y.S. designed and conceived the study, led and supervised all aspects of this study. Y.T. and M.Y. isolated normal epithelial cells from the bronchial or peripheral lung tissues, and conducted cell culture with help from T.S. and T.K. M.T. and A.W. provided ethically approved human lung tissues. Y.T. and S.H. performed western blotting experiments. Y.T. conducted all the RNA work and analysed the data. M.Y. conducted all the flow cytometry work. Y.T., M.Y., T.S., and A.S. performed all the experiments on NSG mice. Y.T. and Y.S. conducted all the immunocytochemistry and immunohistochemistry. H.C., T.K., A.W., and H.T. provided helpful discussions. Y.T. and Y.S. analysed and interpreted data, and wrote the manuscript. All authors commented on and approved the manuscript.

Conflicts of interests

None of the authors of the present study have a conflict of interest to declare.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.yexcr.2018.09.020.

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