

A novel type of serum-free medium for culturing human airway epithelium cells

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Abstract. The present study aimed to evaluate the ability of a novel serum-free medium (SFM) to culture human airway epithelium cells (hAECs). hAECs were cultured in the novel SFM as the experimental group in the PneumaCult-Ex medium and Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) as the control groups. Cell morphology, proliferative capacity, differentiation capacity and expression levels of basal cell markers were assessed accordingly in both culture systems. Optical microscope photos of hAECs were collected for cell morphology assessment. Cell Counting Kit-8 assay was conducted to assess the proliferation ability, and an air-liquid interface (ALI) assay was conducted to assess the differentiation capacity. Markers for proliferating basal and differentiated cells were relatively identified by immunohistochemical and immunofluorescent analysis. The results show that whether grown in the novel SFM or Ex medium, hAECs exhibited similar morphology at every passage, whereas cells could hardly form colonies in the DMEM + FBS group. Cells typically exhibited cobblestone shape, while a proportion

of them in the novel SFM at late passage exhibited a larger shape. White vesicles appeared in the cytoplasm of some control cells at the later stage of culture. Basal cell markers (P63⁺KRT5⁺KI67⁺CC10⁻) for proliferating ability were found in the hAECs cultured by the novel SFM and Ex medium. hAECs at passage 3 cultured in the novel SFM and Ex medium both had the capacity to differentiate into ciliated cells (acetylated tubulin⁺), goblet cells (MUC5AC⁺) and club cells (CC10⁺) in the ALI culture assay. In conclusion, the novel SFM was capable of culturing hAECs. The hAECs cultured by the novel SFM could proliferate and differentiate *in vitro*. The novel SFM does not change the morphological characteristics or biomarkers of hAECs. The novel SFM has the potential for the amplification of hAECs for scientific research and clinical application.

Introduction

Respiratory diseases are one of the most common causes of death worldwide (1) and pose a significant burden on any healthcare system and society. The spread of COVID-19 has further complicated the respiratory disease network and exacerbated the challenges of social healthcare resources (2). Accurate diagnosis and effective treatment of respiratory diseases require an in-depth understanding of their pathogenesis and the pathophysiology of airway epithelial cells. However, the existing airway epithelial models cannot meet the growing scientific needs, mainly due to the lack of models and the poor efficiency of existing model production, making the study of the basis and application of pathogenic processes of respiratory diseases a difficult and tedious task. Considering the structural and physiological differences between animals and humans, using human airway epithelial cells (hAECs) cultured *in vitro* rather than the animal as an airway epithelial model seems to be an ideal choice. Therefore, how to obtain and expand human airway epithelial cells is bound to become a crucial scientific mission in the future.

Basal cell (BC) is considered the central and the key to airway regeneration. As one of the airway progenitor cells responsible for regeneration, BC can regenerate itself, differentiate into other types of airway epithelial cells to maintain airway homeostasis, and participate in emergency repair in situations such as airway injury (3). Thus, the successful

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Abbreviations: SFM, serum-free medium; hAECs, human airway epithelium cells; ALI, air-liquid interface; DMEM, Dulbecco's modified eagle medium; BC, basal cell; CR, conditional reprogramming; FBS, fetal bovine serum; Db-cAMP, N, N-Dibutyladenosine 3',5'-phosphoric acid; PKA, protein kinase A; CREB, cAMP-responsive element binding protein; ATF1, transcriptional activator 1; Epac, exchange protein directly activated by cAMP

Key words: serum-free medium, human airway epithelium cell, basal cell

amplification of BC is significant to the amplification of hAECs.

Researchers cultured BC *in vitro* by the technique named conditional reprogramming (CR) (4,5). CR involves the mouse fibroblast cell line 3T3-J2 as feeder layer cells and culture medium containing fetal bovine serum (FBS). The feeder cells 3T3-J2 and FBS affect the clinical use of hAECs because of containing animal components foreign to humans. Even if patients receive autologous cell therapy, media supplemented with FBS will bring risks of triggering immune rejection reactions, making cell therapy much more uncertain (6). Besides, the preparation of feeder cells is a high workload (7), and FBS contains a number of poorly defined components (e.g., growth factors, antibodies, and other immunologically active substances) with different component concentrations from different production batches (8). Therefore, hAECs should be cultured and expanded in SFM without feeder co-culture.

In the present study, we tested a novel developed SFM for the culture of hAECs. The novel SFM is designed to be able to monoculture hAECs, without FBS and the help of feeder cells. We used PneumaCult™-Ex medium (STEMCELL, Canada) and DMEM + FBS as the control groups and evaluated the cell morphology, cell markers, proliferating, and differentiating accordingly. We hypothesized that hAECs could expand in the novel SFM without altering phenotype, proliferation, and differentiation.

Materials and methods

Media and reagents. To prepare the serum-free medium, the following culture media and reagents were used: MCDB 153 medium, 40 µg/ml L-Histidine, 100 µg/ml L-Isoleucine, 30 µg/ml L-Methionine, 60 µg/ml L-Phenylalanine, 16 µg/ml L-Tryptophan, 100 µg/ml L-Tyrosine, 500 µg/ml Db-cAMP (N, N-Dibutyladenosine 3',5'-phosphoric acid), 10 ng/ml rFGF1 (Recombinant fibroblast growth factor 1), 1 ng/ml rEGF (Recombinant epidermal growth factor), 20 ng/ml rIGF1 (Recombinant insulin growth factor 1), 0.1 µg/ml Hydrocortisone (STEMCELL, Canada), 4 µg/ml Nicotinamide, 5 µmol/l Blebbistatin (Nuwacell, Anhui, China). If not specifically noted, all agents above were purchased from Sigma-Aldrich, USA.

Dulbecco's modified eagle medium (DMEM) and fetal bovine serum (FBS) were from Gibco, USA. PneumaCult-Ex and PneumaCult-ALI medium were from STEMCELL, Canada.

Cell culture. hAECs were collected through bronchoscopic brushing samples. All cell samples were obtained from Shanghai Jiao Tong University Affiliated Sixth People's Hospital (Shanghai, China), with patient informed consent. Table SI includes basic patient information and pulmonary function test results to illustrate the physiological and pathological background of samples. Patient 1 is a 60 years old female diagnosed with bronchiectasis and hypertension, who was sampled on 2022/07/22. Patient 2 is a 56 years old female diagnosed with interstitial pneumonia and no comorbidities, who was sampled on 2022/07/25. Both patients had restrictive hypoventilation. Patient 2 was milder but had diffusion dysfunction and small airway dysfunction. Both patients were

sampled at the basal segment of the lower lobe of the right lobe.

Samples were washed in pre-chilled wash buffer (Ham's F12, 5% fetal bovine serum, 100 µg/ml pen strep, 100 µg/ml Gentamycin, and 0.25 µg/ml Fungizone) and were centrifuged at 1,000 rpm, 4°C for 10 min and the supernatant was discarded. The centrifugal process was repeated three times. The resulting cells were plated into a 12-well plate (Corning, NY, USA) in the serum-free medium containing 100 µg/ml pen strep and 1 µg/ml Blebbistatin (Nuwacell, Anhui, China). The plates were designated as passage 0 (P0) and were transferred into a 37°C, 5% CO₂ saturated humidity incubator for further culturing. The medium was changed every two days, and the cells were passaged at a 1:1 to 1:2 ratio when they reached 80-90% confluency.

Brushing samples carry various cells, including blood cells. Due to the selectivity of the medium, the unwanted cell groups will fade by themselves during the culturing (usually P0 to P2). Only basal cells are suitable to survive and thrive in the medium. In order to avoid the unwanted cell groups affecting experiment results, the subsequent experiments do not apply cells before P3 unless necessary.

CCK-8 cell proliferation assay. hAECs obtained from P3 were seeded on 96-well plates (Corning, NY, USA) at 6×10^3 cells/cm² and incubated at 37°C in 5% CO₂. At 24, 48, 72, 120, 168, and 216 h after cell seeding, hAECs proliferation was measured by CCK-8 assay. The old supernatant was replaced by WST-8 and fresh medium at a 1:10 ratio. WST-8 refers to 2-(4-indophenyl)-3-(4-nitrophenyl)-5-(2,4-disulpho-phenyl)-2H-tetrazolium monosodium salt (Beyotime Biotechnology, Beijing, China). The processed plate was incubated at 37°C in 5% CO₂ for 1.5 h. Staining intensity in the medium was measured by determining the absorbance at 450 nm, and the data were expressed as ratios of the control value. The data are collected from three independent experiments performed in duplicate. The proliferation curve was depicted by Graphpad Prism 8.

Immunohistochemical and immunofluorescent analysis. Cells from P1, P3, P5, and P7 were fixed in 4% (w/v) paraformaldehyde for 8 min, then washed with phosphate-buffered saline (PBS). Cells were proceeded to permeabilization by 0.2% Triton-X for 15 min, washed with PBS. The processed cells were blocked with PBST (0.05% Tween-20, 5% FBS in PBS) for 1 h and then incubated with primary antibodies diluted in PBS overnight at 4°C. Following incubation with primary antibodies, cells were rinsed three times in PBS and incubated with a solution of secondary antibodies diluted at 1:400 in PBS for 1.5 h in the dark. After three further washes in PBS, 4',6-Diamidino-2-phenylindole (DAPI, MilliporeSigma) diluted at 1:500 was added for 5 min to stain cell nuclei. Afterward, the cells underwent confocal microscopy using a laser scanning confocal microscope by Olympus FV1200. Images were analyzed and processed by FV10-ASW 4.2 Viewer and Adobe Photoshop 19.0.

The following antibodies and dilutions were used: mouse anti-P63 (ab735; Abcam, UK) at 1:200; mouse uteroglobin monoclonal antibody (MA5-17170; Thermo Fisher Scientific, USA) at 1:200; rabbit anti-KRT5 (MA5-14473; Thermo

Fisher Scientific, USA) at 1:200; rabbit anti-KI67 (ab16667; Abcam, UK) at 1:250; mouse anti-CDKN2A/P16 (sc-1661; Santa Cruz, USA); rabbit anti-P21 Waf1/Cip1 (#2947, Cell signaling Technology, USA); goat anti-mouse Alexa Fluor 488 and goat anti-rabbit Alexa Fluor 555 (Thermo Fisher Scientific) at 1:400.

As for the immunofluorescence of air-liquid interface (ALI) culture mentioned later, the following antibodies and dilutions were used: mouse anti-acetylated tubulin (T7451; Sigma, USA) at 1:200; mouse uteroglobin monoclonal antibody (MA5-17170; Thermo Fisher Scientific, USA) at 1:200; rabbit anti-MUC5AC (ab198294; Abcam, UK) at 1:200; goat anti-mouse Alexa Fluor 488, goat anti-rabbit Alexa Fluor 555, and goat anti-mouse Alexa Fluor 555 (Thermo Fisher Scientific) at 1:400.

Air-liquid interface (ALI) culture. Cells from P3 were seeded at high density ($5 \times 10^5/\text{cm}^2$) on Costar 6.5 mm Transwell, 0.4 μm Pore Polyester Membrane Inserts. After the seeding cells reached full confluency, the proliferative medium was switched to the differentiation medium (PneumaCult-ALI Medium, STEMCELL, Canada) to induce mucociliary differentiation. Subsequently, the apical medium was removed, and the cells received nutrients only from the basolateral side (air-liquid interface condition) for 3 weeks. When the differentiation was fully performed, pick up the Transwell from the culturing plate. Cut a circle carefully at the bottom membrane with a sterile surgical blade and then separate the membrane. The harvested layers were fixed in 4% (w/v) paraformaldehyde and were paraffin-sectioned to make the monolayer slices of ALI tissue. The slices will show the sagittal plane or coronal plane of the membranes. All cells are incubated under the circumstances of 37°C and 5% CO₂. The cultured ALI tissues were further analyzed by immunohistochemical and immunofluorescent analysis.

Statistical analysis. All experiments were repeated at least three times. The data are shown as the average \pm standard deviation. Comparisons between groups were analyzed using a t-test. Statistical analyses were performed using SPSS 20.0 and Graphpad Prism 8. P-values <0.05 were considered to indicate statistically significant differences.

Cell counting and nuclei mean area measuring were performed via ImageJ. Transform the DAPI image into the 8-bit format and set the threshold by the 'Auto' option without any other adjustments. Use the 'Fill holes' tool to fill the gaps in incomplete cells. Then use the tool of 'Watershed' to split overlapping cells. At last, use the tool 'Analyze Particles.' The number of cells and the nuclei size is calculated automatically by ImageJ.

Results

hAECs expands similarly in the novel SFM and PneumaCult-Ex. Cell samples from bronchoscopic brushing were seeded in the novel SFM and STEMCELL PneumaCult-Ex medium, which was taken as a control group (Fig. 1A). hAECs exhibited similar, consistent morphology at every passage, generally showing the shape of cobblestones (Fig. 1C). Newly seeded cells appeared round initially and

Table I. Duplication time between passages.

Passage	SFM		Ex	
	Patient 1	Patient 2	Patient 1	Patient 2
P0	7 days	7 days	10 days	7 days
P1	6 days	7 days	4 days	3 days
P2	6 days	8 days	3 days	4 days
P3	7 days	7 days	3 days	3 days
P4	9 days	6 days	4 days	5 days
P5	7 days	7 days	6 days	8 days
P6	6 days	/	6 days	
P7	7 days	/	6 days	

began to sprawl into cobblestones-shape. Part of the cells showed a more pointed form, which was like a spindle. A proportion of cells in the novel SFM appeared in a larger form with abundant cytoplasm and large nuclei (Table SII). At late passage, white vesicles appeared in the cytoplasm of cells in Ex medium.

Previous research (9) shows basal cells cultured by CRC retained colony-forming efficiency at P3. Also, during the early culturing phase (P0-P2), unrelated cell groups may interfere with the CCK-8 assay result. When the culturing progressed to P3, there were ample cell materials for the assay. Thus, we applied hAECs from P3 to depict the proliferation curve of cells in the novel SFM and Ex medium (Fig. 1B). From day 1 to day 7, hAEC in the novel SFM grows relatively slowly compared to Ex medium. However, on day 9, the result showed no significant difference in proliferation capacity between hAECs culturing in the novel SFM and Ex medium (P=0.7822).

To clearly illustrate the novel SFM's proliferating ability, we seeded cells from P3 in the novel SFM and DMEM + FBS (Fig. S1). The novel SFM expanded hAECs successfully, whereas DMEM + FBS group can hardly perpetuate the cell lineage (Fig. S1C). The CCK-8 assay result validated the culture result. The difference in the number of cells between the two groups on day 5 was statistically significant (P=0.0024) (Fig. S1B).

As shown in Table I, hAECs in novel SFM proliferated at a relatively constant rate during P0 to P7. hAECs in Ex medium had a slow starter but thrived from P1 to P4. Cells in Ex medium had a shorter duplication time than the novel SFM during P1 to P4. However, the novel SFM had no significant difference with Ex medium in proliferating speed after P4. The proliferation decelerated in the experimental and control groups at late passage.

Immunofluorescent results show basal cell markers strongly positive. In passages 1, 3, and 5, we used hAECs cultured from the monoculture system to conduct immunohistochemical and immunofluorescent analysis to identify whether the novel SFM can maintain the characteristics of hAECs, especially basal cells. As mentioned above in the Introduction, the success of proliferating BCs is of great significance to the amplification

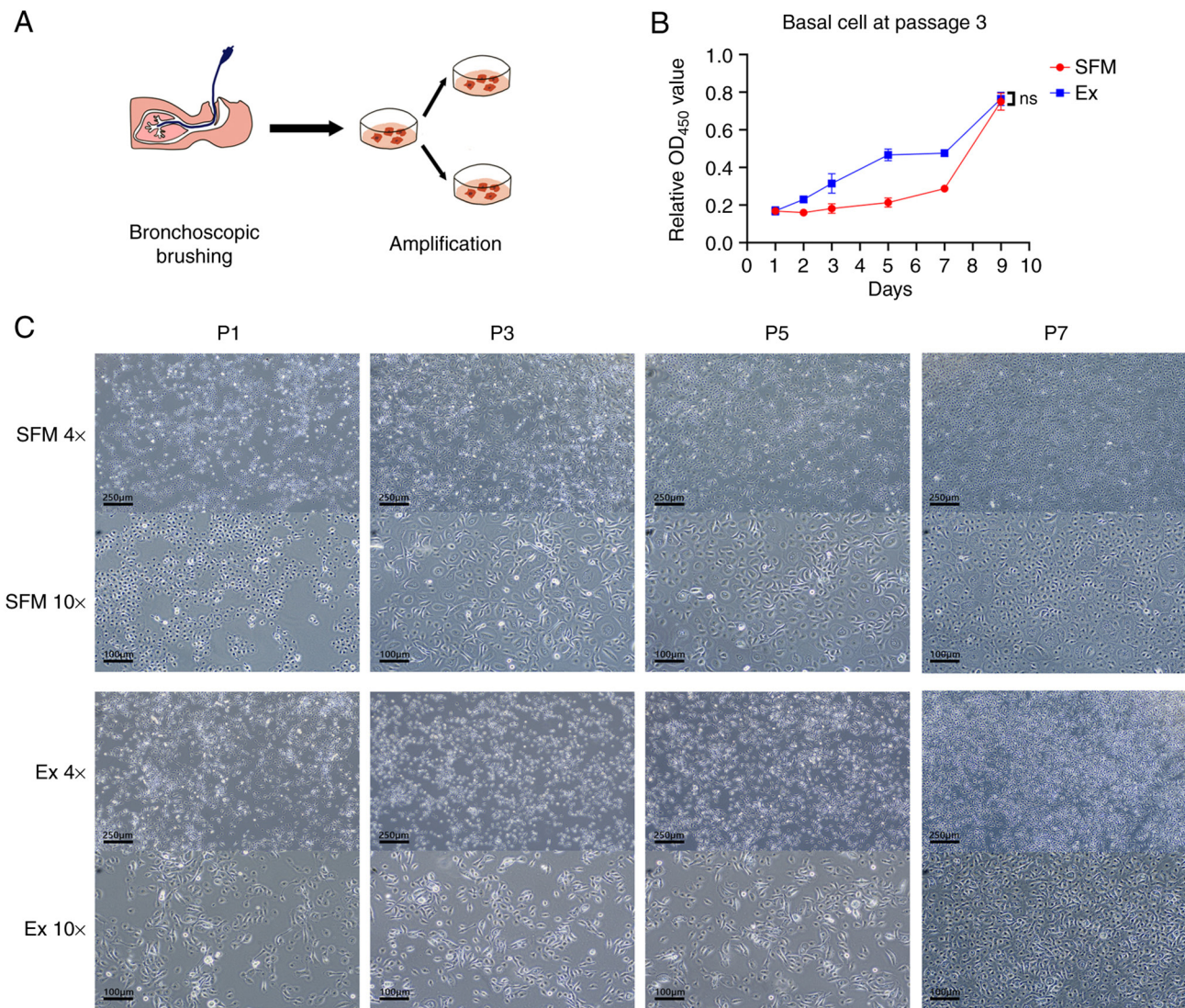


Figure 1. Cell culture results in the experiment and the Ex control group. (A) General design of the experiment. Cell samples from bronchoscopic brushing were seeded in the medium and subsequently amplified. (B) Proliferation curve of basal cells in the novel SFM and Ex medium. (C) Brightfield microscopy images of cell colonies in the novel SFM and Ex medium (scale bar top row, 250 μm ; scale bar bottom row, 100 μm). Ns, no statistical significance; SFM, serum-free medium; Ex, PneumaCult-Ex medium.

of hAECs. Staining for BC marker (P63⁺KRT5⁺) was strongly positive in both novel SFM or Ex medium (Fig. 2). The proliferating marker Ki67 was also positive in both groups (Fig. 3A). Compared with 32, 38, and 36% in the Ex medium, 31, 30, and 30% of P63⁺KRT5⁺ BCs showed Ki67 positive in the novel SFM in passages 1, 3, and 5. (Fig. 3B). CC10, a marker representing club cells, was utterly negative in both groups.

hAECs differentiated into an airway-like structure on the air-liquid interface (ALI). We asked further whether hAECs from the novel SFM can retain the differentiate ability. We chose a three-dimensional platform air-liquid interface (ALI) to investigate possible differences between the novel SFM and Ex medium. Cultured on ALI successfully, the tissues were sent to subsequent immunofluorescence. The result showed that both hAECs from the novel SFM and Ex medium could differentiate into an airway-like structure, which consists of ciliated cells (Acetylated tubulin⁺), goblet cells (MUC5AC⁺), and club cells (CC10⁺) (Fig. 4A).

The ciliated cells (green) formed a line on the top layer, with goblet cells (red, left) forming niches. Sporadic club cells (red, right) lay on the bottom side of the tissue. Differentiated tissues presented similar cell composition: 35~40% ciliated cells (39.8% SFM vs. 36.3% Ex), 15~22% goblet cells (21.8% SFM vs. 16.8% Ex), and 8% club cells (7.7% SFM vs. 8.1% Ex) (Fig. 4B). The composition is comparable with the typical conducting airway differentiation cell ratio.

Immunofluorescent results show cell senescence may initiate after passage 3. To investigate whether cell senescence will appear in hAECs and when it will appear, we stained P16 and P21 markers (both are senescence-indicating markers) in P3, P5, and P7 using cells in the experimental and the Ex control group (Fig. 5). The immunofluorescent results show barely any P16 or P21 positive cells in passage 3. P16 or P21 positive cells were found in passages 5 and 7, and there were more P16 or P21 positive cells in passage 7 than in passage 5.

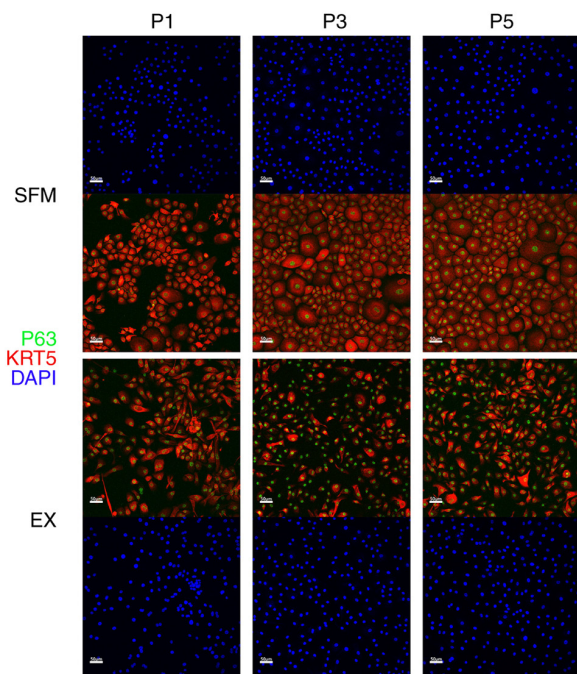


Figure 2. Immunofluorescent results of P63 and KRT5. The figure shows fluorescence microscopy images of basal cell colonies stained for P63 (green), KRT5 (red), and DAPI (blue) at passage 1 (P1), passage 3 (P3), and passage 5 (P5) cultured by the novel SFM and Ex medium. Scale bar, 50 μ m. SFM, serum-free medium; Ex, PneumaCult-Ex medium.

Discussion

The novel serum-free medium provides a new alternative to amplify human airway epithelium cells. Compared with other media used for hAECs amplification, the novel SFM has several characteristics: 1) clear and well-defined components; 2) low cost for ingredients; 3) simple and easy culturing procedure, no need to coating or prepare feeder cells; 4) able to maintain proliferating and differentiating ability 5) can retain lineage marker consistency (4,5).

Other culturing protocols for hAECs usually include the application of FBS and feeder cells. On the one hand, FBS contains a number of poorly defined components (e.g., growth factors, antibodies, and other immunologically active substances). Different production batches of FBS contain different concentrations of components (8). Moreover, FBS contains animal-origin components which are foreign to humans. Even if patients receive autologous cell therapy, cells cultured in a medium supplemented with FBS still carry the risk of triggering immune rejection reactions, making cell therapy much more uncertain (6). On the other hand, feeder co-culture gives a much higher workload compared to monoculture because of the necessity of preparing feeder cells (7). Before co-culturing is launched, feeder cells need to be expanded and adjusted for cell status. The irradiation or mitomycin treatment is also required to stop feeder cell proliferation. Feeder cells need to be removed by manual or culture media change to prevent contamination of animal-origin components. To diminish the workload of preparing feeder cells, a conditioned medium after co-incubation with irradiated feeder cells has been tested, but the result turned out not to be as effective as direct co-culturing

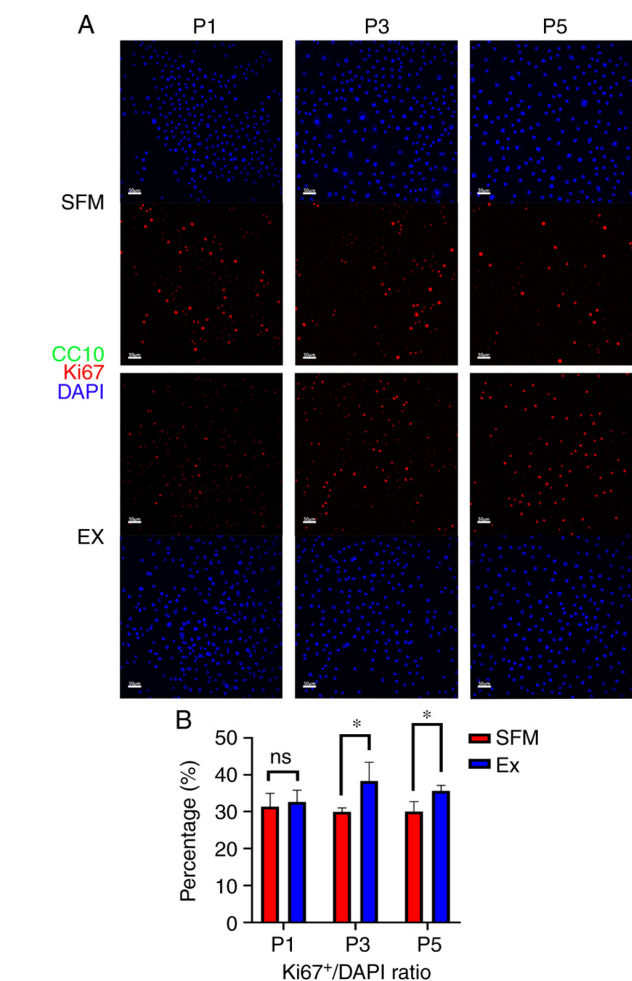


Figure 3. Immunofluorescent results of KI67 and CC10. (A) Fluorescence microscopy images of basal cell colonies stained for Ki67 (red), CC10 (green), and DAPI (blue) at P1, 3, and 5 cultured by the novel SFM and Ex medium. Scale bar, 50 μ m. (B) Ratio of Ki67 positive cells to the total cells. * $P < 0.05$. Ns, no statistical significance; SFM, serum-free medium; Ex, PneumaCult-Ex medium.

with feeder cells (10). Although most feeder cells can be manually removed during the culture process, any slight of very few feeder cells can still render cell agents potentially heterogeneous (11). Therefore, for hAECs, monoculture using a serum-free medium is recommended for high safety and stability.

Due to the reasons above, we have tested various combinations and developed the novel SFM. Based on the basic MCDB 153 medium, we supplemented high amino acid supplement and Db-cAMP, rFGF1, rEGF, rIGF1, hydrocortisone, and nicotinamide. The high amino acid supplement includes histidine, isoleucine, methionine, phenylalanine, tryptophan, and tyrosine, which are all essential amino acids. Cell proliferation needs the support of essential amino acids. Significantly when cells thrive, the consumption of essential amino acids will increase. Isoleucine may play a more important role because when isoleucine is deficient, the cell cycle will be blocked at G or G/M phases, leading to proliferation impairment (12).

Db-cAMP is involved in activating the second messenger (cAMP) signal transduction pathway, whose activating can lead to cell growth (13). Any reagent which can activate the

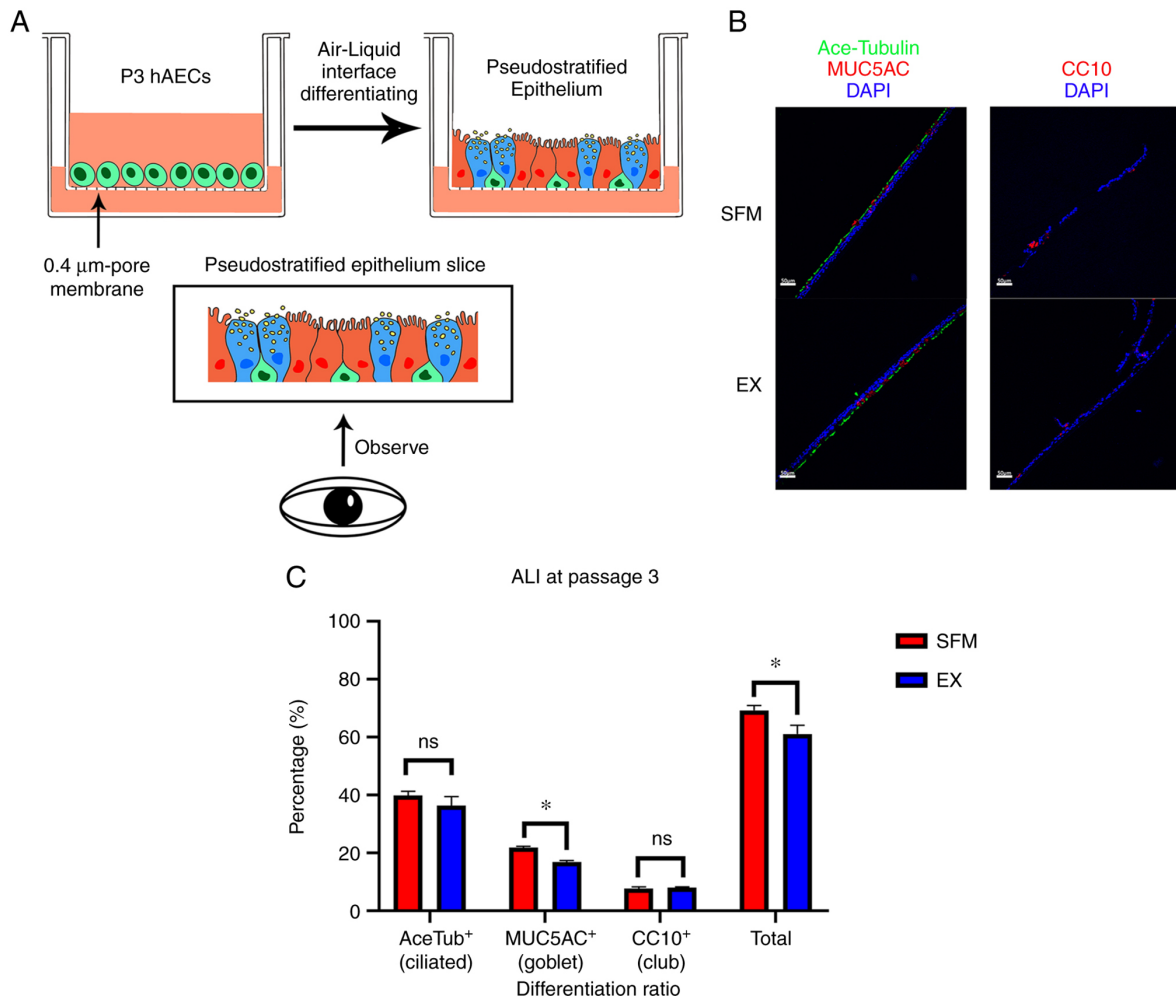


Figure 4. Differentiating results and immunofluorescent results of ALI. (A) General procedures of ALI culturing. hAECs from P3 were seeded in Transwell assay with 0.4- μm pore polyester membrane. When the ALI culturing was fully performed, the differentiated pseudostratified epithelium tissue (basal cell, green; ciliated cell, red; goblet cell, blue) was processed to be sliced. The slices show the sagittal plane or coronal plane of the membranes. (B) Fluorescence micrographs of the differentiation conditions of ALI tissue at day 21. Cell sources of ALI tissues were basal cells at passage 3 cultured from the novel SFM and Ex medium, respectively. Acetylated tubulin, green; MUC5AC, red left; CC10, red right; DAPI, blue. Scale bar, 50 μm . (C) Differentiation ratio of ALI tissue. * $P < 0.05$. Ns, no statistical significance; ALI, air-liquid interface; hAECs, human airway epithelium cells; SFM, serum-free medium; Ex, PneumaCult-Ex medium.

cAMP signal transduction pathway to increase intracellular cAMP levels to activate cAMP-dependent protein kinases is optional for components, including but not limited to 8-Bromo-cAMP, 6-Bnz-cAMP, cAMPs-Sp, 8-CPT-Cyclic AMP, Forskolin or IBMX. According to the previous research (14,15), the increase of cAMP stimulates the growth via the activation of protein kinase A (PKA), activation of the cAMP-responsive element binding protein (CREB), and activation of the extracellular signal-regulated kinases 1/2 (ERK1/2). The CREB family proteins are well-characterized PKA substrates. This family is a group of the basic leucine zipper (bZIP) superfamily transcription factors, including CREB, cAMP-responsive element regulatory protein (CREM), and transcriptional activator 1 (ATF1). Activated ATF1 and CREB can form homodimer or heterodimer, bind to the cAMP response element (CRE) in the promoter region of target genes and initiate gene transcription, thereby regulating cell differentiation and proliferation. cAMP can also activate Epac (Exchange protein directly activated by cAMP). Epac proteins exert their diverse biological functions either

alone and/or in concert with PKA (16). Epac was reported to have effects on cell division and differentiating (17). However, The role of Epac in cell division signaling by cAMP cannot be determined. Epac exerts opposing effects even on the same cellular process (16). Whether Epac affects hAECs in concert with/without PKA in proliferating and differentiating positively or negatively still remains elusive.

FGF1 and EGF are potent growth factors that can stimulate epithelial cell proliferation. They are both involved in the receptor complex kinase and RTK-Ras protein signaling pathways (18). IGF1 belongs to the insulin factor family and is structurally and functionally similar to insulin but has higher growth-promoting activity than insulin (19). Hydrocortisone and nicotinamide are common supplements for epithelial culture. They can increase cell viability and maintain cell morphology (20,21). Noticeably, there are no specific restrictions on the specific type of components. Any component with similar effects is optional. The supplemented ingredients above are all clear and well-defined. In addition, because of the absence of feeder cells, the

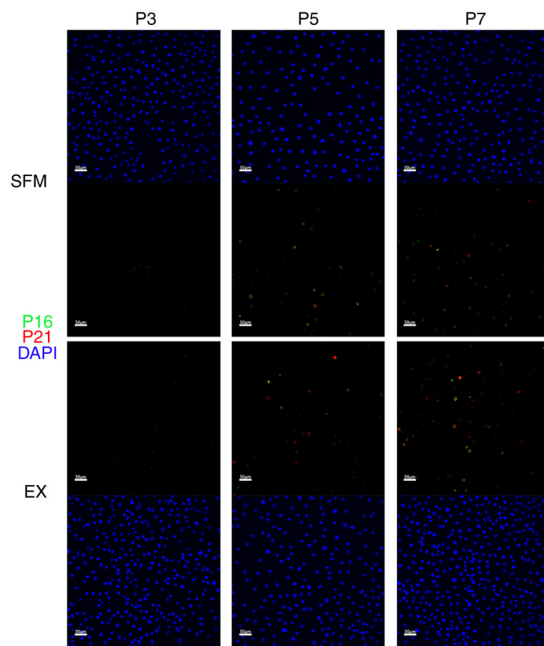


Figure 5. Immunofluorescent results of P16 and P21. The figure shows fluorescence microscopy images of basal cell colonies stained for P16 (green), P21 (red), and DAPI (blue) at passage 3 (P3), passage 5 (P5) and passage 7 (P7) cultured by the novel SFM and Ex medium. Scale bar, 50 μ m. SFM, serum-free medium; Ex, PneumaCult-Ex medium.

culturing procedure using the novel SFM is simple and easy. Preparing feeder cells or pre-coating is of no necessity for the novel SFM.

To investigate the culture potential of the novel SFM, we used cell cultures and took the STEMCELL Ex medium and DMEM + FBS as control groups. The results show that serum-free medium can expand hAECs successfully without involving FBS (Seen Fig. S1). Due to the poor practicability of the DMEM + FBS combination, which can hardly keep basal cells expanding, we chose STEMCELL PneumaCult-Ex medium as the control group in the following research phases. The general morphology of hAECs was consistent from the beginning to the end of the culture process. However, white vesicles appeared in the cytoplasm of cells at late passage (passage 3-5) in Ex medium (Fig. 1). We considered the phenomenon might be the symbol of senescence and apoptosis, which is consistent with the result that the percentage of Ki67⁺ basal cells dropped as the generation counts increased at late culturing phase (Fig. 3).

For primary hAECs, it is natural to find the cell expansion ability declines, representing as cell division slows down and differentiation potential loss (22). Previous research (9) suggests basal cells cultured by CRC methods retained colony-forming efficiency at least to passage 3. To investigate whether cell senescence will appear in hAECs, we stained P16 and P21 (Fig. 5). The results suggested cell senescence may initiate after passage 3. We supplement the novel SFM ROCK-inhibitor (Rho-associated coiled coil protein kinase inhibitor, ROCKi) Blebbistatin to solve the problem. According to previous studies (23-25), ROCK-inhibitor can accelerate the cell cycle and inhibit apoptosis and cell differentiation.

For the next step, we want to confirm whether cells cultured in the different media have differences. We have conducted cell marker immunofluorescence and found no significant difference, suggesting that though the cells isolated from the samples have morphological diversity in different media, they were all basal cells (P63⁺KRT5⁺KI67⁺CC10⁻). The different medium formulas can be responsible for the morphological diversity, but both medium managed to culture primary hAECs into BCs. According to earlier research (26), KRT5 and P63 are markers showing cell stemness. Differentiated cells or cells ready to differentiate will lose these stemness markers. Approximately 30% of cells in the Ex medium did not express the KRT5 marker, whereas 100% of cells in the novel SFM were KRT5⁺. In other words, the novel SFM can retain the basal cell marker better and thus has better stemness retaining ability.

To ask further whether hAECs from the novel SFM can retain the differentiating ability, we used a three-dimensional differentiate platform air-liquid interface (ALI). Obviously, the immunofluorescent result showed similar composition between tissues cultured by the novel SFM or Ex medium. Notably, the composition of ALI tissue is comparable with the cell proportion of conducting airway (27). However, differentiated cells from Ex medium account for only 60% (ideally, it should be 70~80%) in ALI tissue. There were lesser goblet cells in the Ex control group. A possible explanation is that the ALI tissue from the Ex medium was not fully differentiated, or the induction characteristics of the ALI medium should be considered. In other words, hAECs cultured by the novel SFM or Ex medium can be induced to differentiate into airway-like structure tissue, implying adequate differentiating potential and the de-differentiation effect of ROCKi.

There are still limitations and a few questions need to be answered in this study. The reason for the relatively slow growth speed in the early stage of the novel SFM remains unclear. The main task in the future is to focus on increasing proliferating cell ratio in the cell groups. Moreover, how to postpone cell senescence and apoptosis and prolong the available duration are to be addressed. It is also unknown how the cell physiology changes in subsequent generations (such as P9) since we have terminated the culture at P7. We should explore the effect of medium components and specific mechanisms of hAECs in order to promote formula improvements in future studies.

In Conclusion, the novel SFM is capable of culturing hAECs. The hAECs cultured by the novel SFM can proliferate and differentiate *in vivo*. The novel SFM does not change the morphologic characteristics or biomarkers of hAECs. The novel SFM has the potential for the amplification of hAECs for scientific research and clinical application.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

HD, QZ, ZL, SS and TR contributed to the study concept and design. HD, QZ, ZL, SS, YL, JZ, CZ, YX and TR acquired, analyzed and interpreted the data. HD, QZ, ZL and TR drafted the manuscript. HD and QZ confirm the authenticity of all the raw data. HD and QZ performed the statistical analysis. TR obtained funding. HD, QZ and ZL provided administrative, technical and material support. TR supervised the study. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Institutional Review Board of Shanghai Jiao Tong University Affiliated Sixth People's Hospital (approval no. 2020-152).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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