EBioMedicine xxx (2018) xxx–xxx

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Paper

Long-Term Culture of Distal Airway Epithelial Cells Allows Differentiation Towards Alveolar Epithelial Cells Suited for Influenza Virus Studies

Aki Imai-Matsushima [a,1], Laura Martin-Sancho [a,1], Alexander Karlas [a], Seiichiro Imai [a], Tamara Zoranovic [a], Andreas C. Hocke [b], Hans-Joachim Mollenkopf [c], Hilmar Berger [a], Thomas F. Meyer [a,*]

[a] Department of Molecular Biology, Max Planck Institute for Infection Biology, Berlin, Germany
[b] Department of Internal Medicine/Infectious Diseases and Pulmonary Medicine, Charité University Medicine, Berlin, Germany
[c] Max Planck Institute for Infection Biology, Core Facility Microarray/Genomics, Berlin, Germany

ARTICLE INFO

Article history:
Received 22 January 2018
Received in revised form 25 May 2018
Accepted 25 May 2018
Available online xxxx

Keywords:
Distal airway epithelial cells
Influenza A virus
Alveolar epithelial cells
Lung disease

ABSTRACT

As the target organ for numerous pathogens, the lung epithelium exerts critical functions in health and disease. However, research in this area has been hampered by the quiescence of the alveolar epithelium under standard culture conditions. Here, we used human distal airway epithelial cells (DAECs) to generate alveolar epithelial cells. Long-term, robust growth of human DAECs was achieved using co-culture with feeder cells and supplementation with epidermal growth factor (EGF), Rho-associated protein kinase inhibitor Y27632, and the Notch pathway inhibitor dibenzazepine (DBZ). Removal of feeders and priming with DBZ and a cocktail of lung maturation factors prevented the spontaneous differentiation into airway club cells and instead induced differentiation to alveolar epithelial cells. We successfully transferred this approach to chicken distal airway cells, thus generating a zoonotic infection model that enables studies on influenza A virus replication. These cells are also amenable for gene knockdown using RNAi technology, indicating the suitability of the model for mechanistic studies into lung function and disease.

1. Introduction

Lower respiratory infections, chronic obstructive pulmonary disease and lung cancer are amongst the top ten causes of death worldwide. However, research into these conditions has been hampered by the absence of stable primary cell models.

The human alveolar epithelium is lined with non-proliferative type I alveolar epithelial (AEI) cells specialized for gas exchange, and proliferative type II alveolar epithelial (AEII) cells, which produce surfactant proteins with essential roles during innate immune responses [1, 2]. AEII cells are targeted by various pathogens that cause life-threatening pneumonia, several of which have a zoonotic origin [3]. This includes influenza A virus (IAV), whose natural reservoir is birds but which has undergone human adaptation causing serious pandemics [4–9]. Comparative studies using available cell types to study IAV zoonosis, such as human lung adenocarcinoma cell line A549 and the chicken embryonic fibroblast cell line DF-1, are likely to confound species-, tissue-, and cell line-specific effects. Therefore, establishing human and avian primary cell models would enable more physiologically relevant comparison.

Although alveolar epithelial cell culture models have been established previously, these have been hampered by spontaneous transdifferentiation of type II to type I-like cells *in vitro* [10, 11]. Therefore, most experiments with primary human alveolar epithelial cells require isolation from fresh tissue and are limited by the supply of lung specimens, as well as the time and cost associated.

Organoid models allow long-term culture of cells derived from rapidly regenerating organs using dedicated adult stem cells [12–14]. However, the regeneration of adult lung epithelium is only activated upon injury, impeding the identification of the cell types involved. Most of the evidence appoints differentiated alveolar epithelial cells as the progenitor cells of the alveolar epithelium, although other reports suggest that specialized stem cells are recruited upon severe alveolar damage [15]. The potential to differentiate alveolar linages from human distal airway stem cells (DASCs) was addressed previously [16]. Human DASCs were found to express P63 and cytokeratin 5 (CK5), which are markers for progenitor cells of the stratified epithelium, and were able give rise to podoplanin$^+$ AEI cells and CC10$^+$ airway club cells, but not surfactant protein C$^+$ AEII cells [16]. Bove and colleagues grew human AEII cells in culture with feeder cells and the rho kinase inhibitor Y-27632 for >30 population doublings [17]. However, markers of AEII

* Corresponding author at: Max Planck Institute for Infection Biology, Department of Molecular Biology, Charitéplatz, 111017 Berlin, Germany.
E-mail address: meyer@mpiib-berlin.mpg.de (T.F. Meyer).
[1] Authors contributed equally to this work.

https://doi.org/10.1016/j.ebiom.2018.05.032

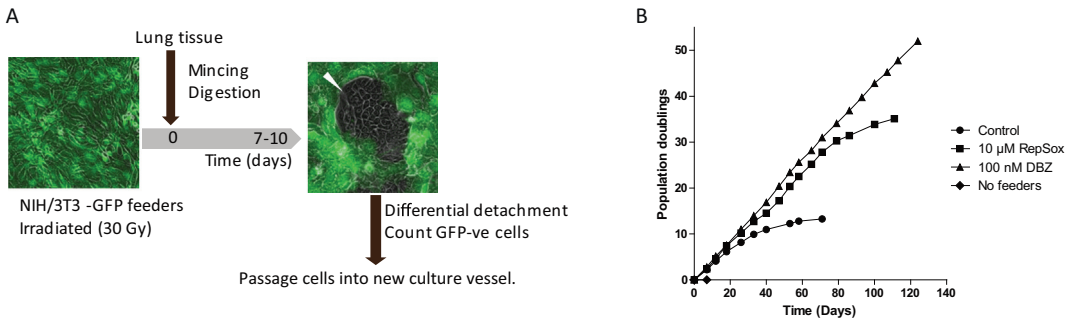

Fig. 1. Successful culture of human distal airway epithelial cells (DAECs) with feeders. (A) Initial workflow of co-culturing human DAECs with irradiated NIH3T3/GFP (feeders). Feeders were plated one day prior to epithelial cell isolation from tissues. A lung tissue sample was finely minced and digested with trypsin and elastase. Liberated cells were plated on feeders in expansion medium. The GFP-negative epithelial cells form discrete colonies (white arrowhead) in 7 to 10 days. Epithelial cells were harvested by differential trypsinization upon passaging. After counting under a fluorescent microscope 2×10 [5] GFP-negative cells were seeded in the next culture plate. (B) The growth curves of human DAECs cultured in different conditions. Cells did not reach enough confluence (80%) after 8 days from initial seeding when they were cultured only with expansion medium (No feeders, ♦). Expansion medium and irradiated feeders (Control, ●) allowed the cells to expand up to approximately 75 population doublings (PDs). Additionally, supplementation with RepSox (10 μM RepSox, ■) and dibenzazepine (DBZ) (100 nM DBZ, ▲) contributed to the longevity of the DAECs compared with feeders alone. Results are representative of three independent experiments using cells from three different donors.

cells were downregulated after the first passage and the phenotype of the cells after feeder removal was not extensively characterized.

The growth-promoting effect of feeders has been linked to activation of apoptosis and secretion of growth factors [18, 19]. The same mechanism has been suggested to orchestrate regeneration after tissue damage and it is likely to underlie the robust growth of lung epithelial cells in feeder co-culture [18]. Additionally, the overlapping marker profile of human distal airway epithelial cells (DAECs) with that of regenerating murine epithelial cells challenged with influenza virus supports this hypothesis [17, 20, 21]. As epithelial cell proliferation is usually followed by coordinated differentiation, we hypothesized that it may be possible to induce differentiation towards alveolar epithelial cells by using factors that induce terminal differentiation of lung progenitors derived from pluripotent stem cells [22].

The result is a novel method that allows expansion of human DAECs using feeder cells. Feeder removal induced a strong inflammatory response and differentiation into an airway club cell phenotype. Addition of small molecules and growth factors at the end of the expansion phase induced differentiation into AEII cells, followed by trans-differentiation into type I cells. We successfully adapted this method to chicken DAECs and compared the growth kinetics of different IAV strains between the two species. Additionally, our model supports siRNA transfection, enabling the application of advanced molecular techniques on primary DAECs to allow physiologically relevant research on various human and zoonotic lung diseases.

2. Material and Methods

2.1. Isolation and Culture of Primary DAECs

2.1.1. Human

Non-malignant tissue samples were obtained from pneumectomy specimens from the Clinic for Infectious Diseases and Pulmonary Medicine, Charité University Hospital, Berlin under signed informed consent. Scientific usage for experimental purposes was approved by the ethics committee of the Charité University Medicine, Berlin (EA2/079/13). Tissue pieces were processed according to the method by Daum et al. [23] with modifications. Briefly, they were washed with balanced salt solution buffer (BSSB:137 mM NaCl/5.0 mM KCl/0.7 mM Na_2HPO_4/10 mM HEPES/5.5 mM glucose/1.2 mM $MgSO_4$/1.8 mM $CaCl_2$, pH 7.4), minced finely, digested with trypsin (Serva) and elastase (Merck Millipore), passed through a 70 μm filter, centrifuged at 300 g for 5 min, washed twice with BSSB, resuspended in culture medium and plated into flasks previously seeded with irradiated NIH/3 T3-GFP feeders. The cell yield was donor dependent and ranged from $1.0–5.0 \times 10^6$ cells/g of tissue.

Expansion medium was based on F-medium [24] with modifications: 3:1 mixture of Ham's F-12 nutrient mix (Life Technologies) and DMEM supplemented with 5% fetal calf serum/0.4 μg/ml hydrocortisone (Sigma-Aldrich)/5 μg/ml recombinant human insulin (Sigma-Aldrich)/8.4 ng/ml cholera toxin (Sigma-Aldrich)/24 μg/ml adenine (Sigma-Aldrich)/10 ng/ml recombinant human epidermal growth factor (Life Technologies)/9 μM Y27632 (Miltenyi Biotec), supplemented with 10 μg/ml ciprofloxacin (Bayer Vital) and 10 μg/ml vancomycin (Serva) for the first three days, and 2.5 μg/ml amphotericin B (Cayman Chemical, Ann Arbor, USA) for the first seven days. Flasks were maintained in a humidified incubator at 37 °C with 5% CO_2, and medium changed and $1.5–2 \times 10^4/cm^2$ freshly irradiated feeders supplemented every 2–3 days.

2.1.2. Chicken

Lungs of 1-year-old roosters were provided by Annett Kannegießer (Albrecht-Daniel-Thaer-Institute of Agricultural and Horticultural Sciences, Berlin, Germany) in accordance with German and European regulations and with approval of the Berlin state authorities. DAECs were isolated as above, and cultured at 39 °C.

2.2. Induction of Differentiation to Alveolar Epithelial Cells

Four days after passaging, DAECs in co-culture were treated with small molecules and growth factors to induce differentiation toward

Fig. 2. Human DAECs show regenerating cell-like phenotype in co-culture. Immunofluorescent micrograph of human DAECs in co-culture with feeders. (A) Most of the cells in the CDH-1 (red) -positive epithelial colony show positive nuclear signal for the N-terminal-truncated form of P63 (ΔN-P63, green). (B) Cytokeratin 5 (CK5, green) is expressed in all epithelial cells. Red: CDH-1. Note that feeder cells are negative for CDH-1 and have large speckled nuclei. Scale bars: 50 μm. (C) Flow cytometry analysis of integrin α6β4 expression on human DAECs in co-culture with feeders. After sequential gating to single cells (Gate 1-3), live (Gate 4) and CDH-1+ (Gate 5) epithelial cells were subjected to analysis. The integrin α6 + β4+ population (Gate 6) consisted of 80.6% of the epithelial cells. (D–E) Immunofluorescence labelling of molecular markers of differentiation. CDH-1 (red)-positive epithelial cells are negative for both proSP-C (D) or SCGB1A1 (E). Scale bar: 50 μm. Results are representative of three independent experiments using cells from one donor. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Please cite this article as: Imai-Matsushima A, Martin-Sancho L, et al, Long-Term Culture of Distal Airway Epithelial Cells Allows Differentiation Towards Alveolar Epithelial Cells Suited for Influenza ..., EBioMedicine (2018), https://doi.org/10.1016/j.ebiom.2018.05.032