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Directed Induction of Functional Multi-ciliated Cells in Proximal Airway Epithelial Spheroids from Human Pluripotent Stem Cells

Satoshi Konishi,[1] Shimpei Gotoh,[1,*] Kazuhiro Tateishi,[2] Yuki Yamamoto,[1] Yohei Korogi,[1] Tadao Nagasaki,[1] Hisako Matsumoto,[1] Shigeo Muro,[1] Toyohiro Hirai,[1] Isao Ito,[1] Sachiko Tsukita,[2] and Michiaki Mishima[1]
[1]Department of Respiratory Medicine, Graduate School of Medicine, Kyoto University, Kyoto 606-8507, Japan
[2]Laboratory of Biological Science, Graduate School of Frontier Biosciences and Graduate School of Medicine, Osaka University, Suita 565-0871, Japan
*Correspondence: a0009650@kuhp.kyoto-u.ac.jp
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SUMMARY

Multi-ciliated airway cells (MCACs) play a role in mucociliary clearance of the lung. However, the efficient induction of functional MCACs from human pluripotent stem cells has not yet been reported. Using carboxypeptidase M (CPM) as a surface marker of NKX2-1$^+$-ventralized anterior foregut endoderm cells (VAFECs), we report a three-dimensional differentiation protocol for generating proximal airway epithelial progenitor cell spheroids from CPM$^+$ VAFECs. These spheroids could be induced to generate MCACs and other airway lineage cells without alveolar epithelial cells. Furthermore, the directed induction of MCACs and of pulmonary neuroendocrine lineage cells was promoted by adding DAPT, a Notch pathway inhibitor. The induced MCACs demonstrated motile cilia with a "9 + 2" microtubule arrangement and dynein arms capable of beating and generating flow for mucociliary transport. This method is expected to be useful for future studies on human airway disease modeling and regenerative medicine.

INTRODUCTION

Proximal airway epithelial cells (PAECs) play a pivotal role in the host defense in the respiratory tract via mucociliary clearance organized by multi-ciliated airway cells (MCACs) and secretory cells. An abnormal function of MCACs is associated with various lung diseases such as primary ciliary dyskinesia (PCD) (Rossman et al., 1980) and cystic fibrosis (CF) (Zhang et al., 2009). It has been reported that PAECs could be generated from human pluripotent stem cells (hPSCs) involving human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) (Mou et al., 2012; Wong et al., 2012; Huang et al., 2014; Firth et al., 2014). The ciliary movement of hPSC-derived MCACs has not yet been reported, although that of murine embryonic stem cell-derived MCACs has been reported (Nishimura et al., 2006; Shojaie et al., 2015). In our previous study, we identified carboxypeptidase M (CPM) as a surface marker of NKX2-1$^+$ "ventralized" anterior foregut endoderm cells (VAFECs) and demonstrated the potency of CPM$^+$ VAFECs to differentiate into alveolar type II cells (Gotoh et al., 2014). We hypothesized that PAECs could also be induced from CPM$^+$ VAFECs, as all lung epithelial lineage cells have been reported to be differentiated from NKX2-1$^+$ VAFECs (Kimura et al., 1996). We herein report a method of directed differentiation of hPSCs into MCACs and pulmonary neuroendocrine cells (PNECs) and functional analyses of the ciliary movement of hPSC-derived MCACs.

RESULTS

Generation of SOX2$^+$NKX2-1$^+$ PAEPC Spheroids from CPM$^+$ VAFECs in Three-Dimensional Culture

Because proximal airways develop as 3D branching structures in vivo, we adopted 3D differentiation from CPM$^+$ VAFECs to proximal airway epithelial progenitor cells (PAEPCs) (Figure 1A). Undifferentiated hPSCs consisting of H9 hESCs (Thomson et al., 1998), 201B7 (Takahashi et al., 2007), 585A1, and 604A1 hiPSCs (Okita et al., 2013), were stepwise differentiated into NKX2-1$^+$FOXA2$^+$ VAFECs as previously reported (Gotoh et al., 2014), with the exception of the dose of BMP4 used in Step 3. We identified the minimal and sufficient dose of BMP4 to be 20 ng/ml for each hPSC line (Figure 1B). Interestingly, *NKX2-1* was downregulated in the presence of Noggin, which inactivates BMP signaling according to a quantitative RT-PCR (qRT-PCR) analysis. On day 14, CPM$^+$ VAFECs were isolated and 3D culture was started in a similar manner as demonstrated in a tracheosphere assay using primary cells (Rock et al., 2009; Supplemental Experimental Procedures). In the hope of generating MCACs at the last step, the optimal medium conditions for proliferating spheroids and inducing *FOXJ1*, a representative marker of MCACs, were screened by combining FGF10, CHIR99021 (a WNT agonist), KGF, and DAPT (a γ-secretase inhibitor that blocks the Notch pathway), which have been considered to be important (Mou et al., 2012; Huang et al., 2014; Firth et al., 2014) (Figure S1A). The growth of the spheroids and *NKX2-1*, *SOX2*, and *FOXJ1* levels were compared on day

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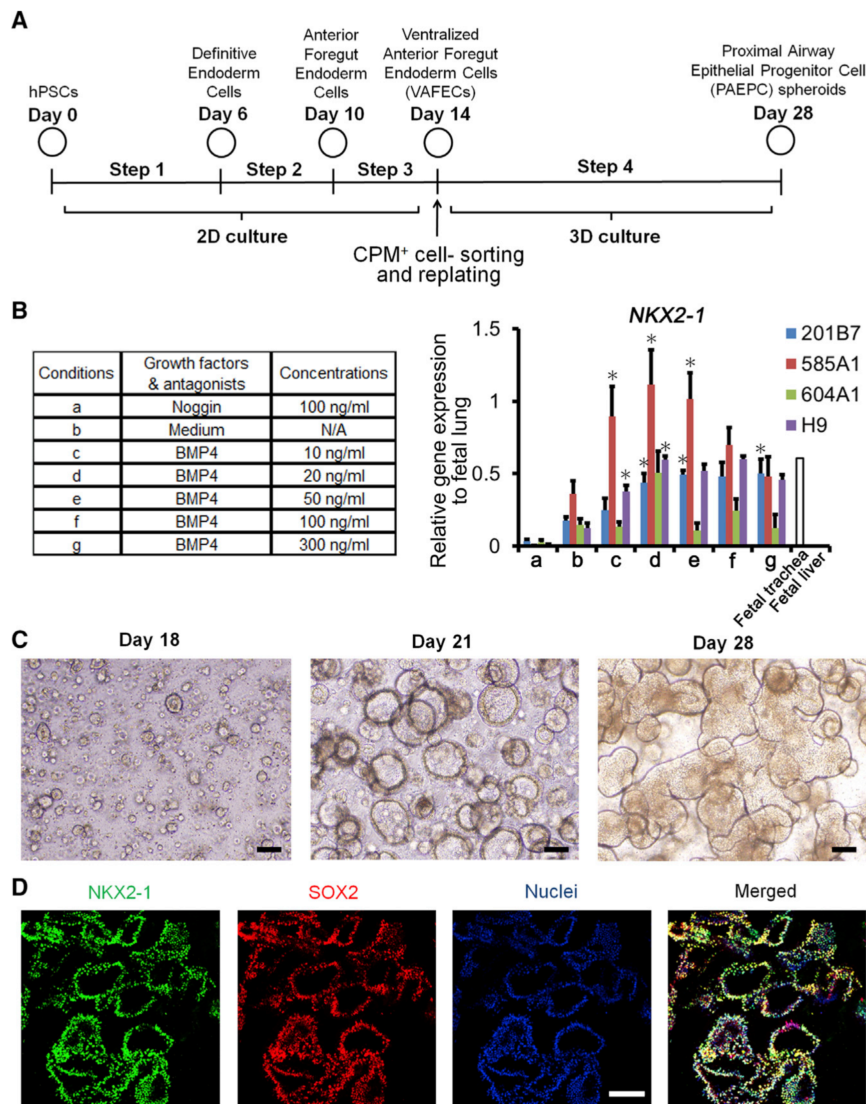

Conditions	Growth factors & antagonists	Concentrations
a	Noggin	100 ng/ml
b	Medium	N/A
c	BMP4	10 ng/ml
d	BMP4	20 ng/ml
e	BMP4	50 ng/ml
f	BMP4	100 ng/ml
g	BMP4	300 ng/ml

Figure 1. Generation of PAEPC Spheroids from CPM$^+$ VAFECs in 3D Culture

(A) Stepwise differentiation to PAEPC spheroids from hPSCs.

(B) qRT-PCR of *NKX2-1* expression in each hPSC line on day 14, according to the dose of BMP4. The concentrations of BMP4 for each condition in Step 3 are shown in the columns. Each value was normalized to *β-ACTIN*. The gene expression level of the fetal lungs was set at 1. Error bars represent the mean ± SEM (n = 3 independent experiments). Each condition was compared with condition b for each hPSC line; $^*p < 0.05$. N/A, not applicable.

(C) CPM$^+$ VAFEC-derived spheroids (201B7 hiPSCs) on days 18, 21, and 28.

(D) CIF imaging shows 201B7 hiPSC-derived PAEPC spheroids coexpressing SOX2 and NKX2-1 on day 28.

Scale bars, 100 μm. See also Figure S1 and Tables S1 and S2.

28 (Figures S1B and S1C), and the medium condition of 3 μM CHIR99021 and 100 ng/ml FGF10 was chosen. Under all conditions, *SOX9* was only slightly detected by qRT-PCR (Figure S1C). In Step 4, the spheroids grew larger and some of them began to fuse by day 28 (Figure 1C). Importantly, confocal immunofluorescence (CIF) imaging studies showed that nearly all the cells forming spheroids were SOX2$^+$NKX2-1$^+$ cells (Figure 1D), whereas SOX9 was not detected (data not shown), indicating that these cells were of PAEC lineage (Que et al., 2009).

Derivation of PAECs from PAEPC Spheroids

At the end of Step 4, no MCACs were observed, which prompted us to hypothesize that there might be another step for inducing MCACs. Therefore, we switched the medium to Step 5 medium based on PneumaCult-ALI medium (P-ALI) (Stemcell Technologies), a medium for primary bronchial epithelial cells (Figure 2A). On day 42, clusters of MCACs were observed by H&E staining (Figure 2B). CIF imaging revealed acetylated tubulin (Ac-Tub)$^+$FOXJ1$^+$ cells and closely aligned Ac-Tub$^+$ cells and MUC5AC$^+$ cells, as observed in the fetal human lung (FHL), while secreted MUC5AC markedly accumulated in the closed lumen of the hPSC-derived spheroids (Figure 2C). A small number of SCGB1A1$^+$cells (club cells), KRT5$^+$cells (basal cells) and chromogranin A (CHGA)$^+$ and synaptophysin (SYP)$^+$cells (PNECs) were also found (Figure 2D). Nearly all the hPSC-derived PAECs expressed NKX2-1 (Figures 2D and S2A), consistent with the previous reports (Bilodeau et al., 2014) and CIF imaging of the FHL (Figure S2A). By triple immunostaining, each representative marker of MCACs, club cells and basal cells was expressed in the different cells

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