Title
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Author(s)
Gotoh, Shimpei; Ito, Isao; Nagasaki, Tadao; Yamamoto, Yuki; Konishi, Satoshi; Korogi, Yohei; Matsumoto, Hisako; Muro, Shigeo; Hirai, Toyohiro; Funato, Michinori; Mae, Shin-Ichi; Toyoda, Taro; Sato-Otsubo, Aiko; Ogawa, Seishi; Osafune, Kenji; Mishima, Michiaki

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Generation of Alveolar Epithelial Spheroids via Isolated Progenitor Cells from Human Pluripotent Stem Cells

Shimpei Gotoh,1,* Isao Ito,1,2,* Tadao Nagasaki,1 Yuki Yamamoto,1 Satoshi Konishi,1 Yohei Korogi,1 Hisako Matsumoto,1 Shigeo Muro,1 Toyohiro Hirai,1 Michinori Funato,3 Shin-Ichi Mae,1 Taro Toyoda,3 Aiko Sato-Otsubo,4 Seishi Ogawa,4 Kenji Osafune,3 and Michiaki Mishima1
1Department of Respiratory Medicine, Graduate School of Medicine
2Institute for Integrated Cell-Material Sciences (iCeMS)
3Center for iPS Cell Research and Application (CiRA)
4Department of Pathology and Tumor Biology, Graduate School of Medicine
Kyoto University, Kyoto 606-8507, Japan
*Correspondence: a0009650@kuhp.kyoto-u.ac.jp (S.G.), isaoito@kuhp.kyoto-u.ac.jp (I.I.)
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SUMMARY
No methods for isolating induced alveolar epithelial progenitor cells (AEPCs) from human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs) have been reported. Based on a study of the stepwise induction of alveolar epithelial cells (AECs), we identified carboxypeptidase M (CPM) as a surface marker of NKX2-1+ “ventralized” anterior foregut endoderm cells (VAFECs) in vitro and in fetal human and murine lungs. Using SFTPC-GFP reporter hiPSCs and a 3D coculture system with fetal human lung fibroblasts, we showed that CPM+ cells isolated from VAFECs differentiate into AECs, demonstrating that CPM is a marker of AEPCs. Moreover, 3D coculture differentiation of CPM+ cells formed spheroids with lamellar-body-like structures and an increased expression of surfactant proteins compared with 2D differentiation. Methods to induce and isolate AEPCs using CPM and consequently generate alveolar epithelial spheroids would aid human pulmonary disease modeling and regenerative medicine.

INTRODUCTION
Type II alveolar epithelial cells (AECs) are a major cellular component of the distal lung epithelium, where they secrete pulmonary surfactant and generate type I AECs that cover most of the surface area of the alveoli (Whitsett et al., 2010; Rock and Hogan, 2011). The stepwise differentiation of human pluripotent stem cells (hPSCs), including human embryonic stem cells (hESCs) and induced pluripotent stem cells (hiPSCs), into lung epithelial cells would help to elucidate the etiologies of human lung diseases and create novel treatments, and has been reported in both proximal airway cells (Mou et al., 2012; Wong et al., 2012; Firth et al., 2014) and distal lung epithelial cells (Green et al., 2011; Ghaedi et al., 2013; Huang et al., 2014). Currently, however, there are no surface markers that can be used to purify human NKX2-1+ “ventralized” anterior foregut endoderm cells (VAFECs) as alveolar epithelial progenitor cells (AEPCs), although NKX2-1 is an early marker of lung and thyroid development (Kimura et al., 1996). Here, we report the efficacy of carboxypeptidase M (CPM) as a surface marker of AEPCs for generating type II AECs.

RESULTS
Identification of CPM as a Marker of NKX2-1+ VAFECs
We hypothesized that identifying a surface marker for NKX2-1+ VAFECs would be helpful for isolating a homogeneous population of AEPCs without establishing NKX2-1 reporter cell lines. We constructed a stepwise protocol to induce hPSCs to AECs (Figure 1A). On day 0, previously established hPSCs were seeded (Thomson et al., 1998; Takahashi et al., 2007; Nakagawa et al., 2008; Okita et al., 2013) following single-cell enzymatic dissociation (Kajiwara et al., 2012), resulting in definitive endodermal cells (DECs) at an efficiency of ≥80% (Figure S1A available online). In step 2, the DECs were differentiated to anterior foregut endodermal cells (AEFCs) (Green et al., 2011) at an efficiency of ≥88% (Figure S1B). In step 3, the concentrations of all-trans retinoic acid, CHIR99021, and BMP4 were optimized for seven hPSC lines for differentiation into NKX2-1+FOXA2+ cells, attaining an efficiency of 57.0%–77.5% (Figures 1C and 1D; Supplemental Experimental Procedures). In step 4, the cells were cultured in medium containing FGF10 for 7 days. In step 5, the cells were differentiated in medium containing dexamethasone, 8-Br-cAMP, 3-isobutyl-1-methylxanthine, and KGF (Gonzales et al., 2002; Longmire et al., 2012). We confirmed induction of AECs by detecting SFTPB and SFTPC using RT-PCR and double staining SFTPC and SFTP with NKX2-1 (figures S1C and S1D). Transcription factors were analyzed by quantitative RT-PCR (qRT-PCR; Figure 1B). SOX17, FOXA2, GATA6, and SOX2 were compatibly changed on day 6 and day 10 as previously described (Green et al., 2011). On day 14, NKX2-1, GATA6, ID2, SOX9, and HOPX levels
simultaneously increased. Interestingly, NKX2-1, GATA6, and HOPX levels decreased on day 21 and then increased again on day 25. The levels of other organ lineage markers were found to be limited from day 0 to day 25 (Figure S1E).

In order to identify candidate markers of VAFECs, we performed a microarray analysis to compare the global gene-expression patterns of AFECs (day 10) and VAFECs (day 14) in 201B7 hiPSCs. CPM and NKX2-1 were remarkably upregulated on day 14 (Figures 1E and S1F). In immunofluorescence (IF) staining, CPM and NKX2-1 increased from day 10 to day 14 (Figure 1F), whereas EPCAM and FOXA2 did not appear to change (Figure S1G). Although CPM was reported to be a marker of type I AECs (Nagae et al., 1993), only CPM drastically increased on day 14 in a similar pattern to NKX2-1, in contrast to other markers of type I AECs (AQPS and CAV1) (Figure S1H). On day 25, the various airway markers, including distal lung transcription factors (SOX9 and NKX2-1), type II AEC markers (SFTPB and SFTPC), and a club cell marker (SCGB3A2), were expressed in the CPM+ cells. KRT5, a marker of basal cells, was not expressed in the CPM+ cells (Figure 1G).

In fetal human lung at 18.5 weeks of gestation, SFTPC was extremely low compared with the observed in the fetal lung. NGFR, a marker of proximal airway basal stem cells (Rock et al., 2009), was significantly decreased in the CPM+ cells (n = 5; Figure 2F).

**Generation of SFTPC-GFP Knockin Reporter hPSCs**

In order to investigate whether CPM is a potential surface marker of AECs, we generated SFTPC-GFP knockin reporter hPSC lines from 201B7 hiPSCs using BAC-based homologous recombination methods (Mae et al., 2013; Figure 3A; Supplemental Experimental Procedures), as SFTPC is the most specific marker of type II AECs. Following electroporation of the targeting vectors, 12 of 55 G418-resistant clones were found to have a heterozygous deletion of the genomic endogenous SFTPC-coding region (Figure 3B). The pgrk-Neo cassette was removed via electroporation of the Cre-expression vector (Figure 3C), and normal karyotypes of the A17-14 and B2-3 clones were confirmed (Figure S3). The genomic copy number was calculated as previously described (Mae et al., 2013). The parental 201B7 (data not shown), A17-14, and B2-3 clones have...
Alveolar Spheroids from Pluripotent Cells

A

<table>
<thead>
<tr>
<th>Stem Cells</th>
<th>Definitive Endoderm</th>
<th>Anterior Foregut Endoderm</th>
<th>Ventralized Anterior Foregut Endoderm</th>
<th>Alveolar and Distal Airway Epithelium</th>
</tr>
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<tr>
<td>hPSCs</td>
<td>Day 0</td>
<td>CXCR4</td>
<td>SOX17</td>
<td>FOXA2</td>
</tr>
<tr>
<td>+ Y-27632 10 μM (Day 0-2)</td>
<td>Day 10</td>
<td>SOX2</td>
<td>FOXA2</td>
<td>Day 14</td>
</tr>
<tr>
<td>Day 21</td>
<td>ID3</td>
<td>SOX9</td>
<td>Day 25</td>
<td>NKK2-1</td>
</tr>
<tr>
<td>Step 2</td>
<td>Noggin</td>
<td>100 ng/ml</td>
<td>1 μM</td>
<td>Step 3</td>
</tr>
<tr>
<td>Step 4</td>
<td>FGF10</td>
<td>100 ng/ml</td>
<td>Step 5</td>
<td></td>
</tr>
</tbody>
</table>

B

Relative gene expression

C

Positive ratio / nuclei (%)

D

FOXA2

NKK2-1

Nuclei

Merged

E

Day 14

NKK2-1

CPM

Merged / Nuclei

F

CPM

NKK2-1

CPM / NKK2-1 / Nuclei

G

NKK2-1

SOX9

SFTPB

SFTPC

SCGB3A2

KRT5

CPM

Merged / Nuclei

H

NKK2-1

SFTPC

T1α

CPM

Merged / Nuclei

I

E12.5

E15.5

E17.5

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two copies of the SFTPC gene loci, in contrast to the A17-13 clone, in which random transgenic integration is supposed to have occurred, as indicated by three copies of the loci (Figure 3D). No copy-number variation was detected for the B2-3 clone, whereas a copy-number loss at chromosome 16 q23.3 and gain at chromosome 20 p13 were detected for the A17-14 clone (data not shown). Both SFTPC-GFP reporter hPSCs were then differentiated to the end of step 5 and GFP+ and GFP− cells were obtained by FACS after the CPM+ cells were sorted using MACS (Figure 3E). We confirmed the correlation between GFP and SFTPC on RT-PCR (Figure 3F). GFP was detected in SFTPC+, SFTPB+, and NKX2-1+ cells for both clones (Figure 3G).

**Alveolar Differentiation from CPM+ VAFECs in 3D Coculture**

We attempted 2D differentiation, reseeding the CPM+ SFTPC-GFP reporter hPSCs purified from VAFECs on day 14 onto Matrigel-coated, 96-well plates. After 14 days of differentiation in step 5 medium, SFTPB became positive in the reseeded CPM+ cells (Figure S2E); however, SFTPC was almost negative (Figure S4D, condition b). We obtained similar results when we sorted and reseeded CPM+ cells on day 23 (Figure S2F). The discrepancy between the expression of SFTPB and SFTPC in developing human lungs was previously reported (Khoor et al., 1994). Therefore, we hypothesized that some missing factors are important for the coexpression of SFTPB and SFTPC. We then adopted a 3D coculture with fetal human lung fibroblasts (FHLFs) obtained at 17.5 weeks of gestation (Figure 4A). CPM+ cells purified from VAFECs on day 14 and FHLFs were mixed at a ratio of 1:50 and reseeded onto cell inserts. After 10 days of differentiation in step 5 medium, GFP became positive in some spheroids (Figure 4B). The spheroids were subsequently examined with a transmission electron microscope and lamellar-body-like structures were noted (Figure 4C). On hematoxylin-and-eosin staining, cyst-like spheroids consisting of pseudostratified, columnar, or cuboidal cells with dark pink cytoplasm were observed in the CPM+ cell-derived spheroids, whereas small pieces of spheroids consisting of cuboidal cells with clear cytoplasm were noted in the CPM− cell-derived spheroids (Figure S4A). On IF staining, CPM and NKX2-1 were double positive in most CPM+ cell-derived spheroids, whereas GFP and SFTPC were double positive in some spheroids (Figure 4D). In the CPM− cell-derived spheroids, EPCAM was positive, whereas no CPM+ or NKX2-1+ cells were identified (Figure S4B). SFTP A, SFTP B, SFTP C, and SFTP D (representative markers of type II AECs) were positive in the CPM+ cell-derived spheroids (Figure S4C). AQP5+ cells were adjacent to SFTPC+ cells in some spheroids (Figure 4D). ID2 and SOX9 (markers of differentiation into the distal lung-lineage fate) were positive in some NKX2-1+ and CPM+ cells, respectively (Figure S4C). Next, we trypanosized the cells in 3D structures and determined the proportion of SFTPC-GFP+ cells, detecting 3.82% ± 0.50% cells obtained from the CPM+ cell-derived 3D structures and 0.29% ± 0.03% cells obtained from the CPM− cell-derived structures including fibroblasts (Figure 4E). Excluding the fibroblasts, the ratio of the number of SFTPC-GFP+ cells to that of EPCAM+ cells was calculated to be 9.81% ± 1.81% in the CPM+ cell-derived spheroids and 1.07% ± 0.16% in the CPM− cell-derived spheroids. Almost all of the GFP+ cells sorted by FACS were SFTPC+, whereas the GFP− cells were SFTPC− (Figure 4F). The levels of alveolar markers (SFTPB and SFTPC), rather than club cell markers (SCGB1A1 and SCGB3A2), were significantly elevated following the 3D coculture differentiation of CPM+ cells derived from three hPSC lines (H9 hESCs and parental 201B7 and 604A1 hiPSCs) compared with the 2D differentiation employing the three protocols separately starting on day 14 (Figure 1A; Green et al., 2011; Longmire et al., 2012) and the 3D coculture differentiation of CPM− cells (Figure 4G). Interestingly, the levels of SFTPB and SFTPC were quite low for 585A1 hiPSCs, suggesting

**Figure 1. Identification of CPM as a Candidate Marker of NKX2-1+ VAFECs**

(A) Stepwise differentiation to AECs from hPSCs.

(B) Gene-expression levels of transcription factors from day 0 to day 25 (n = 3). Each value was normalized to the level of β-ACTIN. The relative expression level was scored with the maximum value set to 1.0.

(C) Induction efficiency of VAFECs analyzed by scoring the number of FOXA2+ and NKX2-1+ cells relative to the total number of nuclei in an average of ten randomly selected images (n = 3).

(D) FOXA2+ NKX2-1+ VAFECs derived from 201B7 hiPSCs.

(E) Scatterplots comparing the global gene-expression profiles of AFECs (day 10) and VAFECs (day 14). CPM (arrows) and NKX2-1 (arrowheads) are noted. The lines beside the diagonal line indicate a 2-fold cutoff change between the AFECs and VAFECs.

(F) Simultaneous increases of CPM and NKX2-1 detected by IF staining of AFECs (day 10) and VAFECs (day 14).

(G) CPM detected in NKX2-1+, SOX9+, SFTP B+, SFTP C+, and SCGB3A2+ cells, but not in KRT5+ cells, on day 25.

(H) CPM detected in NKX2-1+ lung epithelial cells in fetal human lung.

(I) CPM in E12.5, E15.5, and E17.5 murine lungs.

Error bars show SEM. Scale bars, 100 μm. See also Figure S1 and Tables S1 and S2.
Figure 2. Isolation of CPM⁺ VAFECs Using Anti-CPM Antibody

(A) Flow cytometry of VAFECs. EPCAM⁺CPM⁺ (Q2) and EPCAM⁺CPM⁻/C₀ (Q4) cells were isolated on day 14 (n = 3).

(B) Hierarchical clustering heatmaps of 336 genes with differences of >2-fold (FDR-adjusted p < 0.05) comparing EPCAM⁺CPM⁺ cells with EPCAM⁺CPM⁻/C₀ cells. The cluster of genes increased as the greatest fold change was magnified.

(C) Flow cytometry of MACS-sorted CPM⁺ and CPM⁻/C₀ cells from VAFECs (n = 3).

(D) NKX2-1⁺ cells in FACS-sorted CPM⁺ and CPM⁻/C₀ cells derived from VAFECs analyzed by scoring the number of NKX2-1⁺ cells relative to the total number of nuclei in an average of five randomly selected images (n = 3).

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that the concentration of retinoic acid required to induce NKX2-1⁺ VAFECs in step 3 is less important for subsequent differentiation into AECs than the difference in the cell lines or donors. Moreover, the expression of SFTPB and SFTPC was small for the 2D and 3D differentiation of CPM⁺ cells alone or FHLFs alone (Figure S4D). Finally, other cell-type markers (AQP5 [type I AECs], FOXJ1 [ciliated cells], and AGR2 [goblet cells]) appeared to be elevated in the CPM⁺ cell-derived structures rather than in the CPM⁻ cell-derived structures, suggesting that cell-type markers other than club-cell markers were expressed in the CPM⁺ cell-derived spheroids. KRT5 (a basal cell marker, possibly including both airway and esophageal basal cells) was exclusively expressed in the CPM⁺ cell-derived spheroids.

(E) Levels of CPM and NKX2-1 on day 14 before and after MACS-based purification of CPM⁺ cells on qRT-PCR (n = 5).
(F) Levels of AEC and club-cell markers and NGFR, a proximal airway stem cell marker, on day 25 before and after MACS-based purification of CPM⁺ cells (n = 5).

The gene-expression level observed in the fetal lungs was set at one. Values are presented as the mean ± SEM. Error bars show SEM. #p < 0.05, *p < 0.01. Scale bars, 100 μm. See also Figure S2 and Tables S1 and S2.
Figure 4. Alveolar Differentiation from CPM+ VAFECs in 3D Coculture

(A) Strategy for inducing AECs via 3D coculture with FHLFs.
(B) SFTPC-GFP+ cells detected in spheroids derived from isolated CPM+ VAFECs.
(C) Transmission electron microscopy of lamellar-body-like structures observed in 3D coculture differentiation of CPM+ cells compared with those observed in the adult and fetal murine lungs. Lu, lumen.
(D) IF staining of spheroids derived from CPM+ VAFECs.
(E) Flow cytometry of SFTPC-GFP+ cells in 3D coculture differentiation of CPM+ cells or CPM− cells (n = 3).
(F) GFP+ and GFP− cells isolated via FACS, spun down onto slides, and stained by anti-GFP and anti-SFTPC antibodies.

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cell-derived structures. In addition, *PAX8* (a thyroid marker), *PAX6* (a neuron marker), and the other foregut endodermal lineage cells (*FOXN1, ALB, and PDX1*) were only minimally or slightly induced following 3D coculture differentiation (Figure S4E).

**DISCUSSION**

In this work, we identified CPM as a surface marker that is expressed in NKX2-1+ VAFECs, including AEPCs, and demonstrated that the CPM+ cell-derived spheroids obtained via 3D coculture differentiation with FHLFs enabled more efficient differentiation to AECs than did 2D differentiation. The gene-expression pattern of CPM in developing lungs has not received significant attention, although in situ hybridization of *Cpm* in anterior DECs as early as E7.5 in mice has been reported (Tamplin et al., 2008). Our data from IF staining of murine fetal lungs (Figure S1I) also suggest that lineage-tracing studies may provide answers to the following questions: Is Cpm a possible “specific” marker of lung-lineage progenitor cells such as Shh (Harris et al., 2006), Id2 (Rawlins et al., 2009a), and Nkx2-1 (Longmire et al., 2012)? What is the relationship between CPM+ cells and bipotent cells that are capable of generating type I and type II AECs (Desai et al., 2014)? Do CPM+ cells differentiate into type II AECs directly or indirectly via SFTPC+SCGB1A1+ cells (Kim et al., 2005; Rawlins et al., 2009b)? Furthermore, the present study suggests that a 3D microenvironment and coculture with FHLFs are important factors in the differentiation of progenitor cells into AECs rather than club cells. Although maintaining type II AECs in 2D conditions is often difficult (Dobbs, 1990; Yu et al., 2007), 3D conditions have recently been applied with better outcomes (Yu et al., 2007; McQualter et al., 2010; Barkauskas et al., 2013). Therefore, our 3D differentiation protocol appears to be a reasonable approach for maintaining differentiated type II AECs, although methods for expanding such cells for longer periods should be established in the next step.

The limitations of the present study include the fact that we were unable to demonstrate whether CPM is a more appropriate marker for lung-lineage cells than NKX2-1. Future studies focusing on the possible contribution of NKX2-1 CPM+ cells and/or NKX2-1 CPM- cells to the differentiation of lung epithelial cells may resolve this issue, although we found only two isolatable populations of NKX2-1 CPM+ and NKX2-1 CPM- cells using the present protocol. In addition, we were unable to demonstrate the highest induction efficiency of AECs, as recently described (Ghaedi et al., 2013), although we employed a different method for evaluating efficiency using SFTPC-GFP reporter hPSCs. Another limitation is that the functions of the induced AECs remain to be elucidated.

Nevertheless, the methods applied in the present study to induce and isolate AEPCs using CPM and consequently generate alveolar epithelial spheroids in a stepwise fashion may help to elucidate the complicated differentiation of human AECs and open the door for the development of new strategies for in vitro toxicology and cell replacement therapy, as well as screening for therapeutic drug compounds, in the future.

**EXPERIMENTAL PROCEDURES**

**2D Differentiation**

CHIR99021 (Axon Medchem), an activator of canonical Wnt signaling, was substituted for WNT3A (Mae et al., 2013). For details regarding the protocols used for each differentiation medium, see the Supplemental Experimental Procedures.

**3D Differentiation**

The protocol for the 3D culture was modified from a previous report (Barkauskas et al., 2013). For further details, see the Supplemental Experimental Procedures.

**Ethics**

The use of H9 hESCs was approved by the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan. Human ethics approval was obtained from the Institutional Review Board and Ethics Committee of Kyoto University Graduate School and Faculty of Medicine. Animal ethics approval was obtained from the Animal Ethics and Research Committee of Kyoto University.

**Statistical Analysis**

Values are expressed as the mean ± SEM and “n” stands for the number of independent experiments. Two-tailed Student’s t test was performed to identify significant differences between two conditions of qRT-PCR.

**ACCESSION NUMBERS**

The NCBI GEO accession number for the microarray data reported in this paper is GSE53513.

(G) qRT-PCR comparing the 2D and 3D differentiation into AECs in H9 hESCs and 201B7 (parental), 604A1, and 585A1 hiPSCs. Each value of the gene expression was normalized to the level of *β-ACTIN*. The levels of the fetal lungs were set at one. Values are presented as the mean ± SEM. Error bars show SEM. #p < 0.05, *p < 0.01. Scale bars, 100 μm unless otherwise indicated. See also Figure S4 and Tables S1 and S2.
SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.stemcr.2014.07.005.

AUTHOR CONTRIBUTIONS


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