Differentiation of Mouse induced Pluripotent Stem Cells into Alveolar Epithelial Cells In Vitro for Use In Vivo

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Running Title: Lung re-construction by differentiated iPS Cells

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ABSTRACT
Alveolar epithelial cells (AECs) differentiated from induced pluripotent stem cells (iPSCs) represent new opportunities in lung tissue engineering and cell therapy. In this study, we modified a two-step protocol for embryonic stem cells that resulted in a yield of ~9% SPC+ alveolar epithelial type II (AEC II) cells from mouse iPSCs in a 12-day period. The differentiated iPSCs showed morphological characteristics similar to those of AEC II cells. When differentiated iPSCs were seeded and cultured in a mouse decellularized lung scaffold, the cells re-formed an alveolar structure and expressed SPC or T1α protein, markers of AEC II or AEC I cells, respectively. Finally, the differentiated iPSCs were instilled intratracheally into a bleomycin (BLM)-induced mouse acute lung injury model. The transplanted cells integrated into the lung alveolar structure and expressed SPC and T1α. Significantly reduced lung inflammation and decreased collagen deposition were observed following differentiated iPSC transplantation. In conclusion, we report a simple and rapid protocol for in vitro differentiation of mouse iPSCs into AECs. Differentiated iPSCs show potential for regenerating three-dimensional alveolar lung structure and can be used to abrogate lung injury.

INTRODUCTION
In the advanced stage of acute and chronic lung diseases, such as acute lung injuries, idiopathic pulmonary fibrosis (IPF), and chronic obstructive lung disease (COPD), present medications can only stabilize the disease conditions or delay disease progression. Although lung transplantation is the only definitive option for these advanced-stage lung diseases, donor organ shortage is a major problem.
Tissue engineering regenerative medicine is a new multidisciplinary field for exchanging impaired cells or tissues with new functional cells or tissues. The first step in regenerative medicine for end-stage lung diseases is generation of alveolar epithelium containing two cell types: alveolar epithelial type I and type II cells (AEC I and II). AEC I cells are large, flattened cells comprising 95% of the alveolar lining area and are responsible for gas exchange, whereas AEC II cells are cuboidal cells, more abundant but smaller than AEC I cells, which comprise 5% of the alveolar lining area [1,2]. AEC I cells are terminally differentiated, unable to replicate, and are susceptible to environmental toxicants and pathogens. In the event of lung damage, although AEC II cells have been shown to undergo proliferation and/or differentiation to AEC I cells to repair the damaged alveolar epithelium, this repair process often causes inappropriate reconstruction of lung structures [1,3,4].

Embryonic stem cells (ESCs) are self-renewing pluripotent cells that can differentiate into alveolar epithelial cells in vitro [5-16]. The problems of immune rejection and ethical issues restrict clinical application of ESCs. Induced pluripotent stem cells (iPSCs), which are derived from differentiated somatic cells by introduction of several defined transcription factors, display self-renewal properties and pluripotency similar to ESCs [17,18]. Additionally, they have the potential to overcome the abovementioned limitations. Recently, several studies have reported in vitro differentiation to AEC II-like cells from iPSCs [19,20].

In the present study, we differentiated mouse iPSCs into AECs in vitro and investigated the regenerative and therapeutic potential of those differentiated iPSCs in mouse models. We used a previously reported two-step protocol to differentiate iPSCs into AECs. Although most ESC differentiation protocols have used the conventional embryoid body
(EB) method [9], we achieved iPSC differentiation using a dissociated low-density seeding method that is considered more rapid and effective [10]. We tested six medium combinations to identify the most efficient protocol and successfully differentiated mouse iPSCs into AEC-like cells. The regenerative potential was demonstrated through seeding of the differentiated iPSCs into decellularized mouse lung scaffolds. Their therapeutic potential was demonstrated in a bleomycin-induced mouse acute lung injury model.

**MATERIALS AND METHODS**

**Cell Line and Cell Culture**

A mouse iPS cell line (iPS-MEF-Ng-492B-4) obtained from the RIKEN Cell Bank (Tsukuba, Japan) was maintained on 1×10⁴ cells/cm² mitomycin-C (Sigma-Aldrich, St Louis, MO, USA)-inactivated mouse SNL76/7 cells (ECACC; #EC07032801) as a feeder layer on 0.1% gelatin-coated tissue culture dishes in iPS medium containing high-glucose Dulbecco’s modified Eagle’s medium (DMEM; Gibco, Carlsbad, CA, USA) supplemented with 15% ES cell fetal bovine serum (FBS; Gibco), 0.1 mM nonessential amino acids (Gibco), 0.1 mM 2-mercaptoethanol (Gibco), 1000 U/ml mLIF (Chemicon, Temecula, CA, USA), 50 U/mL penicillin, and 50 mg/mL streptomycin (Gibco).

The murine type II pneumocyte cell line MLE12 was obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA; #CRL-2110) and was cultured in medium containing 50% DMEM (Gibco) and 50% Ham’s F12 medium (Gibco) supplemented with 10 nM hydrocortisone (Sigma-Aldrich), 10 nM β-estradiol (Sigma-Aldrich), 10 nM HEPES (Sigma-Aldrich), 2 nM L-glutamine (Gibco), 1% ITS
(Gibco), 2% FBS (Gibco), 50 U/mL penicillin, and 50 mg/mL streptomycin (Gibco).

**Mouse iPSC Differentiation**

Mouse iPSCs were induced to differentiate into AECII-like cells using the dissociated seeding method for murine ESCs described previously, with slight modifications [10]. As shown in Figure 1A, at first, mouse iPSCs were trypsinized, centrifuged, resuspended in iPS medium, and plated at a lower density (1×10^3 cells/cm^2) on 0.1% gelatin-coated tissue culture dishes for 24 h. Next, the iPS medium was changed to basic differentiation medium (Basic DM) supplemented with 20 ng/ml Activin A (R&D Systems, Minneapolis, MN, USA) and 10 ng/ml Wnt3a (R&D Systems), and then incubated for 6 days (D1–D7). Basic DM contains 75% IMDM (Gibco) and 25% Ham’s F12 medium (Gibco) supplemented with 0.5× of both N2 and B27 (without retinoic acid) supplements (Gibco), 50 U/ml penicillin, 50 mg/ml streptomycin, 2 mM glutamine (Gibco), 0.5 mM ascorbic acid (Sigma-Aldrich), 4.5×10^{-4} M 1-thioglycerol (Sigma-Aldrich), and 0.05% bovine serum albumin (BSA; Sigma-Aldrich). Next, the differentiation medium was changed to fresh Basic DM or small-airway basal medium (SABM) (Lonza, Walkersville, MD, USA) supplemented with 50 ng/ml FGF2 (Sigma-Aldrich) and 50 µg/ml heparin sulfate salt (Sigma-Aldrich), followed by incubation for a further 5 days.

**Immunofluorescence**

Mouse lung tissues were frozen or fixed with 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded sections were deparaffinized, and antigen retrieval was subsequently performed by autoclaving the tissue sections in antigen retrieval solution.
(Nichirei, Tokyo, Japan; #415211) at 120°C for 10 min. Sections were blocked with Protein Block Serum-Free (Dako; #X0909) for 20 min at room temperature, and then were incubated with primary antibody at 4°C overnight. Sections were incubated with secondary antibody for 1 h at room temperature. Sections were stained with DAPI for nuclear counterstaining and mounted with VECTASHIELD Mounting Medium (Vector Laboratories, Burlingame, CA, USA; #H-1500). All fluorescent photographs were acquired using a Nikon C1si Confocal Microscope with the Nikon EZ-C1 software.

To quantify SPC-positive cells, five images (400× magnification) were randomly selected in at least two slides per mouse lung (5 mice/group) to be counted visually. For staining of iPSCs, cells were seeded in 0.1% gelatin-coated four-well tissue culture Permanox chamber slides (Thermo Fisher Scientific, Waltham, MA, USA). The cells on days 7 and 12 of the differentiation protocol were fixed in 4% paraformaldehyde for 15 min and permeabilized for 10 min in 0.2% Triton X-100/phosphate-buffered saline (PBS) on ice. Next, the cells were blocked and stained as described above.

Primary antibodies were goat SPC (1:500; Santa Cruz Biotechnology, Santa Cruz, CA, USA; #SC-7706), goat T1α (1:100; Santa Cruz Biotechnology; #SC-23564), mouse SSEA1 (1:100; STEMGENT, San Diego, CA, USA; #09-0005), rabbit OCT3/4 (1:100; STEMGENT; #09-0023), goat Sox17 (1:100; Santa Cruz Biotechnology; #SC-17355), goat Foxa2 (1:100; Santa Cruz Biotechnology; #SC-6554). The appropriate goat, rabbit, and mouse IgG or IgM was used as the isotype control.

Secondary antibodies were donkey anti-goat IgG-Alexa Fluor 488 (1:200; Invitrogen, Carlsbad, CA, USA; #A11055), goat anti-mouse IgM–cy3 (1:200; Biorbyt, San Francisco, CA, USA; #orb14378), donkey anti-goat IgG-Alexa Fluor 594 (1:200; Invitrogen; #A11058), and goat anti-rabbit IgG-Alexa Fluor 594 (1:200; Invitrogen;
Real-time Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA of iPSCs was extracted using an RNeasy kit (Qiagen, Valencia, CA, USA), and reverse transcriptase reactions were performed using aliquots of 2 μg of total RNA using the High-capacity cDNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA) according to the manufacturer’s protocol. Quantitative real-time PCR was performed in triplicate using the TaqMan Universal PCR Master Mix and TaqMan Gene Expression Assay (Applied Biosystems) in an ABI 7900HT sequence detection system (Applied Biosystems). The conditions for real-time PCR were 50°C (2 min), 95°C (10 min), followed by 50 cycles of 95°C (15 s) and 60°C (1 min). The TaqMan Gene Expression Assay IDs of detected genes were Mm01976556_s1 (Foxa2), Mm00488363_m1 (Sox17), Mm00499170_m1 (SPA), Mm00455681_m1 (SPB), Mm00488144_m1 (SPC), Mm00486060_m1 (SPD), Mm00442046_m1 (CCSP), and Mm99999915_g1 (GAPDH). GAPDH was used as an endogenous control gene, and the average value of the undifferentiated iPSCs was used as the calibrator. Calculations were performed using the comparative C<sub>T</sub> method.

Flow Cytometry

Flow cytometry was performed for quantifying the SPC-positive cells after differentiation. Briefly, cells were dissociated into a single-cell suspension in PBS containing 1 mM EDTA and 3% FBS (flow cytometry buffer), fixed and permeabilized for intracellular antigen (SPC) with Intraprep permeabilization reagent (Beckman Coulter, Fullerton, CA, USA; #A07802) according to the manufacturer’s protocol. The
cells were stained for 30 min on ice with goat anti-mouse SPC (Santa Cruz Biotechnology; #sc-7706), followed by phycoerythrin (PE)-conjugated donkey anti-goat IgG. Goat IgG was used as an isotype control. Finally, cells were analyzed by flow cytometry (Cell Lab Quanta SC; Beckman Coulter).

**Transmission Electron Microscopy (TEM)**

The murine type II pneumocyte cell line MLE12 and mouse iPSCs were washed with 1× PBS and centrifuged for 5 min at 300 × g. The cell pellets were fixed with 2% paraformaldehyde/1% glutaraldehyde in 0.1 M PBS at 4°C overnight, washed with 1× PBS, and post-fixed with 1% OsO4 in 0.1 M PBS for 30 min at room temperature. The cell pellets were then washed three times in PBS and dehydrated in graded ethanol solutions. The cells were moved to a 500-µl embedding tube and treated with propylene oxide for 15 min after centrifugation. The cell pellets were infiltrated at 1:1 propylene oxide/Epon at room temperature overnight. The following day, the samples were centrifuged for 3 min at 800 × g, infiltrated at 1:4 propylene oxide/Epon at room temperature for 1 h, centrifuged for 3 min at 800 × g again, exposed to 100% Epon for 1 h at room temperature, and finally incubated at 60°C for 48 h. Ultrathin sections (50 nm) were cut and collected on sheet mesh, stained with uranyl acetate and lead citrate. The stained sections were examined and photographed in a JEM-1230 TEM (JEOL, Tokyo, Japan) at 80-kV voltage.

**Lung Decellularization and Recellularization**

Mouse lung decellularization and recellularization were performed according to methods reported recently [15,21]. Briefly, harvested heart-lung blocs were
decellularized by injection of 0.1% sodium dodecyl sulfate (Sigma-Aldrich) and 1% Triton X-100 (Sigma-Aldrich) through both the right ventricle and trachea. Cells in complete medium were mixed with 2% low-melting-point agarose at 37°C to generate a suspension. One milliliter of cell suspension (2×10⁶ cells/ml) in 2% low-melting-point agarose was injected intratracheally into the decellularized lung. Lungs were then incubated for 5 min on ice until the agarose hardened and were sectioned into approximately 2-mm thick slices using a sterile surgical blade (Fig.3A). The lung sections were cultured in small airway epithelial cell growth medium (Lonza) for 12 days, and then fixed with 10% formaldehyde and embedded in paraffin.

**Intratracheal Transplantation of Differentiated iPSCs into Bleomycin (BLM)-injured Mouse Lungs**

All mouse experiments were approved by the Institutional Animal Care and Use Committee of Niigata University. Pathogen-free female mice in a C57BL/6N genetic background (8-week-old, 18 to 20-g body weight; Charles River Laboratories, Yokohama, Japan) were anesthetized by intraperitoneal injection of 0.5 mg/g Avertin (Sigma-Aldrich), and then were administered either 4 units/kg BLM (Sigma-Aldrich) or sterile normal saline by intratracheal instillation via oropharynx intubation using a liquid aerosol device and a small animal laryngoscope (Penn-Century, Wyndmoor, PA). Oropharynx intubation allows subsequent noninvasive cell transplantation after BLM treatment [11]. The next day, the BLM-injured mice were transplanted with fibroblasts, undifferentiated iPSCs, or differentiated iPSCs (5×10⁵ cells/mouse). The mice were euthanized on day 13, and lungs were harvested. Transplanted cells were identified by labeling with the PKH26 Red Fluorescent Cell Linker Mini Kit (Sigma-Aldrich).
according to the manufacturer’s protocol.

**Bronchoalveolar lavage (BAL) and ELISA**

After euthanasia on day 12, the trachea was cannulated with a 24-gauge catheter. The airway was lavaged with four consecutive washes of 0.5 ml sterile PBS. BAL fluid was centrifuged at 1,200 rpm for 15 min at 4°C, and the supernatants were stored at -80°C until use. Cell pellets were resuspended in 1 ml ice-cold RPMI-1640 media and centrifuged onto glass slides at 600 rpm for 10 min in a Shandon Cytospin 4 cytocentrifuge (Thermo Scientific, Waltham, USA). Cells were stained using the Diff-Quik stain set (Sysmek, Kobe, Japan). TNF-α and IL-6 concentrations in BAL fluid were measured using solid phase sandwich ELISA kits (Invitrogen; #KMC3011 for TNF-α, #KMC0061 for IL-6) according to the manufacturer’s protocol.

**Wet/Dry Weight Ratio**

The wet/dry weight ratio of the lung was measured to quantitatively evaluate the degree of pulmonary inflammation induced by BLM treatment according to a method described previously [22,23]. The wet weight of lungs was measured immediately after sacrifice. The lungs were then placed with a desiccant in an oven at 60°C for 4 days and reweighed to determine the dry weight.

**Measurement of Lung Collagen**

Sirius red/fast green staining was performed to evaluate collagen deposition in lung tissues. Briefly, paraffin sections of lung tissue were deparaffinized, hydrated, and incubated in 0.1% Fast Green (Wako, Tokyo, Japan; #069-00032) for 1 h at room
temperature, washed in 0.5% acetic acid (Wako) for 5 min and rinsed in tap water. Sections were then incubated in 0.1% Sirius red (Sigma-Aldrich; #365548) in saturated aqueous picric acid (Wako) for 1 h at room temperature and washed with 0.5% acetic acid for 5 min twice. After rinsing with tap water, sections were rapidly dehydrated and mounted in xylene. Collagen deposition was also assessed using a Hydroxyproline Assay Kit (BioVision, Milpitas, CA, USA; #K555-100) according to the manufacturer’s protocol. Briefly, 50 mg of homogenized lung sample were incubated in 1 ml of 6 N HCL at 110°C overnight. The hydrolyzed samples were neutralized with 1 ml of 6 N NaOH, filtered through a 0.1-mm filter, and then incubated with 100-µl Chloramine T reagent for 5 min at room temperature. One hundred microliters of the DMAB reagent were then added and incubated for 90 min at 60°C. The absorbance was measured at 570 nm, and the hydroxyproline content was calculated against a standard curve.

**Statistical Analysis**

The data are presented as means ± standard deviation. One-way analysis of variance (ANOVA) and Tukey-Kramer tests were used to assess the significance of differences. A \(P\) value less than 0.05 was deemed to indicate statistical significance.

**RESULTS**

**Decision of Differentiation Medium through mRNA Expression Levels of Endoderm and Lung Epithelium Markers**

We maintained mouse iPSCs and monitored the undifferentiated status of iPSCs until passage 35. iPSC pluripotency was maintained according to alkaline phosphatase (AP) staining and immunostaining for the mouse pluripotency markers SSEA1, Nanog, and
OCT4. iPSCs were positive for all of these pluripotency markers (Fig. S1). Mouse iPSCs from passages 10 to 30 were used in the present study.

Because the GFP gene was knocked-in the mouse iPS cell line under the Nanog promoter, the undifferentiated iPSCs could be surveyed by detecting GFP expression throughout the differentiation procedure [24]. Around 4% GFP+ cells remained at the end of differentiation stage 1 (day 7), whereas no GFP+ cells were observed at the end of differentiation stage 2 (day 12) (data not shown). The definitive endoderm markers Sox17 and Foxa2 were expressed on day 7 (Fig. S2A). The BSA-free condition further enhanced the expression levels of Sox17 and Foxa2 (55.8±6.4-fold vs. 37.9±3.5-fold, respectively; 326.4±33-fold vs. 201.5±45.5-fold, respectively; P<0.05) (Fig. S2B). The mRNA expression levels of the lung epithelial markers surfactant protein A (SPA), surfactant protein B (SPB), surfactant protein C (SPC), surfactant protein D (SPD), Clara cell secretory protein (CCSP), and thyroid transcription factor 1 (TTF-1, also known as Nkx2-1) were markedly increased compared with undifferentiated iPSCs (Fig. 1B) at the end of stage 2 differentiation (day 12). Small airway basic medium (SABM) enhanced the mRNA expression of SPA, SPC, and SPD, but it decreased that of TTF1 compared with Basic DM when applied during stage 2 differentiation (*P<0.05). The presence of BSA in SABM decreased the mRNA expression of SPA and SPC (#P<0.01) compared with its absence. Because SPC is a specific marker of alveolar type 2 cells, we used the medium combination that resulted in the greatest SPC mRNA expression after completion of two-step differentiation. The mRNA expression of SPA was increased by 116.3±26.5-fold, SPB by 28.8±12.6-fold, SPC by 8.3±2.4-fold, SPD by 9.1±0.6-fold, CCSP by 4.8±2.4-fold, and TTF1 by 168.6±88.4 fold in the condition described above. However, the mRNA expression of lung epithelial markers in
differentiated iPSCs was lower than that of positive controls (MLE12 cells and mouse lung tissue) (Figure 1C). Nevertheless, these differentiated iPSCs expressed lung epithelial markers. As shown in Figure 1A, we used Basic DM + Activin A (20 ng/ml) + Wnt3a (10 ng/ml) in stage 1 and SABM+FGF2 (50 ng/ml) in stage 2 as differentiation media in the following experiments.

**Evaluation of Differentiated iPSC Phenotypes**

SPC protein expression in the cytoplasm of differentiated iPSCs was confirmed by immunofluorescence (Fig. 2A). Additionally, 9.3±3.3% SPC-positive differentiated iPSCs were detected by flow cytometry (Fig. 2B). TEM ultrastructural analysis showed characteristic lamellar bodies and microvilli, organelles specific to alveolar type 2 cells, in differentiated iPSCs on day 12 (Fig. 2C). These data demonstrated that a portion of iPSCs differentiated into AECs, particularly alveolar type 2 cells.

**Recellularization of Decellularized Mouse Lung Scaffold with iPSC-Derived AECs**

We assessed the regenerative potential of iPSC-derived AECs in a mouse decellularized lung scaffold model that was recently demonstrated to be useful for studying functional recellularization in stem and progenitor cell populations [15, 25]. After 12 days of incubation, iPSC-derived AECs were observed to integrate into the parenchymal regions and form alveolar structures (Fig. 3B). Some of them adopted the morphology of alveolar epithelia; i.e., they developed a rounded or flattened shape (Figs. 3B and 4). However, the undifferentiated iPSCs tended to proliferate in the alveolar space and form masses of cells, which resembled colonies (Figs. 3B and 4). The fibroblasts survived but were integrated into the stroma of the lung. Immunofluorescence staining showed
strong expression of SPC and T1α in tissues of the iPSC-derived AEC group, whereas few SPC- or T1α-positive cells were detected in the undifferentiated iPSC group (Fig.4). No SPC or T1α expression was detected in the fibroblast group (Fig. 4). When SPC-positive cells were quantified in the recellularized mouse lung scaffolds by iPSC-derived AEC, we observed an increased percentage of SPC-positive cells (13.46±5.59%) after 12 days compared to those in in vitro differentiation D12 (9.3±3.3%). No further improvement in alveolar morphology structure or lung epithelium marker expression was observed when the culture duration was prolonged to 30 days (data not shown).

**Transplantation of Differentiated iPSCs in a Lung Injury Mouse Model**

To examine whether transplanted cells could home to and ameliorate lung injury, iPSC-derived AECs, undifferentiated iPSCs, or fibroblasts were delivered intratracheally into a BLM-induced lung injury mouse model. Using PKH26 staining, we succeeded in providing a convenient strategy to track the donor cells after transplantation into the mouse lung. As shown in Figs. 5 and S3, the transplanted cells (red) showed uniform distribution throughout the distal alveoli on day 12 after transplantation.

After BLM treatment, the numbers of SPC-positive and T1α-positive cells were drastically reduced (Figs. 5 and S3). Transplantation of iPSC-derived AECs recovered the numbers of SPC-positive cells (from 6.6±3.1% to 12.1±3.3%, P<0.05, Fig S4A) and T1α-positive cells in the BLM-treated lung. Transplantation of undifferentiated iPSCs modestly recovered the numbers of SPC-positive (8.2±2.6%, P>0.05, Fig S4A) and T1α-positive cells. No recovery of SPC-positive or T1α-positive cells was observed
after fibroblast transplantation. In addition, SPC+/PKH26+ and T1α+/PKH26+ cells were observed in lung sections only in the iPSC-derived AEC group (Figs. 5 and S3). No SPC+/PKH26+ or T1α+/PKH26+ cells were detected in the fibroblast or undifferentiated iPSC groups. Because of the non-uniform composition of the cell population and longer centrifugation time (four × 10 min) needed in our PKH26 staining protocol, the viability of iPSC-derived AECs might be reduced. Thus, the frequency of double-positive cells was stochastic and might be quantified as being lower. We did not quantify double-positive cells in lung sections. Nonetheless, the presence of SPC+/PKH26+ and T1α+/PKH26+ cells demonstrated the successful derivation of AECs from iPSCs and their engraftment into the mouse lung. We also evaluated the long-term survival of differentiated iPSCs in BLM-injured mouse lungs. We confirmed the presence of SPC+/PKH26+ and T1α+/PKH26+ cells on day 30 after transplantation, although PKH26 fluorescence became very weak (Fig. S4B).

Transplantation of Differentiated iPSCs Reduces Lung Inflammation and Attenuates Lung Fibrosis in BLM-treated Mice

Twelve days after intratracheal exposure of BLM, the lung tissues were severely damaged as shown by hematoxylin and eosin (HE) staining (Fig. 6A). The lungs presented typical injuries such as disorganized epithelium, extensive inflammatory cell infiltration, interstitial thickening, collapse of the alveolar wall, and obvious cystic air spaces. Increased collagen deposition was observed by Sirius red/Fast green staining and hydroxyproline assay (Fig. 6A and 6C). Wet/dry weight ratios indicated that BLM treatment resulted in a significant increase in edema compared with the saline group (6.1±0.4 vs. 4.5±0.2, respectively; **P<0.01) (Fig. 6B). Transplantation of
differentiated iPSCs significantly reduced the extent of fibrosis and recovered the lung tissue structure to similar to that of the saline control (Figs. 5, S3, and 6A). A decrease in lung edema was confirmed in the iPSC-derived AEC group compared with the BLM and BLM/fibroblast groups (Fig. 6B; #P<0.01). Transplantation of differentiated iPSCs reduced inflammatory cell infiltration (Fig. 7A) and decreased TNF-α and IL-6 levels in BLM-treated mice to the same levels as in the saline control (Fig. 7B and 7C; † P>0.05). There was no significant decrease in TNF-α and IL-6 production in the undifferentiated iPSC group compared with the BLM and BLM/fibroblasts groups (Fig. 7B and 7C; ▴ P>0.05). Hydroxyproline assay showed that the collagen content was decreased in the lungs of the differentiated iPSC transplantation group compared with the BLM and BLM/fibroblast groups (Fig. 6C; #P<0.01). However, treatment with iPSCs-derived AECs did not result in a return to the basal collagen level (Fig. 6C; *P<0.05). By contrast, there was no significant decrease in lung edema and collagen deposition in the undifferentiated iPSC group compared with the BLM group (Fig. 6B and 6C; ▴P>0.05). Transplantation of fibroblasts did not rescue the lung injuries.

**DISCUSSION**

In the present study, we report an *in vitro* protocol for direct differentiation of mouse iPSCs into ~9% lung progenitor or epithelial cells in two steps. These differentiated cells express a lung progenitor marker, TTF-1, and a type II alveolar type marker, SP-C. These cells can recellularize a decellularized mouse lung scaffold in three-dimensional culture. Finally, these differentiated cells ameliorated the BLM-induced lung injury and engrafted into the lung.

Stem cell therapy provides a new strategy for repairing severe acute and chronic lung
injuries. Although some studies have demonstrated the therapeutic potential of bone-marrow-derived stem cells in rodent lung injury models [23, 26-28], there is no evidence of a role for these stem cells in populating the lung alveolar epithelium *in vivo* [29-32]. Recent studies have reported efficient and direct derivation of lung alveolar epithelium from murine embryonic stem cells (ESCs) [10,11,14,16] for *in vitro* and *in vivo* applications. However, immune reactions and ethical issues represent barriers to their clinical application.

We modified a protocol for mouse iPSC differentiation into AECs *in vitro* in a 12-day period, and achieved a 9.3±3.3% yield of SPC⁺ AEC II-like cells. However, a recent study developed a more efficient differentiation protocol for lung epithelial cells (20). mRNA expression of type II epithelial markers, such as SPA and SPC, was suppressed in the presence of BSA (Fig. 1B), as reported previously [9,33]. SABM enhanced the mRNA expression of SPA, SPC, and SPD but suppressed that of TTF1 compared with Basic DM when used during stage 2 differentiation. A primordial progenitor stage defined by TTF-1 expression is considered essential for formation of lung epithelia differentiated from endodermal cells [15,34,35]. Therefore, use of SABM results in enhanced differentiation of iPSCs to lung epithelia compared with Basic DM.

The decellularized lung scaffold preserves extracellular matrix proteins intact. The three-dimensional hierarchical branching structures of the airway and vasculature have recently been used to investigate regeneration of lung tissues by stem and progenitor cells. Two initial studies reported regeneration of functional lung tissues by seeding of epithelial and endothelial cells onto this decellularized lung scaffold and successful short-term functional orthotopic transplantation [36, 37]. Upon addition of mouse ESCs, the decellularized lung scaffold guided ESC differentiation toward lung-specific
lineages [12]. However, our studies demonstrated that only a limited number of undifferentiated iPSCs expressed alveolar epithelial markers during culture. This suggests that the efficiency of ESC or iPSC differentiation to lung epithelial cells by the lung scaffold is low. Jensen et al. seeded predifferentiated mouse ESCs into a decellularized whole lung scaffold, and then subcutaneously implanted this scaffold into mice. The implanted scaffold facilitated maintenance of lung-specific differentiation of mouse ESCs [38]. Neovascularization was confirmed in this recellularized lung scaffold after implantation.

We demonstrated the regenerative potential of differentiated iPSCs using a mouse decellularized lung scaffold model in in vitro three-dimensional culture. According to the results of HE staining and immunofluorescence staining, intratracheal transplantation of differentiated iPSCs into decellularized lung scaffolds resulted in formation of three-dimensional alveolar structures and differentiated alveolar epithelial cells expressing SPC or T1α protein. Differentiated iPSCs showed further maturation when cultured for 12 days in the decellularized lung scaffold.

We further investigated the therapeutic potential of differentiated iPSCs in a mouse BLM-induced lung injury model. Although only ~9% of iPSCs differentiated into SPC+ cells in vitro, transplantation of these unsorted differentiated iPSCs contributed to the reconstitution of BLM-injured lung and significantly reduced BLM-induced lung inflammation and fibrosis in mice. In addition to direct replacement of injured lung epithelia by differentiated iPSCs, homed iPSCs may provide beneficial effects in a paracrine manner [16, 20, 39]. Current data indicate that diverse paracrine mechanisms exist in stem cell therapy, such as modulation of cytokines, growth factors, and antimicrobial peptides [40]. In this study, we observed modulation of the inflammatory
cytokines TNF-α and IL-6 upon transplantation of differentiated iPSCs. Indeed, Yang et al. reported that undifferentiated iPSCs reduced endotoxin-induced acute lung injury in mice when delivered through the tail vein [41]. This effect was considered to occur in a paracrine manner and be mediated by reductions in NF-κB activity and neutrophil accumulation [41]. Transfer of MSCs also reduced BLM-induced lung injury and fibrosis partly through down-regulation of inflammatory cytokines, such as TNF-α and IL-6 [42]. However, our data showed that undifferentiated iPSCs did not sufficiently rescue the BLM-induced lung injury. This discrepancy may be due to the different degrees of acute lung injury induced by BLM and endotoxin or the different transplantation methods used.

To our knowledge, this is the first report of the three-dimensional alveolar lung structure regenerative potential and lung injury therapeutic potential of differentiated mouse iPSCs. However, because of the low efficiency of differentiation and the heterogeneity of differentiated iPSCs, the cells generated using this method are unsuitable for clinical application. A recent study reported that primordial lung progenitors can be efficiently differentiated from definitive endoderm cells through inhibition of TGFβ and BMP signaling, followed by stimulation of BMP and FGF signaling [15]. Sorting strategies can be used to enrich differentiated iPSCs. Longmire et al. used ESCs harboring a new Nkx2-1GFP knock-in reporter to derive primordial lung and thyroid progenitors and purified these progenitors using GFP for expansion in culture [15]. Soh et al. reported that lung progenitors could be enriched using the stem cell marker CD166 when differentiated from human ESCs or iPSCs [20].

**CONCLUSION**
We demonstrated that mouse iPSCs could acquire the alveolar epithelial cell phenotype 
in vitro under specific differentiation conditions. The differentiated iPSCs possessed the 
potential for regenerating three-dimensional alveolar lung structures and abrogating 
BLM-induced acute lung injury in the mouse.

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FIGURE LEGENDS

Figure 1. Differentiation media based on mRNA expression levels of endodermal and lung epithelial markers A: Differentiation protocols for the derivation of alveolar epithelial cells from mouse iPSCs using a dissociated seeding method. B: Quantitative polymerase chain reaction analysis of lung epithelial markers on day 12. *P<0.05 versus D12-①; # P<0.01 versus D12-②; ▲P<0.01 versus D12-①.

C: Quantitative polymerase chain reaction (qPCR) analysis of lung epithelial markers in differentiated iPSCs D12-② compared with the murine type II pneumocyte cell line MLE12 and mouse lung tissue. Expression ratios were normalized to the GAPDH expression level. Data are representative of three independent experiments. Note that the y-axis is a logarithmic scale.

Figure 2. Evaluation of differentiated iPSC phenotypes on Day 12
A: iPS cells differentiated for 12 days were immunostained with goat anti-mouse pro-SPC antibody (red) or goat IgG and nuclear-counterstained with DAPI (blue). Scale bars: 50 μm. B: Flow cytometry analysis of SPC expression. C: Transmission electron micrographs of MLE12 cells (a murine type 2 pneumocyte cell line) and differentiated iPSCs. Upper-left: MLE12 cells exhibit characteristic lamellar bodies (black arrows) and apical microvilli (red arrows). Upper-right: Magnified view of the upper-left image. Lower-left: Mouse differentiated iPSCs showing similar lamellar bodies and microvilli to those of MLE12 cells. Lower-right: Magnified view of the lower-left image. Scale bars: 1 μm.

Figure 3. Decellularization and recellularization of mouse lung
A: A photo and schematic illustration of decellularization and recellularization B: HE staining of sections of a decellularized mouse lung scaffold (no cell) and recellularized lung tissues reseeded with fibroblasts, undifferentiated iPSCs, or differentiated iPSCs on D12. Bottom panels show magnified views of the dotted line areas in upper panels. Scale bar: 100 μm for upper panels, 50 μm for bottom panels.

Figure 4. Immunofluorescence staining of the alveolar type-II cell marker SPC and alveolar type-I cell marker T1α in recellularized lung tissues. Decellularized lung scaffolds were reseeded with mouse fibroblasts, undifferentiated iPSCs, or differentiated iPSCs and immunostained with goat anti-mouse pro-SPC or T1α antibodies after 12 days. Yellow arrows indicate SPC⁺ or T1α⁺ cells. Bottom panels show magnified views of the areas marked by the dotted lines in the upper panels. Scale bar: 50 μm for upper panels, 10 μm for lower panels.
**Figure 5. Immunofluorescence staining of the alveolar type-II cell marker SPC in mouse lung tissues**

Following intratracheal exposure to normal saline or 4 U/kg bleomycin (BLM), fibroblasts, undifferentiated iPSCs, or differentiated iPSCs labeled with PKH26 cell tracker (red) were instilled intratracheally into mice on day 2. The lung tissues were excised on day 12 and immunostained with goat anti-mouse pro-SPC antibody (green). Nuclei were counterstained with DAPI (blue). Yellow arrows indicate PKH26+/SPC+ cells. Scale bars: 50 μm. A magnified view of a PKH26+/SPC+ cell is indicated by dotted lines. Bottom panels show magnified views of the areas marked by the dotted lines in the upper panels. Scale bar: 50 μm for upper panels, 10 μm for lower panels.

**Figure 6. Amelioration of lung fibrosis by transplantation of iPSCs-derived AECs in BLM-treated mice**

Following intratracheal exposure of normal saline or 4 U/kg BLM, fibroblasts, undifferentiated iPSCs, or differentiated iPSCs were instilled intratracheally into mice on day 2. The lung tissues were excised on day 12. A: HE and Sirius red/Fast green staining of lung sections. Scale bar: 100 μm. Magnified views of the white dotted line areas were marked with yellow dotted lines. B: The wet/dry ratio of lungs on day 12. Data are expressed as the mean±SD; n= ≥7/group; * * P<0.01 versus the saline control group; •P>0.05 versus the BLM group; *P<0.05 versus the saline control group; # P<0.01 versus the BLM and BLM/fibroblasts groups. C: Collagen deposition in lung tissue was evaluated qualitatively by hydroxyproline assay. Data are expressed as the mean±SD; n= ≥5/group; * * P<0.01 versus the saline control group; •P>0.05 versus
the BLM group; *$P<0.05$ versus the saline control group; # $P<0.01$ versus the BLM and BLM/fibroblasts groups.

**Figure 7. Effect of differentiated iPSC transplantation on the BALF profile in BLM-treated mice**

Following intratracheal exposure to normal saline or 4 U/kg BLM, fibroblasts, undifferentiated iPSCs, or differentiated iPSCs were instilled intratracheally into mice on day 2. BAL was performed on day 12. A: BAL fluids from the four groups were cytopspined, and the cell pallets were stained using Diff-Quik. Representative images are shown. Yellow arrows indicate neutrophils and black arrows lymphocytes. Scale bar: 50 μm. IL-6 (B) and TNF-α (C) concentrations in BAL fluids were measured using sandwich ELISA kits. Data are expressed as the means ± SD; n ≥ 5 per group; B: * $P<0.05$ versus the saline control group; ▲ $P>0.05$ versus the BLM group and BLM/fibroblasts group; # $P<0.05$ versus the BLM group; † $P>0.05$ versus the saline control group; C: ** $P<0.01$ versus the saline control group; * $P<0.05$ versus the saline control group; ▲ $P>0.05$ versus the BLM group and BLM/fibroblasts group; # $P<0.01$ versus the BLM group; P$<0.05$ versus the BLM/fibroblasts group; †P$>0.05$ versus the saline control group.

**Figure S1. The pluripotency of undifferentiated iPS cells (D0) at passage 30**

A: Immunofluorescence staining of mouse iPSCs with the stem cell markers OCT4 and SSEA1. The GFP gene was knocked-in under the Nanog promoter, allowing detection of GFP (green) in undifferentiated cells. Scale bar: 200 μm for upper panels, 100 μm for bottom panels. B: Alkaline phosphatase staining. Bottom panel show magnified view of
the area marked by the dotted lines in the upper panel. Scale bar: 100 μm for upper panel, 50 μm for bottom panel. C: Flow cytometry analysis of mouse iPS cells using SSEA1 and OCT4.

**Figure S2. Expression of the endoderm markers Sox17 and Foxa2 in iPSCs on day 7 of differentiation**

A: iPS cells differentiated for 6 days were immunostained using goat anti-mouse Sox17 and Foxa2 antibodies (red) and were nuclear-counterstained with DAPI (blue). Scale bar: 50 μm. B: qRT-PCR analysis of Sox17 and Foxa2 on day 7 of differentiation. *P<0.05 versus Basic DM.

**Figure S3. Immunofluorescence staining of the alveolar type-I cell marker T1α in mouse lung tissues**

Following intratracheal exposure to normal saline or 4 U/kg BLM, fibroblasts, undifferentiated iPSCs, or differentiated iPSCs labeled with PKH26 cell tracker (red) were instilled intratracheally into mice on day 2. The lung tissues were excised on day 12 and immunostained with a goat anti-mouse T1α antibody (membrane staining, green). Nuclei were counterstained with DAPI (blue). Yellow arrows indicate PKH26+/T1α+ cells. Scale bars: 50 μm. A magnified view of a PKH26+/T1α+ cell is indicated by dotted lines. Bottom panels show magnified views of the areas marked by the dotted lines in the upper panels. Scale bar: 50 μm for upper panels, 10 μm for lower panels.

**Figure S4. Recovery of SPC+ cells and identification of transplanted differentiated iPSCs in the BLM-injured lung**
A: Quantification of SPC+ cells in lung tissues after differentiated iPSC transplantation in the BLM-injured lung on day 12. ** P<0.01 versus the saline control group; * P<0.05 versus the saline control group; †P>0.05 versus the BLM group; # P<0.05 versus the BLM group; ▴ P>0.05 versus the saline control group. B: SPC and T1α immunofluorescence in lung tissues transplanted with PKH26-stained differentiated iPSCs after BLM challenge on day 30. Yellow arrows indicate PKH26+/SPC+ or PKH26+/T1α+ cells. Scale bar: 30 μm
Figures 1

**Stage 1**

- Day 0 (D0)
- Day 1 (D1)
- Basic DM+ Activin A (20ng/ml)+Wnt3a (10ng/ml)
-undefinitive endoderm iPScs

**Stage 2**

- Day 7 (D7)
- Basic DM+ FGF2 (50ng/ml)
- SABM+ FGF2 (50ng/ml)
- SABM+0.5% BSA+FGF2 (50ng/ml)

**Figure 1**

A

<table>
<thead>
<tr>
<th>Day 0 (D0)</th>
<th>Day 1 (D1)</th>
<th>Stage 1</th>
<th>Day 7 (D7)</th>
<th>Stage 2</th>
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B

- mRNA relative expression levels
- D0, D12-①, D12-②, D12-③

C

- mRNA relative expression levels normalized to GAPDH
- D0, D12-②, MLE, Lung
Figure 2

A

DAPI  SPC  Merge  IgG+

B

counts

SPC-PE

counts

IgG-PE

9.3 ± 3.3%

C

MLE12 cell
differentiated iPScs
**Figure 3**

A. Decellularized lung

- 2×10⁶ cells/mouse
- Recellularization of lung
- Lung slices were cultured in Small Airway Epithelial Cell Growth Medium (SAGM) for 12 days

B. Images showing:
- No cell
- Fibroblasts
- Undifferentiated iPSCs
- Differentiated iPSCs
Figure 4

SPC

- fibroblasts
- undifferentiated iPScs
- differentiated iPScs

T1α

IgG

undifferentiated iPScs

SPC

undifferentiated iPScs

differentiated iPScs

SPC

SPC+DAPI

T1α

T1α+DAPI

IgG+DAPI

undifferentiated iPScs

SPC

SPC+DAPI

T1α

T1α+DAPI

IgG+DAPI

differentiated iPScs

SPC

SPC+DAPI

T1α

T1α+DAPI

IgG+DAPI
Figure 5

Control

BLM

BLM / fibroblasts

BLM / undifferentiated iPScs

BLM / differentiated iPScs

PKH26-

PKH26-

PKH26-

PKH26-

PKH26-

PKH26-

PKH26-

PKH26-

Merge+ DAPI

Merge+ DAPI

Merge+ DAPI

Merge+ DAPI

Merge+ DAPI

Merge+ DAPI

Merge+ DAPI

Merge+ DAPI
**Figure 6**

A. Microscopic images showing HE staining and sirius red/fast green staining for different groups: Control, BLM, BLM/fibroblasts, BLM/undifferentiated iPSCs, and BLM/differentiated iPSCs. The images are scaled to 100 um.

B. Bar graph showing the wet/dry weight ratio for different groups: Control, BLM, BLM/fibroblasts, BLM/undifferentiated iPSCs, and BLM/differentiated iPSCs. The bars are labeled with statistical significance indicators.

C. Bar graph showing the hydroxyproline (ug/lung) for different groups: Control, BLM, BLM/fibroblasts, BLM/undifferentiated iPSCs, and BLM/differentiated iPSCs. The bars are labeled with statistical significance indicators.
Figure 7

A

Control  BLM  BLM / undifferentiated iPScs  BLM / differentiated iPScs

B

IL-6 in BALF (pg/ml)

C

TNF-α in BALF (pg/ml)

Control  BLM  BLM / undifferentiated iPScs  BLM / differentiated iPScs  Control  BLM  BLM / fibroblasts  BLM / undifferentiated iPScs  BLM / differentiated iPScs
**Figure S1**

**A**

![Images of DAPI, Nanog-GFP, OCT4, and Merge](image1)

**B**

![Image of cells with scale bar](image2)

**C**

![Histograms of IgG and SSEA1-PE with percentages](image3)

- DAPI: 0.13%
- Nanog-GFP: 91.3%
- OCT4: 82.4%
- IgG: 0.96%
Figure S2

A

DAPI  Sox17  Merge

DAPI  Foxa2  Merge

B

mRNA relative expression levels

- D0
- Basic DM
- Basic DM (no BSA)

SOX17  Foxa2
Figure S4

A

Percentages of SPC+ cells(%)

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<th>Percentage</th>
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</tr>
<tr>
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</tr>
<tr>
<td>BLM differentiated iPSCs</td>
<td><strong>16</strong></td>
</tr>
</tbody>
</table>

B

PKH26

SPC

Merge+DAPI

PKH26

T1c

Merge+DAPI