A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells

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Alveolar epithelial type II (ATII) cells are small, cuboidal cells that constitute ≈60% of the pulmonary alveolar epithelium. These cells are crucial for repair of the injured alveolus by differentiating into alveolar epithelial type I cells. ATII cells derived from human ES (hES) cells are a promising source of cells that could be used therapeutically to treat distal lung diseases. We have developed a reliable transfection and culture procedure, which facilitates, via genetic selection, the differentiation of hES cells into an essentially pure (>99%) population of ATII cells (hES-ATII). Purity, as well as biological features and morphological characteristics of normal ATII cells, was demonstrated for the hES-ATII cells, including lamellar body formation, expression of surfactant proteins A, B, and C, α-1-antitrypsin, and the cystic fibrosis transmembrane conductance receptor, as well as the synthesis and secretion of complement proteins C3 and C5. Collectively, these data document the successful generation of a pure population of ATII cells derived from hES cells, providing a practical source of ATII cells to explore in disease models their potential in the regeneration and repair of the injured alveolus and in the therapeutic treatment of genetic diseases affecting the lung.

complement | differentiation | surfactant proteins | α-1-antitrypsin | cystic fibrosis transmembrane conductance receptor

The alveolar epithelium covers >99% of the internal surface area of the lung and is composed of two major cell types, the alveolar type I (ATI) cell and the alveolar type II (ATII) cell. ATI cells are large flat cells through which exchange of CO_2/O_2 takes place. They cover ≈95% of the alveolar surface and comprise ≈40% of the alveolar epithelium and 8% of the peripheral lung cells. In contrast, ATII cells are small, cuboidal cells that cover ≈5% of the alveolar surface and comprise 60% of the alveolar epithelium and 15% of the peripheral lung cells. They are characterized by the unique ability to synthesize and secrete surfactant protein C (SPC) and by the distinct morphological appearance of inclusion bodies, known as lamellar bodies. Important functions of ATII cells are (*i*) to synthesize, store, and secrete surfactant, which reduces surface tension, preventing collapse of the alveolus; (*ii*) to transport ions from the alveolar fluid into the interstitium, thereby minimizing alveolar fluid and maximizing gas exchange; (*iii*) to serve as progenitor cells for ATI cells, which is particularly important during reepithelialization of the alveolus after lung injury; and (*iv*) to provide pulmonary host defense by synthesizing and secreting several complement proteins including C3 and C5 (1–3) as well as numerous cytokines and interleukins that modulate lymphocyte, macrophage, and neutrophil functions (4). Severe pulmonary diseases can be caused by deficiencies or genetic mutations in proteins synthesized by ATII cells that are important in maintaining normal lung homeostasis. For example, complete deficiency of surfactant protein B (SPB) is caused by genetic mutations in the SPB gene. This deficiency results in impaired pulmonary surfactant composition and function and is a major cause of fatal neonatal respiratory disease (5, 6). In addition, ATII cells synthesize and secrete the serine protease inhibitor α-1-antitrypsin (α-1AT), which also plays a key role in alveolar homeostasis by regulating protease imbalance and adjusting fluid clearance (7, 8), the importance of which is supported by the association of α-1AT deficiency with the development of pulmonary emphysema (9). Cystic fibrosis is thought to be primarily a disease of the upper airway and submucosal epithelia and is caused by mutations in the cystic fibrosis transmembrane conductance receptor (CFTR) (10). CFTR is an important regulator of Cl^- and liquid transport in the lung (11–14) and is functionally expressed by human ATII cells, strongly suggesting a critical role for CFTR in regulating ion and fluid transport in the lung alveolus in addition to the upper airway (13).

ES cells isolated from the inner cell mass of blastocyst-stage embryos are undifferentiated, pluripotent cells (15, 16), which can be induced to differentiate *in vitro* into a wide range of different cell types (17–25). The potential clinical use of ES cells to regenerate or repair damaged tissue has fueled a tremendous amount of research activity to develop methods that promote the differentiation of ES cells into specific cell lineages. Because of its numerous important functions, including its ability to proliferate and differentiate into the easily damaged ATI cell, human ES (hES) cell-derived ATII (hES-ATII) cells are promising as a source of cells that could be used therapeutically to treat distal lung injury as well as pulmonary genetic disorders. Recently published data demonstrated that ES cells can be differentiated into ATII cells via embryonic body (EB) formation (26, 27) or coculture of EBs with pulmonary mesenchyme (28). However, these procedures were not efficient, generating only a very small percentage of ES cell-derived ATII cells (29). A mixed population of cell derivatives, as those generated in these reports, will not be suitable for transplantation into the lung. In addition, the pluripotent cells in the differentiating cultures carry a significant risk of producing teratomas after transplantation *in vivo*. Therefore, a major prerequisite for using ATII cells therapeutically is to achieve a pure population of hES-ATII cells. Selection strategies such as those employing protein markers or drug-resistance genes under the control of cell-specific promoters may be highly effective in producing a pure culture of ES cell-derived donor cells (30). The aim of the following study was to achieve a pure population of hES-ATII cells based on the hypothesis that hES cells cultured on Matrigel-coated dishes can be efficiently

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The authors declare no conflict of interest.

Abbreviations: α-1AT, α-1-antitrypsin; ATI, alveolar type I; ATII, alveolar type II; CFTR, cystic fibrosis transmembrane conductance receptor; EB, embryonic body; hES, human ES; hES-ATII, hES cell-derived ATII; SPA, surfactant protein A; SPB, surfactant protein B; SPC, surfactant protein C; DM, differentiation medium.

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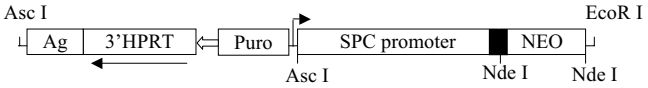

Fig. 1. Structure of the SPC promoter-NEO transgene 3′-HPRT vector. A 3.8-kb human genomic DNA fragment containing the SPC promoter and 170 bp of noncoding sequence of exon 1 was cloned into the 3′-hprt targeting vector containing the puromycin-resistance gene. The Neo^r gene was added downstream of the SPC promoter. The EcoRI site located between the Agouti and Neo^r gene was used to linearize the plasmid before transfection.

differentiated into ATII cells without EB formation and that a highly pure population of ATII cells can be generated by genetic selection of hES cells transfected with a SPC promoter/NEO^r fusion gene.

Results

Derivation and Selection of ATII Cells from hES Cell Lines. The structure of the human SPC promoter-neomycin transgene (3′-hprt-SPCP.NEO) is depicted in Fig. 1. The hES cell line H9.2 was transfected with the linearized transgene as described in *Methods*. Of the ≈1 million hES cells subjected to transfection, 216 survived puromycin selection. Of these, 12 were found to contain a single copy of the correctly inserted transgene, of which one (SPCP/NEO.74) was selected for further investigation. To induce spontaneous differentiation *in vitro*, H9.2 and SPCP/NEO.74 cell lines were cultured in six-well extra-low-attachment plates for 5 days to form EBs. The presence of ATII cells in the differentiating cultures of both hES cell lines was determined by RT-PCR specific for SPC RNA. No SPC RNA was detected in the undifferentiated hES cells (day 0) or in the differentiating cultures on day 10. SPC RNA was detected in differentiating cultures of H9.2 and SPCP/NEO.74 hES cells by day 15, with significant increases of SPC RNA observed in both hES cultures on day 25 (Fig. 2 *Upper Left*). The ability of hES cells to directly differentiate into ATII cells *in vitro* without EB formation was examined by culturing the cells on Matrigel-coated plates in differentiation medium (DM). SPC RNA expression was detected as early as day 10 in both hES cell lines under these culture conditions (Fig. 2 *Upper Right*). Therefore, compared with cultures differentiated via EB formation, ATII cells appeared 5 days earlier in differentiating hES cells cultured on Matrigel-coated plates. To examine whether hES cell-derived ATII cells can be enriched by genetic selection, differentiating cultures of SPCP/NEO.74, with or without EB formation, were subjected to G418 treatment (20 μg/ml). SPC RNA expression was detected in G418-selected cultures with EB formation on day 10 (Fig. 2 *Lower Left*) and without EB formation on day 5 (Fig. 2 *Lower Right*), but not in nonselected cultures at corresponding time points. In addition, significantly higher levels of SPC RNA in G418-selected cells were observed compared with nonselected cells at the longer time points of differentiation (Fig. 2 *Lower*). Collectively, these results indicate that the SPCP/NEO.74 differentiating cell cultures can be enriched in ATII cells after selection with G418 treatment and that hES cell-derived ATII cells can be generated efficiently on Matrigel-coated plates without EB formation.

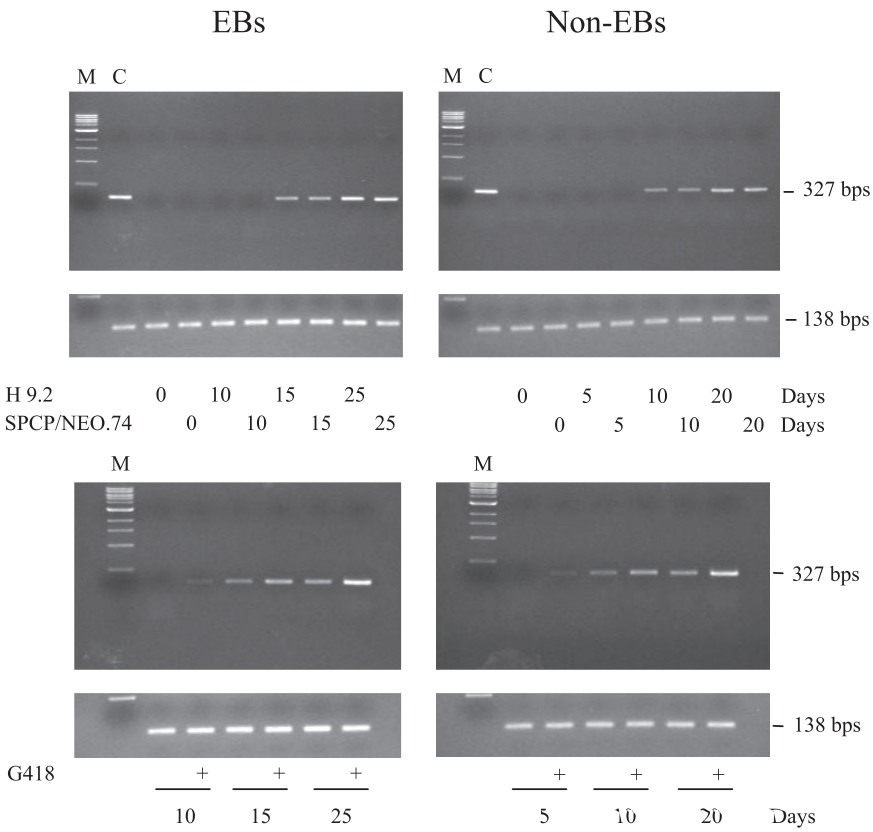

Fig. 2. Relative RNA levels of SPC in G418-selected and nonselected differentiating hES cells. SPC-specific RT-PCR was performed by using total RNA isolated from differentiating cultures of hES cell lines H9.2 and SPCP/NEO.74. (*Left*) Data obtained from differentiating cells subjected to EB formation. (*Right*) Data obtained from differentiating cells without EB formation. (*Lower*) The results from G418-selected SPCP/NEO.74 hES cells. Lanes M, 1-kb DNA ladder. Lanes C, the SPC-specific RT-PCR positive control using RNA isolated from the ATII cell line A549. The lower blot in each panel shows the 18S-specific RT-PCR, demonstrating that changes in the amount of SPC-specific 327-bp RT-PCR product was due to corresponding changes in SPC RNA expression. Total days of differentiation at which the RNA samples were obtained are indicated.

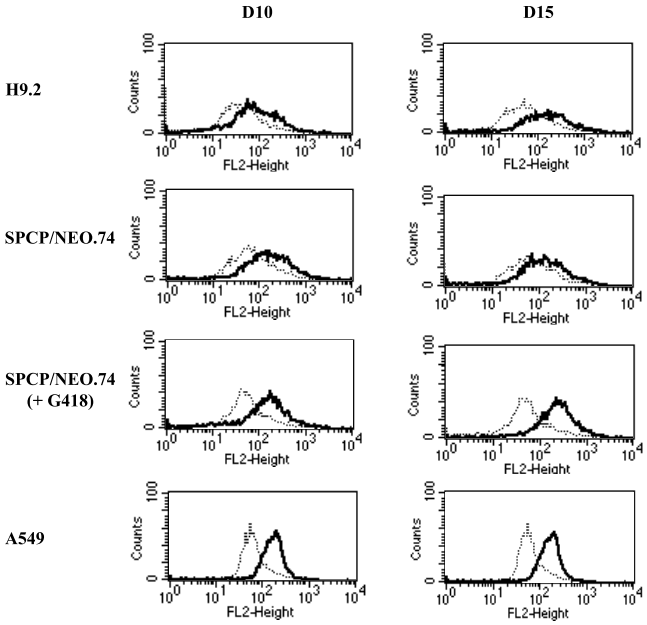

Fig. 3. Flow cytometry examining SPC expression in G418-selected and nonselected cultures of differentiating hES cells. hES cell lines H9.2 and SPCP/NEO.74 were induced to differentiate by culturing on Matrigel-coated plates with DM for 10 days (*Left*) or 15 days (*Right*). The differentiated cells were dissociated and immunostained by rabbit anti-human SPC antibody for flow cytometry analysis as described in *Methods*. Results using the SPC antibody are depicted by solid lines, and nonimmune rabbit serum controls are illustrated by dashed lines.

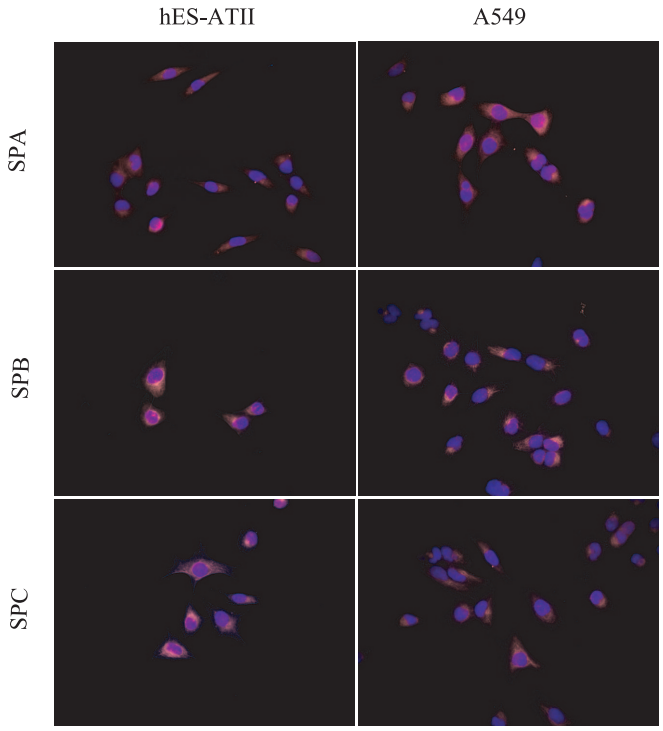

Fig. 4. Immunofluorescence of SPA, SPB, and SPC in SPCP/NEO.74-derived ATII cells. The hES cell-derived ATII cells generated by G418 selection from the SPCP/NEO.74 cell line and A549 cells were immunostained by rabbit anti-human SPA, SPB, and SPC antibodies (red) and nuclear-counterstained with DAPI (blue). (Magnification: ×400.)

Flow Cytometry Analysis of SPC Protein Expression. The differentiated hES cell cultures without EB formation (days 10 and 15) were examined for ATII-specific SPC protein expression by flow cytometry. These studies revealed low level SPC protein expression in the differentiated culture of H9.2 cells on day 10, which did not increase on prolonged culture (15 days) (Fig. 3). Similar SPC protein levels were observed in the differentiated cultures of the SPCP/NEO.74 cell line. In contrast, when genetically selected by G418 treatment for 10 and 15 days, differentiated cultures of SPCP/NEO.74 exhibited significantly higher levels of SPC protein expression (Fig. 3). The levels of SPC protein in the genetically selected SPCP/NEO.74 cultures were comparable to that of the human ATII cell line A549.

Immunofluorescent Staining of SPC, Surfactant Protein A (SPA), and SPB in hES-ATII Cells. The synthesis of SPC is a unique feature of ATII cells and is commonly used to identify these cells from other lung parenchymal cells. Undifferentiated hES cells, H9.2 and SPCP/NEO.74, did not stain positive for SPC. As expected, however, the G418-selected hES-ATII cells, as well as the A549 cells, displayed intense staining with rabbit anti-human SPC antibody (Fig. 4). At higher magnification (×630), SPC-specific staining can be seen throughout the cytoplasm, with more intense staining observed in the perinuclear region, suggesting the presence of SPC in the Golgi/ER compartments. In addition to SPC, the hES-ATII and A549 cells were shown to express SPA and SPB (Fig. 4).

Percentage of hES-ATII Cells in Differentiated Cell Cultures. SPC staining was used to determine the percentage of ATII cells in differentiated hES cell cultures. SPC-positive staining was displayed in only 11.2% of the differentiated hES cells in the nonselected H9.2 ES cell culture (Table 1). Similar results were observed in the nonselected SPCP/NEO.74 cell culture (12.6%). In stark contrast, 99.6% of cells in the G418-selected differentiated culture of SPCP/NEO.74 cells expressed SPC protein, indicating that G418 selection of the transfected hES cells produced an essentially pure culture of ATII cells.

Identification of Lamellar Bodies in hES-ATII Cells by Transmission Electron Microscopy and Papanicolaous Staining. Lamellar bodies are intracellular organelles that contain pulmonary surfactant proteins and lipids. The presence of lamellar bodies is a criterion traditionally used for the identification of ATII cells. To determine whether the hES-ATII cells contained these intracellular organelles, G418-selected hES-ATII cells were examined by transmission electron microscopy. The hES-ATII cells exhibited ultrastructural features characteristic of human type II cells, including cytoplasmic lamellar bodies as seen in A549 cells (31) (Fig. 5). Supporting the electron microscopy data, lamellar bodies were also detected in the differentiated hES cultures by Papanicolaous staining, which is another procedure routinely used for the identification of lamellar bodies (32).

Expression of Human CFTR, α-1AT, and Complement Proteins C3 and C5. Because, ATII cells express CFTR and α-1AT *in vivo* and play an important role in alveolar homeostasis, the hES-ATII cells

Table 1. Relative ATII cell content in nonselected and G418-selected cultures of differentiating hES cells

Cell	G418 selection	SPC-positive cells	SPC-negative cells	% of SPC-positive cells
H9.2	−	58	442	11.2
SPC/NEO.74	−	63	437	12.6
SPC/NEO.74	+	498	2	99.6

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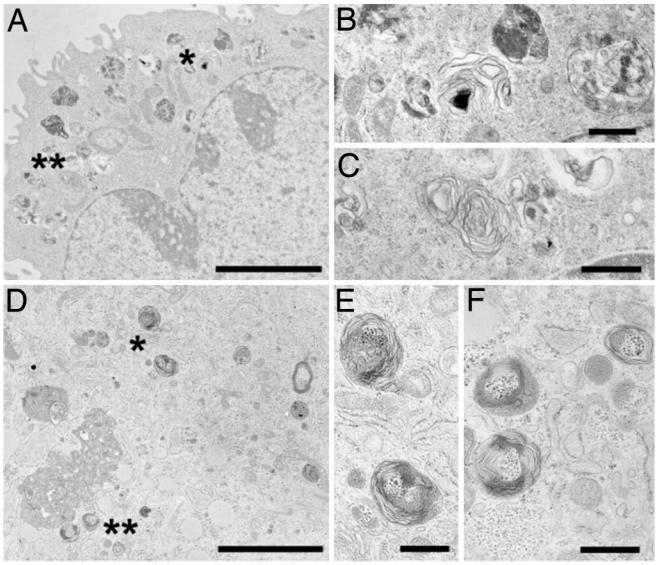

Fig. 5. Transmission electron micrographs of A549 and hES-ATII cells. (*A*) A549 cells with characteristic cytoplasmic electron-dense and loose lamellar bodies. (*B* and *C*) Magnified views of regions * and **, respectively, more clearly showing the structure of the lamellar bodies. (*D*) hES cell-derived ATII cells showing similar lamellar bodies and other morphological characteristics as the A549 cells. (*E* and *F*) Magnified views of regions * and ** in *D*, respectively, showing clear lamellar structures. (Scale bars: 5 μm in *A* and *D* and 0.5 μm in *B*, *C*, *E*, and *F*.)

were examined for expression of CFTR and α-1AT RNA by RT-PCR. As anticipated, specific RNA transcripts of CFTR and α-1AT were observed in the hES-ATII cells and A549 cells, but not in the starting undifferentiated hES cell lines (Fig. 6). ATII cells are thought to be a major cell source of local production of complement proteins in the lung. Therefore, we also tested whether hES-ATII cells have the ability to synthesize and secrete C3 and C5, major components of the complement system. ELISA measurements of the cell culture supernatants indicated that early differentiated ATII cells (day 10) synthesized and secreted C3 at a rate of 33 $\pm$ 3 ng/mg every 24 h, which was comparable to that produced by the human ATII cell line A549 (data not shown). Similar levels of C3 were also observed on day 12 and day 15 (Fig. 6 *Upper Right*). C5 was also detected but only in the day 15 cultures (1.6 ng/mg every 24 h) (Fig. 6 *Lower Right*). The amount of C5 produced by the hES-ATII cells was similar to the quantity of C5 produced by primary cultures of rat and human ATII cells (1).

Discussion

Lung injury due to chronic pulmonary diseases, such as chronic obstructive pulmonary disease and asthma, and inherited genetic disorders, such as cystic fibrosis and α1-AT deficiency are leading causes of morbidity and mortality worldwide. Cystic fibrosis and α1-AT deficiency are two of the most common inherited genetic defects affecting Caucasians. In addition, SPB deficiency is a major cause of respiratory disease and fatality in neonates. All three of these diseases are caused by single-gene defects and therefore have been logical candidates for gene therapy. However, efficient vector delivery and sufficient transgene expression needed for therapeutic benefit have remained elusive. Recent research advances indicate that gene delivery via transplantation of cells derived from human stem cells may provide an attractive alternative to viral or liposome vector-based gene therapies. Moreover, transplantation of cells derived from human stem cells may prove ideal for the repair and regeneration of injured lung tissue.

Because of its ability to proliferate as well as to differentiate into ATI cells, the ATII cell is an excellent choice of lung cell for possible therapeutic use in gene delivery and repair of the alveolus. However, the generation of hES-ATII cells in sufficient purity and number for transplantation purposes has proven difficult. Until now, only one laboratory has reported the differentiation of ATII cells from hES cells (27). In this report multiple steps were used to derive ATII cells through EB formation. This approach required prolonged time periods to develop the endoderm from which the ATII cells are derived; however, only a scarce number of ATII cells were produced. Because the direct differentiation of hES cells into ATII cells had yet to be investigated, we examined the possibility of differentiating hES cells to ATII cells without EB formation. As

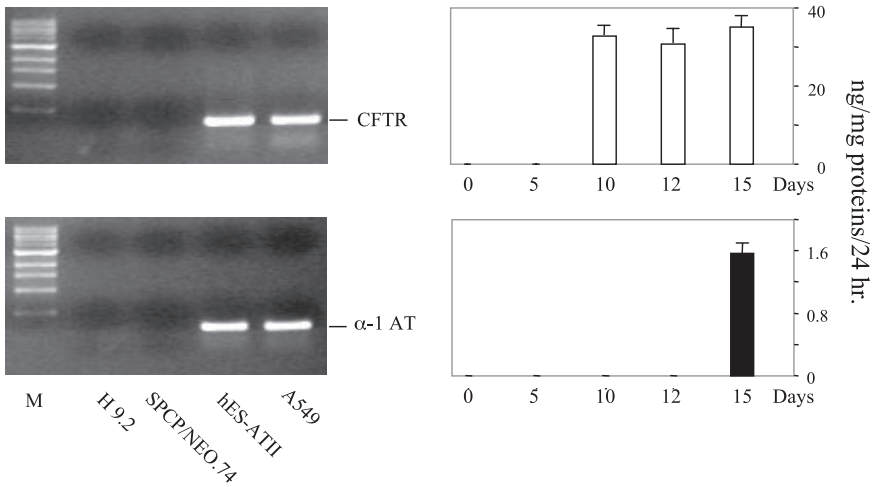

Fig. 6. Expression of CFTR, α-1AT, and the complement proteins C3 and C5 by hES-ATII cells. Total RNA isolated from hES cell-derived ATII cells was used to examine CFTR and α-1AT expression by RT-PCR as described in *Methods*. The RNA expression levels of CFTR and α-1AT in hES cell-derived ATII cells were comparable with that in A549 cells but were not detectable in the undifferentiated hES cell lines H9.2 and SPCP/NEO.74 (*Left*). C3 and C5 proteins produced by hES cell-derived ATII cells were determined by ELISA as described in *Methods*. (*Upper Right*) Bar graph depicting C3 protein levels from the hES cell-derived ATII cultures on days 10, 12, and 15. The numerical values on these days were 33 $\pm$ 3, 32 $\pm$ 3, and 35 $\pm$ 3 ng/mg total protein per 24 h, respectively. (*Lower Right*) Bar graph depicting C5 protein levels from the hES cell-derived ATII cultures. C5 protein in the hES cell-derived ATII cells was measurable in the day-15 culture (1.6 $\pm$ 0.1 ng/mg total protein per 24 h).

documented by RT-PCR, flow cytometric analysis, and immunostaining, hES cells cultured on Matrigel-coated dishes did in fact differentiate directly into ATII cells without EB formation. In addition, SPC expression indicating the presence of ATII cells in the differentiating hES cell cultures occurred 5 days sooner in the absence of EB formation. Moreover, 11.2% of differentiated cells cultured on the Matrigel-coated dishes were determined to express SPC protein on day 15 compared with 2.8% on day 33, when the EB formation approach was used (33). Collectively, these results suggest that the components of Matrigel, such as laminin and collagen IV, may not only efficiently maintain the biological characteristics of ATII cells but also encourage differentiation of hES cells to ATII cells.

The use of ES cells as a source of transplantable cells in the lung alveolus will require the generation of significant quantities of highly pure ATII cells. To achieve this goal, we chose to genetically modify hES cells so that resulting differentiated ATII cells could be enriched through antibiotic selection. Our approach was to establish a stable transfected hES cell line containing a single copy of the human SPC promoter-Neo^r fusion gene. When subjected to differentiation *in vitro*, it was hypothesized that ATII cells derived from this genetically modified hES cell line (SPCP/NEO.74) would express the Neo^r gene and would therefore survive G418 antibiotic selection, whereas, all of the other differentiated cell lineages as well as the pluripotent cells would be eliminated by G418 selection. Immunocytochemical and flow cytometric analysis of the surviving G418-selected cells supported this hypothesis, indicating that this genetic selection approach resulted in an enrichment of hES-ATII cells to >99% when cultured on Matrigel-coated plates. Our protocol reproducibly produced from each 10-cm culture dish $>10^6$ essentially pure ATII cells within 15 days of differentiation. These differentiated ATII cells survive for at least 2 days in culture in the absence of G418 and will provide in a timely manner sufficient numbers of pure ATII cells for future transplantation investigations.

Ultrastructural examination by transmission electron microscopy and Papanicolaous staining demonstrated that the hES-ATII cells are morphologically normal and exhibit typical lamellar bodies, which are a characteristic hallmark of primary ATII cells. The hES-ATII cells were shown to exhibit normal important biological functions, such as the synthesis of SPA, SPB, and SPC. Moreover, these cells expressed RNA specific for CFTR and α-1AT, suggesting that they may have therapeutic value in the treatment of patients with cystic fibrosis or α-1AT deficiency. The hES-ATII cells also synthesized and secreted complement proteins C3 and C5, which are important in inflammation and host defense in the lung. Activation of C3 and C5 produces the potent complement anaphylatoxins, C3a and C5a. Recent reports indicate that C3a and C5a have novel and important roles in tissue regeneration (34), and neurogenesis (35). These findings suggest that C3 and C5 synthesized and secreted by ATII cells are not only important in mediating pulmonary inflammation and host defense but could also play critical biological roles in alveolus regeneration and repair. In conclusion, this study provides a description of a unique single-step procedure that can be used reliably to drive the differentiation of hES cells into a highly pure population of ATII cells. These hES-ATII cells are morphologically normal, express the characteristic SPA, SPB, and SPC, CFTR and α-1AT RNA, as well as synthesize and secrete complement proteins C3 and C5, and thereby provide a practical transplantable source of ATII cells that could be used in the future to repair damaged lung alveolus as well as to treat genetic diseases that affect the lung.

Methods

Human SPC Promoter-Neomycin Vector. A 3.8-kb fragment of human genomic DNA containing the human SPC promoter and 170 bp of noncoding sequence of exon 1 (36) was cloned into the AscI site of the 3′-hprt insertion targeting vector (37) (a gift from Allan Bradley, The Wellcome Trust Sanger Institute, Cambridge, U.K.). The Neo^r cDNA-poly(A) fragment was subcloned into an engineered NdeI restriction site downstream of the SPC promoter. The resulting vector (3′-hprt-SPCP.NEO) is depicted in Fig. 1 and was linearized by EcoRI before transfection.

Transfection and Selection of hES Cells. The National Institutes of Health-approved hES cell line H9.2 (passages 45–65) (WiCell, Madison, WI) was used throughout this study. Undifferentiated hES cells were cultured on mitotically inactivated mouse embryonic fibroblasts in six-well plates with hES cell culture medium containing 80% DMEM/F12, 20% knockout serum replacement (Gibco Invitrogen, Carlsbad, CA), 1% nonessential amino acid, 1 mM L-glutamine (Chemicon, Temecula, CA), 0.1 mM 2-mercaptoethanol, and 4 ng/ml basic FGF (Gibco Invitrogen). The hES cells from one six-well plate were resuspended in 100 μl of supplemented Mouse ES Cell Nucleofector Solution, mixed with 5 μg of the linearized 3′hprt-SPCP.NEO vector, and transfected by using the cell Nucleofector II (Amaxa, Gaithersburg, MD). The hES cells were then plated on Matrigel-coated 10-cm plates with mouse embryonic fibroblast conditioned hES cell medium (MEF-CM) (38). The 3′-hprt-SPCP.NEO transfected hES cells were selected in the presence of 0.25 μg/ml puromycin (Sigma, St. Louis, MO) for 14 days. Surviving hES clones were examined for the Neo^r gene by PCR analysis, and a positive clone (SPCP/Neo.74) containing a single copy of the transgene was selected for further analysis.

***In Vitro* Differentiation and Selection of hES Cell-Derived ATII Cells.** To induce spontaneous differentiation via EB formation, collagenase IV dissociated hES cells were plated on six-well ultra-low-attachment plates in hES cell medium. On day 2, the resultant EBs were collected, washed, and cultured on fresh six-well ultra-low-attachment plates with DM, composed of 80% knockout DMEM (Gibco Invitrogen), 20% FBS, 1% nonessential amino acid, 1 mM L-glutamine, 100 μg/ml penicillin, and 100 μg/ml streptomycin. On day 6, the EBs were collected and seeded on gelatin-coated six-well culture plates in DM (15 EBs per well) and allowed to expand. Selection of hES cell-derived ATII cells was started on day 6 by adding 20 μg/ml G418 (Gibco). To promote the differentiation without EB formation, the collagenase IV dissociated hES cells were seeded on Matrigel-coated six-well plates with MEF-CM (day 0). On day 1, the medium was replaced by DM with or without G418 (20 μg/ml).

RT-PCR. Total RNA was isolated from the hES cultures by using RNA Bee (Tel-Test, Friendswood, TX) following the manufacturer's protocol. The following primer pairs were used in the RT-PCRs, employing 0.5 μg of total RNA and the OneStep RT-PCR kit (Qiagen, Valencia, CA): (*i*) SPC forward (5′-TGG TCC TCA TCG TCG TGG TGA TTG-3′) and SPC reverse (5′-CCT GCA GAG AGC ATT CCA TCT GGA AG-3′), (*ii*) CFTR forward (5′-GGA GGG ATT TGG GGA ATT ATT TGA GAA AGC-3′) and CFTR reverse (5′-CTA TAT TCA TCA TAG GAA ACA CCA AAG ATG-3′), (*iii*) α1-AT forward (5′-TGA CAC TCA CGA TGA AAT CCT GGA G-3′) and α1-AT reverse (5′-CCT TGA GTA CCC TTC TCC ACG TAA TC-3′), and (*iv*) 18S forward (5′-TAA CGA ACG AGA CTC TGG CAT-3′) and 18S reverse (5′-CGG ACA TCT AAG GGC ATC ACA G-3′).

Immunofluorescence and Flow Cytometry. Differentiated hES cells were dissociated into single-cell suspensions by incubation with 0.25% trypsin for 2 min. The dissociated cells were resuspended (0.3×10^6 cells) in 250 μl of fixation/permeabilization solution (Cytofix/Cytoperm kit; BD Biosciences, Franklin Lakes, NJ),

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kept on ice for 20 min, and washed twice with Perm/Wash buffer. After blocking with 10% goat serum in 300 μl Perm/Wash buffer for 45 min on ice, the cells were incubated with rabbit anti-human SPC antibody (1:200 dilution; Chemicon) in the block solution for 45 min on ice. The cells were resuspended in 350 μl of Perm/Wash buffer after incubation with goat anti-rabbit IgG conjugated by R-Phycoerythrin (Sigma, St. Louis, MO) for 45 min on ice, washed twice, and analyzed by flow cytometry. For immunofluorescent staining, the differentiated hES cells, with or without G418 selection, were dissociated and seeded on polyD-lysine-coated coverslips, cultured for 24 h, and stained with the rabbit anti-human SPC antibody following the manufacturer's directions. The SPC-positive cells were visualized with Alexa Fluor 546 conjugated goat anti-rabbit IgG (1:1,000; Molecular Probes, Eugene, OR) with DAPI counterstaining. The number of SPC-positive cells was counted per 500 cells based on the DAPI staining on each slide. This procedure was also used for immunostaining of SPA and SPB using rabbit anti-human SPA and anti-human proSPB (1:1,000; Chemicon).

Electron Microscopy. The G418-selected hES cell-derived ATII cells and A549 cells were trypsinized and fixed (2 h) in suspension with 0.1 M sodium cacodylate buffer containing 2.5% glutaraldehyde and then postfixed in 1% tannic acid (5 min) followed by 1% osmium tetroxide (1 h) and then aqueous uranyl acetate (1 h). Samples were subsequently dehydrated in a graded ethanol series and embedded in Araldite resin, and ultrathin serial sections (100 nm) were obtained by using an ultramicrotome (RMC 7000; RMC, Tucson, AZ) equipped with a diamond knife. Sections were stained with uranyl acetate and lead citrate before photographing with a JEOL 200CX electron microscope.

ELISA Analysis. Cultures (4, 9, 11, or 14 days) of differentiating hES cell-derived ATII cells and A549 cells were switched to DMEM with 15% FBS and incubated for 24 h, and 100 μl samples of each culture were added to 96-well plates that had been coated with either anti-human C3c or anti-human C5 antibodies (2 μg/ml; Quidel, La Jolla, CA). After incubation at room temperature for 2 h, the plates were exhaustively washed, incubated for 2 h with the primary goat anti-human C3 and anti-human C5 antibodies (Complement Technology, Tyler, TX), washed, and incubated for 1 h with alkaline phosphatase-conjugated rabbit anti-goat IgG (Sigma). Samples were developed by using the Alkaline Phosphatase Yellow Liquid Substrate System (Sigma). Results were normalized based on total cell protein content by lysis of the cells with 50 mM Tris·HCl (pH 7.5), 150 mM NaCl, 1.25 mM protease inhibitor (Sigma), and 0.2% SDS. Protein concentrations were determined by using a protein assay kit (catalog no. 500-0111; Bio-Rad, Hercules, CA).

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