Isolation and Characterization of Alveolar Epithelial Type II Cells Derived from Mouse Embryonic Stem Cells

Huanhuan Sun, MD, PhD,^{1,*} Yuan Quan, MD, PhD,^{1,*} Qing Yan, PhD,¹ Xinmiao Peng, PhD,²
Zhengmei Mao, MD, PhD,³ Rick A. Wetsel, PhD,¹ and Dachun Wang, MD¹

The use of embryonic stem cells (ESCs) to regenerate distal lung epithelia damaged by injuries or diseases requires development of safe and efficient methodologies that direct ESC differentiation into transplantable distal lung epithelial progenitors. Time-consuming culture procedure and low differentiation efficiency are major problems that are associated with conventional differentiation approaches via embryoid body formation. The use of a growth factor cocktail or a lung-specific cell-conditioned medium to enrich definitive endoderm for efficient differentiation of mouse ESCs (mESC) into alveolar epithelial progenitor type II cells (ATIICs) has been reported, but not yet successful for generating a homogenous population of ATIICs for tissue regeneration purpose, and it remains unclear whether or not those mESC-derived ATIICs possess normal biological functions. Here, we report a novel method using a genetically modified mESC line harboring an ATIIC-specific neomycin^R transgene in Rosa 26 locus. We showed that ATIICs can be efficiently differentiated from mESCs as early as day 7 by culturing them directly on Matrigel-coated plates in DMEM containing 15% knockout serum replacement. With this culture condition, the genetically modified mESCs can be selectively differentiated into a homogenous population (>99%) of ATIICs. Importantly, the mESC-derived ATIICs (mESC-ATIICs) exhibited typical lamellar bodies and expressed surfactant protein A, B, and C as normal control ATIICs. When cultured with an air-liquid-interface culture system in Small Airway Epithelial Cell Growth Medium, the mESC-ATIICs can be induced to secrete surfactant proteins after being treated with dibutyryl cAMP + dexamethasone. These mESC-ATIICs can synthesize and secrete surfactant lipid in response to secretagogue, demonstrating active surfactant metabolism in mESC-ATIICs as that seen in normal control ATIICs. In addition, we demonstrated that the selected mESC-ATIICs can be maintained on Matrigel-coated plates for at least 4 days with robust proliferative capacity. When cultured in DMEM medium containing 10% FBS, mESC-ATIICs spontaneously differentiated into alveolar epithelial type I cells. Collectively, these data demonstrate that the genetically modified mESCs can be selectively differentiated into a homogenous population of functional ATIICs, providing a reliable cell source to explore their therapeutic potential in lung tissue regeneration.

Introduction

ALVEOLAR EPITHELIAL PROGENITOR type II cells (ATIICs) are critical for maintaining the homeostasis of alveoli, which are constantly exposed to injurious stimuli from the environment. Failure to repair injured alveoli has been implicated in the development of idiopathic pulmonary fibrosis,¹ which is associated with high morbidity and mortality. In addition, many types of severe pulmonary diseases can be caused by deficiencies in proteins that are synthesized by ATIICs. For example, complete deficiency of surfactant

protein B (SPB) results in impaired pulmonary surfactant composition and function, causing fatal neonatal respiratory distress syndrome.^{2,3} Surfactant metabolism disorder due to complete surfactant protein C (SPC) deficiency is the major cause of progressive familial and sporadic interstitial lung disease.⁴ No established therapies are effective in preventing the progression of these pulmonary diseases. For most patients, the only therapeutic option is lung transplantation. Therefore, novel therapeutic options are in urgent need. One promising option is to use embryonic stem cell (ESC)-derived ATIICs to regenerate damaged or diseased alveoli.

¹Research Center for Immunology and Autoimmune Diseases, The Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas Medical School at Houston, Houston, Texas.

²Department of Neuroscience, Baylor College of Medicine, Houston, Texas.

³Microscopy Service Center, The Brown Foundation Institute of Molecular Medicine for the Prevention of Human Diseases, University of Texas Medical School at Houston, Texas.

*These authors contributed equally to the work.

TABLE 1. RELATIVE ATIIC CONTENT IN THE DIFFERENTIATING CULTURES OF mESCs

	SPC ⁺ /SPC ⁻ (% , #) day 7	SPC ⁺ /SPC ⁻ (% , #) day 9	SPC ⁺ /SPC ⁻ (% , #) day 11
mES -G418	166/834 (14.6%, —)	182/818 (16.2%, —)	204/796 (18.4%, —)
mESC-32 -G418	158/842 (13.8%, —)	190/810 (15.4%, —)	220/780 (19.1%, —)
mESC-32 + G418	992/8 (99.2%, 2.3×10^5)	994/6 (99.4%, 4.8×10^5)	994/6 (99.4%, 1.4×10^6)

SPC⁺, mES cell-derived ATIICs; %, percentage of SPC-positive cells; #, the number of SPC⁺ cells in one 6-cm plate; mESC, mouse embryonic stem cell; ATIIC, alveolar epithelial progenitor type II cell; SPC, surfactant protein C.

Recently published data have demonstrated that ESCs can be differentiated into ATIICs via embryonic body (EB) formation^{5,6} or co-culture of EBs with pulmonary mesenchyme.⁷ However, these procedures are not efficient, generating only a very small percentage of ESC-derived ATIICs.⁸ Strategies using a growth factor cocktail or a lung-specific cell-conditioned medium^{9,10} to enrich definitive endoderm for efficient differentiation of ESCs into ATIICs have been developed, but not yet successful for generating a homogenous population of ATIICs. A mixed population of cell derivatives in the differentiated cultures of ESCs is not suitable for lung tissue regeneration. The remaining pluripotent cells in the differentiating cultures carry a significant risk of producing teratomas after transplantation *in vivo*. Hence, a major prerequisite for using ESCs therapeutically is to achieve a pure population of ESC-derived cells. Recently, an essentially pure population of ATIICs has been derived from genetically modified human ESCs (hESCs).¹¹ However, random genetic insertions may limit their therapeutic application. In addition, poor long-term retention/survival of engrafted hESC-derived ATIICs in injured lungs of SCID mice¹² indicates that the mouse lung may reject hESC-derived ATIICs or not provide an appropriate microenvironment for hESC-derived ATIICs to rebuild the progenitor cell pools after injury. Therefore, mouse ESCs (mESCs) should be used to explore the possible long-term therapeutic benefit provided by ESC-derived ATIICs in mouse injury models.

In this study, we generated a mESC line harboring an ATIIC-specific neomycin^R (NEO^R) transgene in Rosa 26 gene locus to avoid possible genetic mutations caused by random vector insertions. With the genetically modified mESCs, we developed a reliable differentiation procedure to efficiently generate a homogenous population of ATIICs and characterized their biological functions *in vitro*.

Materials and Methods

Construction of 3'hprr.mSPCP-NEO^R.Rosa26 targeting vector

One 4.8 kb mouse SPC promoter (mSPCP) fragment was digested with *AscI* and *BssHII*, and cloned into *AscI* site of the 3'hprr insertion targeting vector (a gift from Dr. Allan Bradley, The Wellcome Trust Sanger Institute, Cambridge, United Kingdom). The NEO^R cDNA-poly(A) fragment digested with *AseI* and *EcoRI* was then added into the engineered *NdeI* and *EcoRI* site downstream of mSPCP. To knock-in the mSPCP-NEO^R transgene into Rosa 26 gene locus, one 5 kb DNA fragment homologous to Rosa 26 gene was subsequently cut with *MluI* and *EcoRI*, and cloned into *AscI* and *EcoRI* site of the targeting vector. The resulting

3'hprr.mSPCP-NEO^R.Rosa 26 targeting vector (Fig. 1) was then linearized by *NdeI* before transfection.

Transfection and culture of mESCs

The mESCs with C57BL/6 genetic background were routinely maintained in mitomycin-treated mouse embryonic fibroblast (MEF) feeder cells on six-well plates using mESC culture medium. The medium contains 85% DMEM (Millipore), 15% FBS (HyClone), 10 mM HEPES, 1 mM sodium pyruvate, 1% nonessential amino acid, 2 mM L-glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin (Millipore), 1.52×10^4 M 1-thioglycerol (Sigma-Aldrich), and 1000 U/mL leukemia inhibitory factor (LIF; Millipore). Approximately 1×10^6 mESCs were re-suspended in 100 µL of supplemented mESC Nucleofactor Solution (VAPH-1001; Lonza), mixed with 5 µg of the linearized targeting vector, and then transfected by using Nucleofactor II as previously described.^{11,12} The transfected cells were then placed on gelatin-coated 10-cm plates in mESC culture medium. Puromycin (0.5 µg/mL; Sigma) was added the next day to select stably transfected mESCs. The transfected mESC colonies that had survived puromycin selection were picked up 6 days after transfection, and then maintained in mESC culture medium for the next study.

PCR and Southern blot analysis of site-specifically targeted mESC clones

PCR was performed using primer A and B (all primers used in the study were listed in Table 2) to screen Rosa 26-targeted mESC clones, which was carried out at 94°C for 3 min followed by 30 cycles at 94°C for 30 s, 60°C for 30 s, and 72°C for 1.5 min using AmpliTaq Gold DNA polymerase (AB Applied Biosystems). For Southern blot analysis, genomic DNA samples isolated from Rosa 26-targeted mESC clones (32, 33, and 34) were digested with *BamHI*, *HindIII*, and *NheI*, respectively, for Southern blot hybridization using a 425 bp NEO^R gene fragment as a probe.

In vitro derivation and culture of mESC-derived ATIICs

To spontaneously differentiate mESCs into ATIICs, the dissociated mESCs were cultured on Matrigel-coated or Collagen IV-coated six-well plates at a density of 2×10^4 cells/cm² in hESC differentiation medium (hDM),¹¹ LIF-free mESC culture medium (mESC differentiation medium [mDM]), or SR medium. The SR medium contains 85% DMEM, 15% KnockOutTM serum replacement (Gibco), 10 mM HEPES, 1 mM sodium pyruvate, 1% nonessential amino acid, 2 mM L-glutamine, 100 µg/mL penicillin, 100 µg/mL streptomycin, and 1.52×10^4 M 1-thioglycerol. From day 6, the differentiating cultures were either

TABLE 2. PRIMERS USED IN THE STUDY FOR PCR, RT-PCR, OR QRT-PCR

Primer A	5'-CTG ATC ATG TGC TAG GCC TAC CTT AGG-3'
Primer B	5'-CTT CTG AGG CGG AAA GAA CCA GCT G-3'
SPC forward primer	5'-GCA GGT CCC AGG AGC CAG TTC-3'
SPC reverse primer	5'-GGA GCT GGC TTA TAG GCC GTC AG-3'
AQP5 forward primer	5'-CTT TGG CCT GGC CAT AGG TAC CT-3'
AQP5 reverse primer	5'-CCA GAG TAA TGG CCG GAT TGA TGT G-3'
AQP5 probe	5'-6-FAM-CCC AAG CCC TGG GAC CTG TGA GT-TAMRA-3'
T1 α forward primer	5'-GCA CCT CTG GTA CCA ACG CAG A-3'
T1 α reverse primer	5'-TCT GAG GTT GCT GAG GTG GAC AGT-3'
T1 α probe	5'-6-FAM-CTC TAA GGG AGG CTT CGT CCC ACG CTC T-TAMRA-3'
SPC forward primer II	5'-AAG CCC TCC ACA CCC ACC TCT A-3'
SPC reverse primer II	5'-CTC TCC CGG AAG AAT CGG ACT CG-3'
SPC probe	5'-6-FAM-ACC AGT ATC ATG CCC TTC CTC CTG GCC CAG-TAMRA-3'
18S forward primer	5'-TAA CGA ACG AGA CTCTGG CAT-3'
18S reverse primer	5'-CGG ACA TCT AAG GGC ATC ACA G-3'
18S probe	5'-6-FAM-TGG CTG AAC GCC ACT TGT CCC TCT AA-TAMRA-3'

maintained in the original medium (hDM, mDM or SR medium) or transferred into a serum-free medium¹⁰ for an additional 2, 4, or 6 days (Fig. 3A). The serum-free medium contained 75% Iscove's-modified Dulbecco's medium, 25% Ham's F12 medium (Gibco), 0.05% BSA, 2 mM glutamine, 0.5 mM ascorbic acid (Sigma-Aldrich), 4.5×10^4 M 1-thioglycerol, 50 U/mL penicillin, 50 mg/mL streptomycin, 50 mg/mL heparin sulfate salt (porcine intestinal mucosal; Sigma-Aldrich), $0.5 \times$ of both N2 and B27 (without retinoic acid) supplements (Invitrogen), and 50 ng/mL FGF2 (Sigma-Aldrich). To select the mESC-derived ATIICs (mESC-ATIICs), the mESC-32 cells were cultured on Matrigel-coated 6-cm plates in SR medium in the presence of G418 (25 μ g/mL; GIBCO) for 7, 9, or 11 days. For spontaneous differentiation of mESC-ATIICs into the alveolar epithelial type I cells (ATICs), G418-selected mESC-ATIICs from day 11 as well as isolated mouse primary ATIICs (mATIICs) were cultured in six-well plates with DMEM containing 10% FBS for 8 days.¹² The mATIICs were isolated by using the procedure described by Corti *et al.*¹³ To examine the capacity of mESC-ATIICs to proliferate *in vitro*, G418-selected mESC-ATIICs were cultured on Matrigel-coated six-well plates (2.0×10^4 /well) in MEF-conditioned SR medium with or without 30 ng/mL of Recombinant keratinocyte growth factor (rhKGF; R&D Systems) for 4 days.¹²

RT-PCR and QRT-PCR analysis

To evaluate the differentiation of mESCs into ATIICs, the total RNA samples were isolated from the differentiating cultures of mESCs on days 7, 9, and 11 by using RNA Bee (Tel-Test). RT-PCR was performed to analyze the expression of ATIIC-specific SPC with SPC forward primer and reverse primer by using OneStep RT-PCR Kit (Qiagen). To demonstrate the ability of mESC-ATIICs to differentiate into ATICs, the total RNA samples were isolated from the differentiating cultures of mESC-ATIICs and mATIICs on days 0, 2, 4, 6, and 8 to analyze expression levels of Aquaporin-5 (AQP5), Podoplanin (T1 α) and SPC. QRT-PCR was carried out by using TaqMan One-Step RT-PCR Master Mix Kit (AB Applied Biosystems)¹² with the following primer pairs and probes: (i) AQP5 forward primer, AQP5 reverse primer, and AQP5 probe; (ii) T1 α forward primer, T1 α reverse primer,

and T1 α probe; (iii) SPC forward primer II, SPC reverse primer II, and SPC probe; and (iv) 18S forward primer, 18S reverse primer, and 18S probe.

Immunofluorescent staining

The G418-selected mESC-ATIICs were dissociated on days 7, 9, or 11, and seeded on poly-D-lysine-coated cover slips. After being cultured in SR medium overnight, the cells were washed with PBS and fixed in 4% paraformaldehyde at room temperature for 10 min. Cells were then permeabilized in 0.5% Triton X for 30 min. After being blocked in 5% goat serum for 2 h, mESC-ATIICs were stained with 1:200 diluted rabbit anti-human proSPC, 1:500 diluted rabbit anti-human proSPB, or 1:500 diluted rabbit anti-human SPA antibody (Chemicon) as previously described.¹¹ The surfactant protein expression was visualized with Alexa Fluor 488 conjugated goat anti-rabbit IgG (1:1000; Molecular Probes) with DraQ5 (1:500; Biostatus) counterstaining.

Electron microscopy

The ultra-structural examination of G418-selected mESC-ATIICs was performed by Electron Microscopy Laboratory, Department of Pathology, University of Texas Medical School at Houston.

Secretion of surfactant from cultured mESC-ATIICs

G418-selected mESC-ATIICs on day 11 were trypsinized and then seeded back onto fresh Matrigel-coated 10-cm culture plates with SR medium containing ^3H -choline (1 mCi/plate; PerkinElmer) for 24 h. Cells were washed with PBS and cultured in fresh SR medium with or without secretagogue (TPA, 50 ng/mL; Sigma-Aldrich) for 2 h at 37°C.¹⁴ The medium was collected and combined with rinses from cell wash. The ^3H -labeled phosphatidylcholine (PC) in the medium and cells were extracted and counted, respectively, as reported.¹⁵ To examine surfactant protein secretion, the mESC-ATIICs were trypsinized on day 6 and then cultured by using air-liquid-interface culture system in Small Airway Epithelial Cell Growth Medium (SAGM™; Chemicon Millipore) containing G418, with or without dibutyryl cAMP (Bt₂cAMP, 1 mM) and dexamethasone (Dex, 10^{-10} M)

for 5 days.¹⁶ The proteins were harvested from culture medium and analyzed by Western blot with mouse monoclonal antibody against mature SPB (abcam) and rabbit anti-mature-SPC (Seven Hills Bioreagents) by using the previously described procedure.¹⁷

Statistical analysis

The data shown in Figure 5B and C were analyzed with Student's *t*-test (two tailed) using Microsoft Excel software. Results with *p*-values less than 0.05 (alpha level=0.05) were considered statistically significant.

Results

Site-specific modification of mESCs

To generate site-specifically modified mESC lines, the targeting vector, 3'hprr.mSPCP-NEO^R.Rosa26, was generated as depicted in Figure 1. The *NdeI* was used to linearize the vector before transfection. Of $\sim 1.0 \times 10^6$ mESCs subjected to the transfection, 312 survived the puromycin selection. PCR using primer A and B (Fig. 1) was performed to identify Rosa 26-

targeted mESC clones. Since the primer A is located within Rosa 26 gene region upstream of *NdeI* site and the primer B in the NEO^R gene downstream of the *NdeI* site in the vector, the PCR should only detect the Rosa 26-targeted transgene, but not those randomly inserted transgene fragments. Of 312 stably transfected clones, 29 were found to be Rosa 26-targeted mESC clones. As shown in Figure 2A, the stably transfected mESC clones 32, 33, 34, and 39 contain one 1.35 kb targeted transgene fragment, indicating a site-specific targeting at Rosa 26 gene locus. To select random vector-integration-free mESC clones, Rosa 26-targeted mESC clones 32, 33, and 34 were analyzed by Southern blot hybridization using a 425 bp NEO^R gene fragment as a probe. The probe can recognize a 6.4 kb-targeted *Bam*HI fragment, a 7.2 kb-targeted *Hind*III fragment (Fig. 1), and a randomly inserted targeting vector. Southern blot analysis demonstrated that all of the three Rosa 26-targeted mESC clones contain the targeted *Bam*HI fragment, *Hind*III fragment, and *NheI* fragment (Fig. 2B). No randomly inserted vector DNA was detected in mESC clone 32 (mESC-32), demonstrating only a single copy of the inserted transgene in Rosa 26 gene locus. Therefore, the mESC-32 was selected for the next study.

FIG. 1. 3'hprr.mSPCP-NEO^R.Rosa26 targeting vector. Schematic diagram of 3'hprr.mSPCP-NEO^R.Rosa26 targeting vector (showing relevant positional information, not scaled proportionally based on sequence lengths). The NEO^R transgene controlled by mATIIC-specific SPC promoter (mSPCP-NEO^R) was cloned into 3'hprr targeting vector backbone, containing the puromycin^R gene (PURO), the K14Agouti transgene (Ag), and an LoxP site (arrow). One 5.0 kb DNA fragment homologous to Rosa 26 gene was included in the vector for site-specific targeting by homologous recombination. To generate Rosa 26-targeted mESCs, *NdeI* was used to linearize the vector before transfection of mESCs. The primer A is located within Rosa 26 gene region upstream of the *NdeI* site and the primer B within the NEO^R gene downstream of the *NdeI* site in the targeting vector. Thus, PCR can be performed by using primers A and B to identify Rosa 26-targeted mESC clones. mESC, mouse embryonic stem cell; ATIIC, alveolar epithelial progenitor type II cell; mATIIC, mouse primary ATIIC; NEO^R, neomycin^R; SPC, surfactant protein C.

FIG. 2. Identification of Rosa 26-targeted mESC clones. **(A)** PCR analysis using primer A and B was performed to screen Rosa 26-targeted mESC clones and demonstrated a 1.35 kb Rosa 26-targeted transgene fragment in mESC clones 32, 33, 34, and 39. **(B)** Southern blot analysis of integration sites of targeting vector. A 425 bp NEO^R gene DNA fragment was used as a hybridization probe to analyze *Bam*HI-, *Hind*III-, and *Nhe*I-digested genomic DNA samples of Rosa 26-targeted mESC clones (32, 33, and 34). The probe can recognize a 6.4 kb-targeted *Bam*HI fragment, a 7.2 kb-targeted *Hind*III fragment, a 5.7 kb-targeted *Nhe*I fragment, and a randomly inserted targeting vector (Fig. 1). Southern blot demonstrated that mESC clones 32, 33, and 34 contain the 6.4 kb-targeted *Bam*HI fragment, the 7.2 kb-targeted *Hind*III fragment, and the 5.7 kb-targeted *Nhe*I fragment, indicating a site-specific targeting. Although the randomly inserted targeting vector was also demonstrated in mESC clones 33 and 34, the result showed that mESC clone 32 only has a single copy of targeting vector in Rosa 26 gene locus.

Derivation and purification of mESC-ATIICs

To establish an optimal differentiation procedure for direct derivation of mESCs into ATIICs, mESCs were dissociated and seeded on either Matrigel-coated or Collagen IV-coated six-well plates in hDM, mDM, or SR medium for the first 5 days. The differentiating cultures were then either maintained in the same differentiation medium or switched to the serum-free medium supplemented with 50 ng/mL FGF2 for an additional 2, 4, or 6 days (Fig. 3A). The differentiation of mESCs into ATIICs was evaluated by RT-PCR to analyze SPC expression in the differentiating cultures of mESCs. The expression of SPC RNA was detected in the differentiating cultures of mESCs in the Collagen IV-coated plates only when mDM was used for the first 5 days' differentiation (Fig. 3B). In comparison, significantly higher expression levels of SPC RNA were detected in the differentiating mESC cultures in Matrigel-coated plates as early as on day 7 when either SR medium or mDM was used to initiate the differentiation during the first 5 days (Fig. 3B). Supplements with 50 ng/mL FGF2 significantly enhanced the SPC expression in the differentiating cultures in Matrigel-coated plates on days 7 and 9 when mDM was used in the first 5 days. However, the expression level of SPC RNA declined dramatically on day 11. The FGF2-induced SPC expression was not observed in the differentiating cultures of mESCs in Matrigel-coated plates when SR medium was used for the first 5 days' differentiation. Interestingly, a high expression level of SPC in

the differentiating mESC cultures in Matrigel-coated plates was maintained over time in SR medium without a supplement of FGF2 (Fig. 3B). Therefore, the differentiation procedure by culturing mESCs on Matrigel-coated plates in SR medium was used for the next study. Approximately 13.8% of cells in differentiating cultures of mESC-32 spontaneously differentiated into ATIICs on day 7, which was demonstrated by immunostaining for ATIIC-specific SPC expression (Table 1). The SPC-positive cells in the differentiating cultures increased to 15.4% on day 9 and 19.1% on day 11. The percentages of SPC-positive cells in the differentiating cultures of mESC-32 were comparable to those in the differentiating cultures of control mESCs. To isolate mESC-ATIICs, mESC-32 cells were subjected to spontaneous differentiation in the presence of 25 μ g/mL of G418 for 7, 9, or 11 days. Since the SPC protein is specifically expressed by ATIICs, NEO^R should be only expressed when mESC-32 cells are differentiated into ATIICs. Therefore, only ATIICs derived from mESC-32 can survive G418-selection. Immunostaining for SPC expression was carried out to determine the purity of selected mESC-ATIICs in the differentiating cultures. The number of SPC-positive cells was counted per 1000 cells based on DAPI staining on each plate. The results showed that more than 99% of cells were SPC positive in the differentiating cultures as early as on day 7 (Table 1), indicating that the G418 selection had efficiently eliminated the SPC-negative cell types derived from mESC-32 cells in the cultures. A similar percentage of SPC-positive cells was also present in the differentiating cultures on days 9 and 11. Interestingly, the number of SPC-positive cells in the G418-selected differentiating cultures significantly increased from 2.3×10^5 /plate on day 7 to 4.8×10^5 /plate on day 9 and to 1.4×10^6 /plate on day 11 (Table 1), suggesting that G418-selected mESC-ATIICs continued to proliferate during the differentiation time period from day 7 to day 11.

Lamellar bodies and surfactant protein expression in mESC-ATIICs

ATIICs synthesize and secrete pulmonary surfactant, which are essential for maintaining homeostasis of alveoli. The surfactant lipids and proteins are stored in lamellar bodies, which are characteristic inclusion organelles in ATIICs. To examine whether these ATIIC-specific secretory organelles were present in the mESC-ATIICs, the ultrastructure of mESC-ATIICs was analyzed by transmission electron microscopy. The mESC-ATIICs exhibited typical lamellar bodies containing tightly packed multi-lamellar lipid membranes as observed in mATIICs (Fig. 4B). In addition, immunostaining was performed to examine the surfactant protein expression in mESC-ATIICs. As expected, the G418-selected mESC-ATIICs expressed surfactant proteins A, B, and C on day 7, 9, and 11 as mATIICs (Fig. 4A).

In vitro proliferation and differentiation of mESC-ATIICs

One of the important biological functions of ATIICs is to serve as progenitor cells for repairing injured alveoli in response to injury. It is critical for developing cell-based therapies by using mESC-ATIICs to check whether the mESC-derived ATIICs possess the progenitor capacity. To demonstrate their ability to differentiate into ATIICs, the

FIG. 3. *In vitro* differentiation of mESCs into ATIICs. **(A)** Diagram of differentiation protocols. Dissociated mESCs were cultured in either Matrigel-coated (Gel) or Collagen IV-coated (C4) plates in hDM, mDM, or SR medium for the first 5 days. From day 6, the differentiating cultures were either maintained in the original medium (hDM*, mDM*, or SR* procedure) or transferred into a serum-free medium supplemented with FGF2 (hDM-Sf*, mDM-Sf*, or SR-Sf* procedure) for an additional 2, 4, or 6 days. **(B)** Expression of SPC RNA in differentiating cultures of mESCs. Total RNA samples were isolated from differentiating cultures of mESCs on days 7, 9, and 11, respectively, for analysis of SPC expression by RT-PCR. The 18S expression in each sample was shown in the lower blot of each panel as a control to demonstrate that changes in the amount of SPC-specific RT-PCR product was due to corresponding changes in SPC RNA expression. hDM, hESC differentiation medium; mDM, mESC differentiation medium.

mESC-ATIICs were subjected to spontaneous differentiation in DMEM containing 10% FBS for approximately 8 days. Total RNA was isolated from the differentiating cultures on days 0, 2, 4, 6, and 8 to analyze the expression levels of AQP5, T1 α , and SPC. QRT-PCR demonstrated that the expression levels of AQP5 and T1 α , the ATIC-specific markers,^{18–22} in the differentiating cultures of mESC-ATIICs significantly increased over time as those observed in the differentiating cultures of mATIICs (Fig. 5A). In support of the increased expression of the ATIC-specific markers, AQP5

and T1 α , the expression levels of ATIIC-specific SPC dramatically decreased over time in the differentiating cultures (Fig. 5A). These data demonstrated the ability of mESC-ATIICs to spontaneously differentiate into ATIICs *in vitro*. To examine their proliferative capacity, the G418-selected mESC-ATIICs were dissociated on day 11 and seeded back to Matrigel-coated six-well plates in MEF-conditioned SR medium. The rhKGF2, an ATIIC growth factor,²³ was added into some cultures to test whether rhKGF2 could enhance the proliferation of mESC-ATIICs. Many colonies were

FIG. 4. Surfactant protein expression and lamellar body formation in mESC-ATIICs. **(A)** The G418-selected mESC-ATIICs on days 7, 9, and 11, as well as control mATIICs, were immunostained by rabbit anti-human SPA, proSPB, and proSPC antibodies (Green) with Draq5 counterstaining (Scale bar = 25 μ m). **(B)** Transmission electron micrographs showed well-developed lamellar bodies with normal concentric, tightly packed multi-lamellar lipid membranes in selected mESC-ATIICs on day 11 (**b**, at 4000 magnification) as observed in normal mATIICs (**a**, at 3000 magnification). (**c**) Magnified view (25,000 magnification) of * region in (**a**). (**d**) Magnified view (25,000 magnification) of ** region in (**b**). SPB, surfactant protein B.

FIG. 5. Biological functions of mESC-derived ATIICs. **(A)** QRT-PCR was performed to analyze the expression levels of ATIIC markers, AQP5 and T1 α , as well as the expression levels of ATIIC-specific SPC in the differentiating cultures of mESC-ATIICs on days 0, 2, 4, 6, and 8 ($n=6$). **(B)** To examine the proliferation capacity of mESC-ATIICs, G418-selected mESC-ATIICs from day 11 were cultured on Matrigel-coated six-well plates in MEF-conditioned SR medium, with or without 30 ng/mL of rhKGF, for 4 days. The number of cells/well was counted on days 2 and 4 ($n=6$). $^{##}p<0.01$, $^{#}p<0.05$ versus the groups on day 0. $^{**}p<0.01$ versus the cultures in the absence of rhKGF. **(C)** Surfactant lipid secretion from cultured mESC-ATIICs in response to secretagogue ($n=6$). Secretion of ^3H -labeled PC was shown as percent of total ^3H -labeled PC. $^{**}p<0.001$ versus the cultures in the absence of secretagogue. **(D)** Surfactant protein secretion from cultured mESC-ATIICs in response to the treatment of Bt $_2$ cAMP+Dex. Media collected from cultured mESC-ATIICs and mATIICs were analyzed by immunoblotting for the secretion of SPB and SPC. AQP5, Aquaporin-5; ATIIC, alveolar epithelial type I cell; Dex, dexamethasone; MEF, mouse embryonic fibroblast; PC, phosphatidylcholine.

observed, and the number of mESC-ATIICs significantly increased from 2.0×10^4 /well on day 0 to 3.5×10^4 /well on day 2 and 4.9×10^4 /well on day 4 in the cultures (Fig. 5B), suggesting that mESC-ATIICs proliferated *in vitro*. As expected, addition of rhKGF2 in the cultures promoted the proliferation of mESC-ATIICs, leading to a significantly greater number of cells (6.2×10^4 /well on day 2 and 9.5×10^4 /well on day 4) in the cultures compared with those in the culture in the absence of rhKGF2. Taken together, these data have demonstrated that G418-selected mESC-ATIICs cells possess the progenitor capacity of mATIICs.

Surfactant secretion from cultured mESC-ATIICs

It has been reported that cultured ATIICs recapitulate the expression and secretion of surfactant in a manner found in lungs.¹⁶ To examine whether the mESC-ATIICs have acquired the ability, the cultured mESC-ATIICs were labeled with ^3H -choline to examine the surfactant lipid secretion in response to secretagogue. As seen in the cultures of mATIICs, the secretion of the ^3H -labeled PC (as a percent of total ^3H -labeled PC)

from cultured mESC-ATIICs significantly increased in the presence of secretagogue (Fig. 5C). To further examine whether the expression and secretion of surfactant proteins from mESC-ATIICs can be regulated, the mESC-ATIICs were cultured with air-liquid-interface culture system in SAGMTM with or without Bt $_2$ cAMP and Dex for 5 days as described in the "Materials and methods" section. The protein samples harvested from culture medium were analyzed by Western blot. We found that control mATIICs and mESC-ATIICs synthesized and secreted SPC and SPB at very similar levels (Fig. 5D), whereas the secretion of SPC and SPB significantly increased in response to the treatment of Bt $_2$ cAMP and Dex. These data showed that the mESC-ATIICs synthesized and secreted surfactant in a regulated fashion, demonstrating active surfactant metabolism pathways in the mESC-ATIICs.

Discussion

Due to their numerous crucial physiological functions, ATIICs are a key target for developing therapeutic strategies to restore normal alveolar architecture and function that has

been damaged by injury or disease. However, how to target ATIICs for correcting a mutated gene or expressing a therapeutic gene has remained elusive. Although bone marrow-derived stem cells (BMSCs) are being used to treat human heart failure and myocardial infarction,²⁴ recent investigations have demonstrated that either rare or no engraftment of BMSC-differentiated cells within injured lung epithelium.^{25–30} ESCs can be induced to differentiate into a wide range of different cell types *in vitro*,^{31–38} and, undoubtedly, can offer an attractive cell source for repair of injured/diseased alveoli.

Many methods have been reported for differentiating mESCs into ATIICs, yet all these approaches require complex or laborious culture conditions. Recently, Roszel *et al.* used Activin A and A549-conditioned medium to enrich definitive endoderm and differentiated mESCs seeded on Collagen IV-coated plates into ATIICs.¹⁰ They found SPC expression on day 11 when the differentiation medium was supplemented with FGF2. We have previously reported that components of Matrigel facilitate differentiation of hESCs into ATIICs.¹¹ Here, we showed that significantly higher expression levels of SPC RNA were detected in the differentiating cultures of mESCs seeded on Matrigel-coated plates as early as day 7 in either mDM or SR medium, when compared with that in differentiating cultures of mESCs seeded on Collagen IV-coated plates. FGF2-induced SPC expression was observed in the differentiating cultures in Matrigel-coated plates on days 7 and 9 when mDM was used for the initial 5 days. However, this FGF2-induced SPC expression significantly declined on day 11. In comparison, a high expression level of SPC RNA in the differentiating cultures of mESCs in Matrigel-coated plates was well maintained in SR medium from day 7 to 11. Our results suggest that the choice of differentiation medium and coating matrix is key to efficient differentiation of mESCs into ATIICs. Characterization of protein components of Matrigel for identifying the particular proteins required to direct mESC differentiation into ATIICs can provide a significant insight into the mechanisms underlying mESC differentiation, and merits further investigation.

Expression of a fusion gene comprised a cell type-specific promoter and sequences encoding a resistance to neomycin, puromycin, or bleomycin in an ESC-derived cell lineage is a potential strategy to isolate tissue stem/progenitor cells derived from ESCs.^{11,39} However, the genetic abnormalities caused by genetic manipulation with random vector integrations are the major concern for the technique to be used in regenerative medicine. In this study, we used the site-specific targeting strategy to knock-in mSPCP-NEO^R transgene into Rosa 26 gene locus of mESCs to avoid possible gene mutation caused by random vector insertions. With this single site-specifically modified mESC line, a homogenous population of mESC-ATIICs can be derived as early as on day 7 when mESCs were cultured on Matrigel-coated plates in SR medium. These mESC-ATIICs expressed SPA, SPB, and SPC with a characteristic ultra structure of normal ATIICs. The purity of derived ATIICs was well maintained in the presence of G418, as derived ATIICs were eliminated immediately. It was observed in the study that these early-derived ATIICs continued to robustly proliferate in the differentiating cultures in the presence of G418, leading to a significantly increased number of mESC-ATIICs in differentiating culture (1.4×10^6 mESC-ATIICs/6-cm plate) on day 11. Since SPC expression has been observed in the more primitive lung precursor

cells,⁴⁰ whether these early mESC-derived SPC positive cells on day 7 are the progenitor for ATIICs needs to be further characterized. However, this differentiation condition combined with use of the site-specifically modified mESCs can be scaled up to produce mESC-ATIICs in sufficient number to explore their therapeutic potential in alveolar regeneration.

It is critical to determine whether the mESC-derived ATIICs possess normal biological functions of control mATIICs for exploring their therapeutic potential. Previously reported data demonstrated the differentiation of mESCs into ATIICs by the evidence of surfactant protein expression and lamellar bodies in the derived cells. However, due to the lack of high purity of mESC-derived ATIICs for further characterization, it remains unclear whether or not those mESC-derived ATIICs possess normal biological functions. The site-specifically modified mESCs established in the present study can be selectively differentiated into a homogenous population of ATIICs *in vitro*, providing a sufficient number of mESC-ATIICs for us to characterize their biological functions. Our data demonstrated for the first time that the mESC-ATIICs possess progenitor capacity, and recapitulate the expression and secretion of surfactant in a manner found in the lungs. For example, they synthesized and secreted surfactant lipids, and can be induced to secrete surfactant proteins as control mATIICs. These biological functions are particularly important for ATIICs to maintain alveolar homeostasis. Thus, our results demonstrated that this novel strategy is reliable for producing a sufficient number of functional mESC-ATIICs to explore their therapeutic potential in lung tissue regeneration. The SPCP-NEO^R transgene located in one known gene locus may still be a potential safety concern in practical application. "Clinical grade" ESC-derived ATIICs need to be generated for tissue regeneration in future.

In conclusion, we have successfully established an mESC line with a single copy of mSPCP-NEO^R transgene in Rosa 26 gene locus. The genetically modified mESCs can be selectively differentiated into a homogenous population of functional ATIICs in a sufficient number when they were cultured on Matrigel-coated plates in SR medium. This novel strategy represents an important step toward exploring the therapeutic potential of mESC-derived ATIICs in lung tissue regeneration.

Acknowledgments

This work was supported by The Brown Foundation Institute of Molecular Medicine, University of Texas Medical School at Houston, and U.S. Public Health Service National Institutes Health Grants R21 HL102833-01(D.W). The authors thank Dr. Eva M. Zsigmond for providing the mESCs.

Disclosure Statement

No competing financial interests exist.

References

1. Selman, M., *et al.* Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. *Ann Intern Med* **134**, 136, 2001.
2. Nogee, L.M., *et al.* A mutation in the surfactant protein B gene responsible for fatal neonatal respiratory disease in multiple kindreds. *J Clin Invest* **93**, 1860, 1994.

3. Nogee, L.M., *et al.* Brief report: deficiency of pulmonary surfactant protein B in congenital alveolar proteinosis. *N Engl J Med* **328**, 406, 1993.
4. Hamvas, A., *et al.* Progressive lung disease and surfactant dysfunction with a deletion in surfactant protein C gene. *Am J Respir Cell Mol Biol* **30**, 771, 2004.
5. Ali, N.N., *et al.* Derivation of type II alveolar epithelial cells from murine embryonic stem cells. *Tissue Eng* **8**, 541, 2002.
6. Samadikuchaksaraei, A., *et al.* Derivation of distal airway epithelium from human embryonic stem cells. *Tissue Eng* **12**, 867, 2006.
7. Van Vranken, B.E., *et al.* Coculture of embryonic stem cells with pulmonary mesenchyme: a microenvironment that promotes differentiation of pulmonary epithelium. *Tissue Eng* **11**, 1177, 2005.
8. Rippon, H.J., *et al.* Derivation of distal lung epithelial progenitors from murine embryonic stem cells using a novel three-step differentiation protocol. *Stem Cells* **24**, 1389, 2006.
9. Rippon, H.J., *et al.* Embryonic stem cells as a source of pulmonary epithelium in vitro and in vivo. *Proc Am Thorac Soc* **5**, 717, 2008.
10. Roszell, B., *et al.* Efficient derivation of alveolar type II cells from embryonic stem cells for in vivo application. *Tissue Eng Part A* **15**, 3351, 2009.
11. Wang, D., *et al.* A pure population of lung alveolar epithelial type II cells derived from human embryonic stem cells. *Proc Natl Acad Sci U S A* **104**, 4449, 2007.
12. Wang, D., *et al.* Transplantation of human embryonic stem cell-derived alveolar epithelial type II cells abrogates acute lung injury in mice. *Mol Ther* **18**, 625, 2010.
13. Corti, M., Brody A.R., and Harrison J.H. Isolation and primary culture of murine alveolar type II cells. *Am J Respir Cell Mol Biol* **14**, 309, 1996.
14. Sano, K., Voelker D.R., and Mason R.J. Effect of secretagogues on cytoplasmic free calcium in alveolar type II epithelial cells. *Am J Physiol* **253**(5 Pt 1), C679, 1987.
15. Romero, E.J., *et al.* Interaction of an artificial surfactant in human pulmonary epithelial cells. *Pediatr Pulmonol* **39**, 167, 2005.
16. Alcorn, J.L., *et al.* Primary cell culture of human type II pneumocytes: maintenance of a differentiated phenotype and transfection with recombinant adenoviruses. *Am J Respir Cell Mol Biol* **17**, 672, 1997.
17. Wang, D., *et al.* Response of the neonatal rat cardiomyocyte in culture to energy depletion: effects of cytokines, nitric oxide, and heat shock proteins. *Lab Invest* **75**, 809, 1996.
18. Kreda, S.M., *et al.* Expression and localization of epithelial aquaporins in the adult human lung. *Am J Respir Cell Mol Biol* **24**, 224, 2001.
19. Ramirez, M.I., *et al.* T1alpha, a lung type I cell differentiation gene, is required for normal lung cell proliferation and alveolus formation at birth. *Dev Biol* **256**, 61, 2003.
20. Vanderbilt, J.N., *et al.* Directed expression of transgenes to alveolar type I cells in the mouse. *Am J Respir Cell Mol Biol* **39**, 253, 2008.
21. Chen, J., *et al.* Isolation of highly pure alveolar epithelial type I and type II cells from rat lungs. *Lab Invest* **84**, 727, 2004.
22. Millien, G., *et al.* Alterations in gene expression in T1 alpha null lung: a model of deficient alveolar sac development. *BMC Dev Biol* **6**, 35, 2006.
23. Ulich, T.R., *et al.* Keratinocyte growth factor is a growth factor for type II pneumocytes in vivo. *J Clin Invest* **93**, 1298, 1994.
24. Wollert, K.C., and Drexler, H. Clinical applications of stem cells for the heart. *Circ Res* **96**, 151, 2005.
25. Kubit, V., *et al.* Mixed allogeneic chimerism in lung allograft recipients. *Hum Pathol* **25**, 408, 1994.
26. Bittmann, I., *et al.* Cellular chimerism of the lung after transplantation. An interphase cytogenetic study. *Am J Clin Pathol* **115**, 525, 2001.
27. Kleeberger, W., *et al.* Increased chimerism of bronchial and alveolar epithelium in human lung allografts undergoing chronic injury. *Am J Pathol* **162**, 1487, 2003.
28. Suratt, B.T., *et al.* Human pulmonary chimerism after hematopoietic stem cell transplantation. *Am J Respir Crit Care Med* **168**, 318, 2003.
29. Albera, C., *et al.* Repopulation of human pulmonary epithelium by bone marrow cells: a potential means to promote repair. *Tissue Eng* **11**, 1115, 2005.
30. Zander, D.S., *et al.* Bone marrow-derived stem-cell repopulation contributes minimally to the Type II pneumocyte pool in transplanted human lungs. *Transplantation* **80**, 206, 2005.
31. Wobus, A.M., *et al.* Retinoic acid accelerates embryonic stem cell-derived cardiac differentiation and enhances development of ventricular cardiomyocytes. *J Mol Cell Cardiol* **29**, 1525, 1997.
32. Muller, M., *et al.* Selection of ventricular-like cardiomyocytes from ES cells in vitro. *FASEB J* **14**, 2540, 2000.
33. Okabe, S., *et al.* Development of neuronal precursor cells and functional postmitotic neurons from embryonic stem cells in vitro. *Mech Dev* **59**, 89, 1996.
34. Brustle, O., *et al.* Embryonic stem cell-derived glial precursors: a source of myelinating transplants. *Science* **285**, 754, 1999.
35. Kramer, J., *et al.* Embryonic stem cell-derived chondrogenic differentiation in vitro: activation by BMP-2 and BMP-4. *Mech Dev* **92**, 193, 2000.
36. Buttery, L.D., *et al.* Differentiation of osteoblasts and in vitro bone formation from murine embryonic stem cells. *Tissue Eng* **7**, 89, 2001.
37. Dani, C., *et al.* Differentiation of embryonic stem cells into adipocytes in vitro. *J Cell Sci* **110** (Pt 11), 1279, 1997.
38. Segev, H., *et al.* Differentiation of human embryonic stem cells into insulin-producing clusters. *Stem Cells* **22**, 265, 2004.
39. Klug, M.G., *et al.* Genetically selected cardiomyocytes from differentiating embryonic stem cells form stable intracardiac grafts. *J Clin Invest* **98**, 216, 1996.
40. Kim, C.F., *et al.* Identification of bronchioalveolar stem cells in normal lung and lung cancer. *Cell* **121**, 823, 2005.

Address correspondence to:

Dachun Wang, MD

Research Center for Immunology and Autoimmune Diseases

The Brown Foundation Institute of Molecular Medicine

for the Prevention of Human Diseases

University of Texas Medical School at Houston

1825 Pressler St./IMM 437D

Houston, TX 77030

E-mail: dachun.wang@uth.tmc.edu

Received: July 10, 2013

Accepted: October 7, 2013

Online Publication Date: November 11, 2013