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Original Article

Efficient Derivation of Alveolar Type II Cells from Embryonic Stem Cells for *In Vivo* Application

Blair Roszell, M.S.,[1,2] Mark J. Mondrinos, B.S.,[1] Ariel Seaton, B.S.,[2] Donald M. Simons, Ph.D.,[1] Sirma H. Koutzaki, M.S.,[1] Guo-Hua Fong, Ph.D.,[2] Peter I. Lelkes, Ph.D.,[1] and Christine M. Finck, M.D.[2,3]

In the present study, mouse embryonic stem cells (ESCs) were differentiated into alveolar epithelial type II (AEII) cells for endotracheal injection. These enriched lung-like populations expressed lung epithelial markers SP-A, SP-B, SP-C, and CC10. First we show that rapid differentiation of ESCs requires a dissociated seeding method instead of an embryoid body culture method. We then investigated a two-step differentiation of ESCs into definitive endoderm by activin or A549-conditioned medium as a precursor to lung epithelial cells. When conditioned medium from A549 cells was used to derive endoderm, yield was increased above that of activin alone. Further studies showed that Wnt3a may be one of the secreted factors produced by A549 cells and promotes definitive endoderm differentiation, in part, through suppression of primitive endoderm. Activin and Wnt3a together at appropriate doses with dissociated cell seeding promoted greater endoderm yield than activin alone. Next, fibroblast growth factor 2 was shown to induce a dose-dependent expression of SPC, and these cells contained lamellar bodies characteristic of mature AEII cells from ESC-derived endoderm. Finally, ES-derived lung cells were endotracheally injected into preterm mice with evidence of AEII distribution within the lung parenchyma. This study concludes that a recapitulation of development may enhance derivation of an enriched population of lung-like cells for use in cell-based therapy.

Introduction

Preterm delivery with resultant pulmonary hypoplasia is a major problem in obstetrics and accounts for more than 70% of perinatal mortality.[1] Premature infants treated with surfactant therapy and ventilator strategies still often suffer from permanent impairment of lung function.[2,3] While the use of steroids to promote the maturation of fetal lungs is often effective at promoting long-term survival, it also leads to decreased alveolarization and mesenchymal thinning in some animal models, while its effects in humans are not completely understood.[4,5] Stem cell–based therapy is a promising option as an alternative treatment, due to the cells' ability to orchestrate physiological processes in response to local signaling cues. One possible cell source for cell-based treatment is embryonic stem cells (ESCs) derived from the inner cell mass of a preimplantation blastocyst. These cells can self-renew indefinitely while retaining their capacity to differentiate into cell types of all three primitive germ layers.[6] The aim of our study was to use developmental biology–based strategies to efficiently direct the differentiation of ESCs toward lung alveolar epithelial type II (AEII) cells. AEII cells are an attractive cell type for ES-directed differentiation since these cells specialize in secreting a variety of surfactants that coat the distal lung epithelium, thereby reducing surface tension. Moreover, these cells are involved in the repair and maintenance by differentiating into alveolar type I cells in response to injury, and would provide a useful tool for cell-based therapy for lung disease.[7]

Efficient directed differentiation of many cell types of the ectodermal, mesodermal, and even endodermal origin has relied on a recapitulate *in vitro* of some of the critical differentiation cues that promote cell lineage commitment *in vivo*. With the aid of Green Fluorescent Protein (GFP)-tagged markers, protocols were developed to promote the commitment of undifferentiated ESCs to a primitive streak-like stage and required balanced signaling of both activin and Wnt3a.[8] Based on activin-induced endoderm commitment of ESCs, prior studies have established reproducible methods for generating cell populations enriched in definitive endoderm.[9–11] Others have reported the derivation of proximal and distal lung epithelial cell lineages that sometimes include an endoderm enrichment step.[12–19] The most recent report of growth factor–defined distal alveolar epithelial differentiation combined the

[1]School of Biomedical Engineering, Sciences, and Health Systems, Drexel University, Philadelphia, Pennsylvania.
[2]University of Connecticut Health Center, Farmington, Connecticut.
[3]Connecticut Children's Medical Center, Hartford, Connecticut.

use of a GFP reporter system to monitor endoderm induction while borrowing previous distal lung differentiation protocols to derive AEII cells in the most systematic and developmentally accurate way yet. Still, in the best of cases the efficiency of these techniques is very low (~4%), or requires strategies that would be difficult to implement clinically such as antibiotic selection, genetic manipulation, or use of fetal cells.

Fibroblast growth factors (FGFs) are important regulators of embryonic processes such as morphogenesis and differentiation. After gastrulation, the primitive gut tube is regionalized into an anterior and posterior region established by a gradient of FGFs *in vivo*. As many of these mechanisms were discovered in explants from fetal endoderm, we hypothesized that these same cues might specify lung lineages from ESC-derived endoderm in a similar manner. The lung endoderm develops proximal to the cardiac mesoderm, an inductive tissue that secretes high levels of FGF2 at the 7–8 somite stage, preceding the induction of lung-specific gene expression. Thus, FGF2 is a possible candidate to instruct the differentiation of ES-derived endoderm into lung epithelial cell types.

In this study, we compare existing protocols of both defined and undefined medium composition that have previously been shown to promote endoderm differentiation. Then, using culture conditions known to induce differentiation of lung from multi-potent endoderm, we report on a novel protocol for lung epithelial differentiation with specific additives that improve yield and reduce culture time required for AEII differentiation. In extending our previous research clarifying the role of FGFs in distal lung morphogenesis, we now established FGF2 as an important factor in distal lung differentiation of ESCs.

Here we describe a novel protocol for directed differentiation of ESCs to AEII cells and investigate the ability of FGF2 to specify lung lineages.

Materials and Methods

Maintenance of murine ESCs

Murine ESCs, E14tg2a (American Type Culture Collection, Manassas, VA), were maintained under feeder-free conditions in culture medium (maintenance medium), which was comprised of Glasgow Minimum Essential Medium (GMEM) (Invitrogen, Carlsbad, CA), 10% Batch-tested fetal bovine serum (FBS) (Biowest, Miami, FL), 5 mL sodium pyruvate (1 mM; Gibco, Carlsbad, CA), 5 mL nonessential amino acids (1 mM; Gibco), 5 mL glutamine (2 mM; Invitrogen), 50 U/mL penicillin, 50 μg/mL streptomycin (Invitrogen, Carlsbad, CA), leukemia inhibitory factor 1000 U/mL (ESGRO; Millipore, Billerica, MA), and 0.1 mM β-mercaptoethanol. For routine maintenance and propagation, the cells were split anywhere from 1:2 to 1:6 in 0.1% gelatin-coated T-25 flasks (Falcon–Beckton Dickinson, San Jose, CA) every 2–3 days using 1 mL of TrypLE (Gibco) for 1 min, resuspended in maintenance medium, and replated.

Differentiation of ESCs into definitive endoderm and AEII cells

E14tg2a cells were induced to differentiate either using dissociated seeding or embryoid body (EB) seeding method (Fig. 1). For dissociated seeding, ESCs were trypsinized, disaggregated into single cells, centrifuged, and resuspended in differentiation medium (see below) and plated at a low density (3×10^4 cells/cm^2). This density allowed expansion for up to 8 days without passaging. For EB formation, ESCs were dissociated as described above. Then, 4 mL of a cell suspension (8.0×10^4 cells in differentiation medium) was pipetted into 5-cm culture dishes and shaken on a Belly Dancer (Stovall, Greensboro, NC) in the incubator for 2.5 days. EBs were

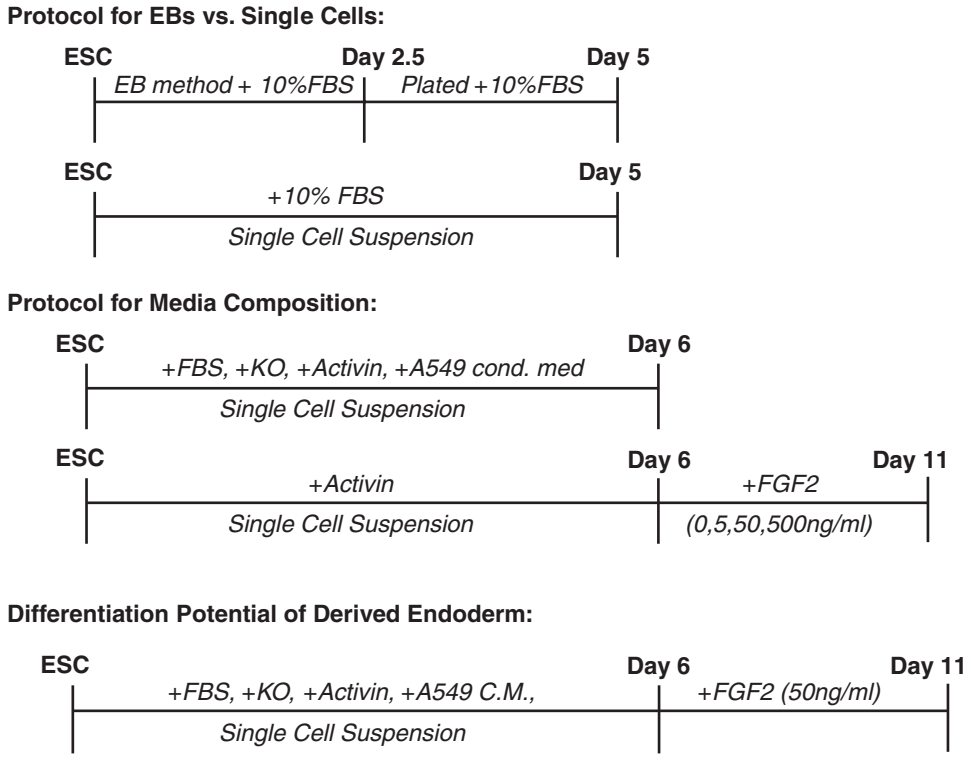


FIG. 1. Schematic of differentiation protocols. The protocol for optimal immunoreactivity to SP-C involves 6 days exposure of low-density single cells seeded in activin-containing medium, and then switched to high doses (50 or 500 ng/mL) of fibroblast growth factor 2 (FGF2) for an additional 5 days.

then harvested and plated for an additional 2.5 days on collagen IV–coated six-well plates.

To induce differentiation in two-dimensional culture, ESCs were seeded on collagen IV–coated six-well plates (25 ng/mL in 0.1M HCl) in a humidified incubator in 5% CO_2 at 37°C. All differentiation media contained 75% (v/v) Iscove's modified Dulbecco's medium (Invitrogen) and 25% (v/v) Ham's F12 medium (Invitrogen) supplemented with 0.5× of both N2 and B27(without retinoic acid) supplements (Invitrogen), penicillin 50 U/mL, streptomycin 50 μg/mL, 0.05% bovine serum albumin (BSA), 2 mM glutamine (Invitrogen), 0.5 mM ascorbic acid (Sigma), and 4.5×10^{-4} M 1-thioglycerol (Sigma, St. Louis, MO). Media were further supplemented with 10% FBS for the initial differentiation studies.

For directed differentiation to endoderm, all media were serum-free and supplemented with activin A (20 ng/mL; R&D Systems, Minneapolis, MN), A549-conditioned medium (filtered and added at 50:50 v/v to serum-free medium as the working solution), or Wnt3a (10 ng/mL; R&D systems).

As an additional control, we used an alternative serum-free medium, Knock Out (KO) medium, made of Dulbecco's modified Eagle's medium (Gibco) supplemented with 10% KO (Knockout Serum Replacer; Invitrogen, Paisley, United Kingdom), penicillin 50 U/mL, streptomycin 50 μg/mL. All differentiation media were replenished every 24–48 h. For lung differentiation, medium was changed to serum-free medium supplemented with 50 μg/mL heparin sulfate salt (porcine intestinal mucosal; Sigma) and 0–500 ng/mL of FGF2 (Cat# F-0291; Sigma).

Our lung differentiation protocol required a total of 11 days in culture: 6 for endoderm induction, and 5 for lung epithelial induction. All studies characterizing lung epithelial differentiation used serum-free medium and supplemented with 50 ng/mL FGF2 and 500 ng/mL chimeric protein FGFR1 (IIIc/Fc) (Axxora; LLC, San Diego, CA) where indicated.

Microscopy and immunofluorescence imaging

For immunostaining, cells (or EBs) were seeded in eight-well tissue culture Permanox chamber slides (Thermo Fisher Scientific, Waltham, MA). At the end of the differentiation protocol, cells were washed twice in PBS, fixed in 4% paraformaldehyde for 10 min, and washed twice more with 1× tris-buffered saline (TBS). At this stage, samples were imaged using a Leica DM1000 equipped with a Leica DFC320 camera (Leica, Wetzlar, Germany). For immunostaining, the samples were permeabilized for 15 min in a TBS solution with 0.1% Triton-x (Sigma). Primary antibodies were Brachyury T 1:100 (Santa Cruz, Santa Cruz, CA), Foxa2 1:150 (Santa Cruz), GATA4 1:100 (Santa Cruz), Oct4 1:100 (Santa Cruz), Surfactant Protein-C (SPC) 1:1000 (Chemicon), and Sox17 1:150 (Santa Cruz) and applied for 90 min at room temperature. As a negative control, the primary antibodies were omitted or species-specific IgG (1:100) was used to assess nonspecific binding. Cells were then washed before the secondary antibody (Donkey Anti-Rabbit IgG-R or Donkey Anti-Goat IgG-FITC; Santa Cruz) was applied at a dilution of 1:1000 for 30 min at room temperature in a solution of TBS with 1% BSA and 0.1% Triton-X. Finally, cells were mounted with Vectashield mounting medium plus 4′,6-diamidino-2-phenylindole (Vector, Burlingame, CA) as a nuclear counterstain. All fluorescent images were acquired using a Leica DMRX upright microscope equipped with a DFC 300 FX camera (Leica) with Leica imaging software and processed using Adobe Photoshop CS2 (Adobe Systems, San Jose, CA). To quantify immunofluorescence, the percent positive cells in random fluorescent micrographs were counted using ImageJ software. Each condition was performed in triplicate for every experiment, and at least 10 independent microscopic fields were collected per condition. Each experiment was repeated three times.

Reverse transcriptase–polymerase chain reaction and quantitative polymerase chain reaction

Total RNA was extracted using the TriReagent protocol supplied by the manufacturer (Sigma). Two micrograms of RNA was used in each condition for first-strand cDNA synthesis, using random primers supplied in the All Access reverse transcriptase–polymerase chain reaction (RT-PCR) kit (Promega). AMV reverse transcriptase enzyme or sample was omitted (water control) as a negative control for each condition. Samples were cycled 35 times at 95°C for 30 s, 55–66°C for 45 s (see Table 1), and 72°C for 60 s on a GeneAmp PCR system 2400 (Perkin Elmer, Waltham, MA). The primer sequences and annealing temperatures and amplicon length are listed in Table 1. For quantitative PCR (qPCR), cDNA stocks were diluted 1:12.5 in DEPC-H_2O. The final qPCR reactions contain 5 μL of diluted sample, 0.5μM of forward and reverse primers, and 12.5 μL SYBR green mix (Applied Biosystems, Carlsbad, CA), and were adjusted to 25 μL with ddH_20. Relative quantification was calculated using the 2-(delta-delta c(T)) method described previously. Samples were normalized to β-actin for all conditions.

Flow cytometry

ES CD4-Foxa2/GFP-Bry cells (a gift from Dr. G. Keller, Mount Sinai School of Medicine, New York City, NY) were used to characterize endodermal differentiation by Fluorescence Activated Cell Sorting (FACS) analysis because of the easily detectable surrogate surface marker for Foxa2 expression. These ESCs were maintained using a medium comprised of 50/50 (v/v) mixture of Neurobasal medium (Invitrogen) and Dulbecco's modified Eagle's medium/F12 medium (Invitrogen) supplemented with 0.5× of both N2 and B27 supplements (Invitrogen), penicillin 50 U/mL, streptomycin 50 μg/mL (Invitrogen), 0.05% BSA (Sigma), leukemia inhibitory factor 1000 U/mL (ESGRO; Millipore), human bone morphogenic protein 4 (10 ng/mL; R&D Systems), and 1.5×10^{-4} M 1-thioglycerol (Sigma) and differentiated using the same protocols described above. For FACS analysis, cell nuclei were stained using TO-PRO 3 iodide (Molecular Probes, Eugene, OR) according to the manufacturer's protocol. Only cells with stained nuclei were gated and counted to exclude debris. Undifferentiated cells served as the negative control for all experiments. A phycoerythrin-conjugated anti CD4-antibody (Santa Cruz), diluted to 1:100 in TBS supplemented with 1% serum, was detected using the FACS Calibur in the UConn Health Center core facility. All FACS data were analyzed with FlowJo software (Tree Star, Inc., Ashland, OR). Percent of positive differentiated cell population was calculated as the percentage area under the curve past 99% of undifferentiated ESCs.

Table 1. Reverse Transcriptase–Polymerase Chain Reaction Primers

Gene	*Primer sequence (5′–3′)*	*Tm (°C)*	*Amplicon length (bp)*
β-Actin	F: GCTCCGGCATGTGCAA R: AGGATCTTCATGAGGTAGT	55	542
Foxa2	F: TGGTCACTGGGGACAAGGGAA R: GCAACAACAGCAATAGAGAAC	56	289
GAPDH	F: GCACCGTCAAGGCTGAGAAC R: GCCTTCTCCATGGTGGTGAA	55	150
Gata6	F: GCCGGAGGAAATGTACCAGAC R: CCCCTTGAAGGTAGGGCAG	55	191
Goosecoid	F: ATGCTGCCCTACATGAACGT R: CAGTCCTGGGCCTGTACATT	55	517
Hnf4a	F: GTGGCGAGTCCTTATGACACG R: GCTGTTGGATGAATTGAGGTTGG	55	60
Mixl1	F: GCACGTCGTTCAGCTCGGAGCAGC R: AGTCATGCTGGGATCCGGAACGTGG	55	305
Oct4	F: GGCTTCAGACTTCGCCTCC R: AACCTGAGGTCCACAGTATGC	60	211
Pdx-1	F: CCCCAGTTTACAAGCTCGCT R: CTCGGTTCCATTCGGGAAAGG	55	177
Sox17	F: GCCAAAGACGAACGCAAGCGGT R: TCATGCGCTTCACCTGCTTG	53	211
SP-C	F: GAGGTCCTGATGGAGAGC R: GCTTAGACGTAGGCACTG	55	463

Tm, melting temperature; bp, base pair.

Electron microscopy protocol for murine lung epithelial cells (MLE) and ESCs

Cultures were rinsed briefly with phosphate buffered saline, and then fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 1 h at room temperature. After rinsing in 0.1 M cacodylate buffer, the cultures were postfixed for 1 h in 1% OsO_4/0.8% potassium ferricyanide in 0.1 M cacodylate buffer, rinsed in distilled water, and treated for 1 h with 1% aqueous uranyl acetate. They were then dehydrated in graded ethanol solutions and embedded in Polybed epoxy resin (Polysciences, Warrington, PA). Ultrathin (~70 nm) sections were cut either parallel with or perpendicular to the cell layer with a diamond knife on a Reichert Ultracut E ultramicrotome. The sections were collected on 200-mesh copper/rhodium grids, stained with uranyl acetate and lead citrate, and examined and photographed in a Philips (Andover, MA) CM10 transmission electron microscope at an accelerating voltage of 80 kV.

Endotracheal instillation

All procedures were carried out using an Institutional Animal Care and Use Committee (IACUC)-approved protocol. Timed-pregnant 129/sv mice were anesthetized with isoflurane, while the preterm E18.5 pups were extracted through cesarean section surgery. About 1×10^5 E14tg2a or ES CD-4Foxa2/Bry-GFP cells were labeled with CellTracker™ Green BODIPY(R) dye (Molecular Probes) according to the manufacturer's instructions and endotracheally instilled into the E18.5 preterm pups as previously described. As a control, unlabeled E14tg2a cells were instilled and lungs were stained for CD-4. After surgery, the animals were housed for 5 days. Upon termination of the experiment, mice were euthanized and lungs were harvested and perfused with PBS. Lungs were inflated and fixed with 4% paraformaldehyde overnight at 4°C. Lungs were then incubated overnight in sucrose, embedded, sectioned, and immunostained for SPC and CD-4 expression.

Statistics

Data are presented as the mean ± standard deviation. One-way analysis of variance (Microsoft Office Excel, 2003) was used to determine significant differences, with $p < 0.05$ regarded as statistically significant. Bars indicate standard deviation, and statistical significance was calculated using a *t*-test assuming unequal variance.

Results

ESC differentiation with EBs versus dissociated cells

Although initial reports establishing protocols for definitive (and not visceral) endoderm differentiation employed a low-density single-cell seeding method, most lung epithelial differentiation protocols that include an endoderm enrichment step instead use an EB differentiation protocol. To investigate initial ESC differentiation either during the EB stage or upon seeding as dissociated cells, we cultured undifferentiated ESCs using the two methods over a 5-day period in a serum-containing differentiation medium (as described in the Materials and Methods). While undifferentiated ESCs form compacted colonies in maintenance medium (Fig. 2A), dissociated cells seeded and cultured 5 days in a serum-based differentiation medium (Fig. 2B) displayed cuboidal morphology typical of epithelial cells. Dissociated seeding resulted in cells that were uniformly positive for GATA4, a rather ubiquitous (early) mesodermal/endodermal marker, which is present in definitive endoderm, ventral mesoderm, and yolk sac endoderm (Fig. 2C). These cells retained only sparse expression of Oct4 (Fig. 2D). Alternatively, we also

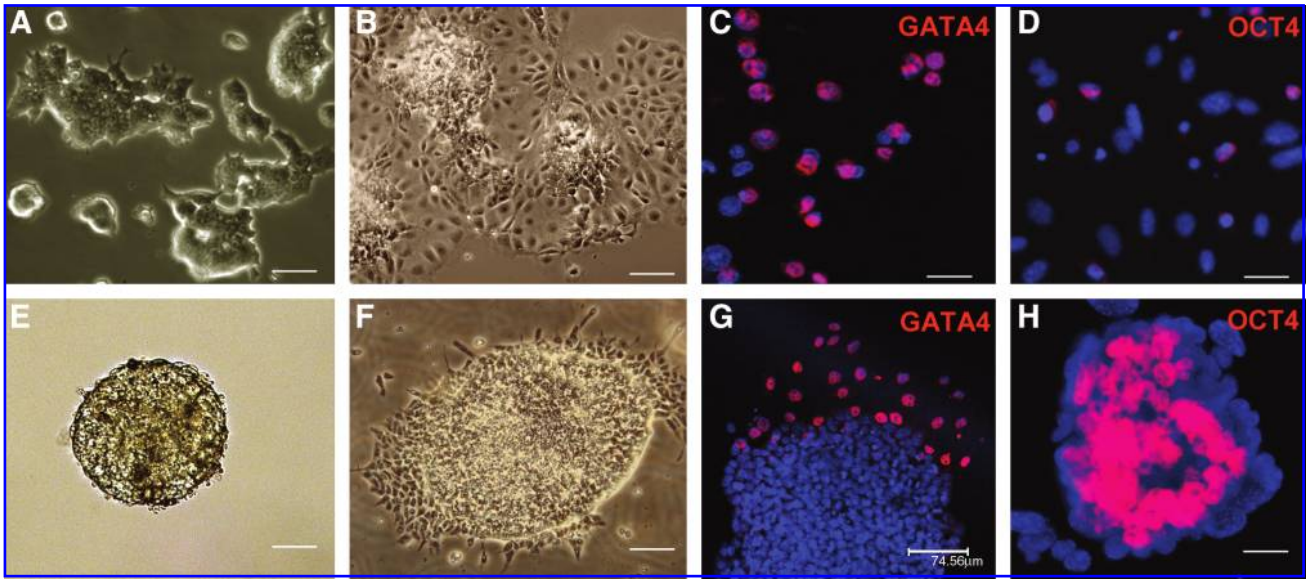


FIG. 2. ES cells differentiate uniformly when plated as disaggregated cells. Phase contrast image of undifferentiated ES cells maintained in LIF-containing medium (**A**). ES cells in serum-containing medium plated as disaggregated cells on collagen IV–coated surface for 5 days. After 5 days, cells seeded in a single-cell suspension spread and took on a cobblestone epithelial morphology (**B**). Dissociated ES cells express Gata4 uniformly after 5 days (**C**), but not Oct4 (**D**). Alternatively, embryonic stem cells (ESCs) were aggregated into embryoid bodies (EBs) for 2.5 days (**E**), and then plated out on a collagen IV–coated dish for 2.5 days (**F**). At day 5, cells formed into EBs spread from clusters (**F**). Only ES cells that migrated away from the EB express Gata4 (**G**). Cells within EBs retain Oct4 expression after 5 days (**H**). Bars = 75 µm (**A, B, F, G**) and 25 µm (**C–E, H**). Color images available online at www.liebertonline.com/ten.

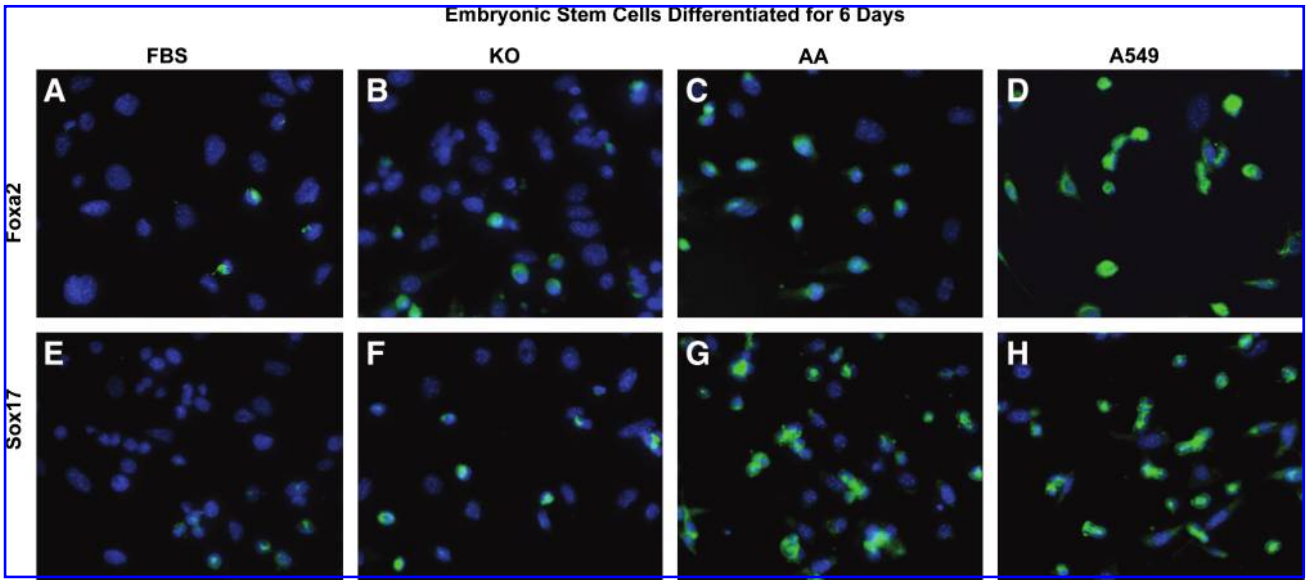
differentiated ESCs by aggregating them into EBs for 2.5 days (Fig. 2E) and then plating them for another 2.5 days (Fig. 2F). Only the spreading cells were positive for GATA4 (Fig. 2G). Instead, the EBs appeared to retain an undifferentiated core after 5 days of differentiation (Fig. 2H) as inferred from the continued expression of Oct4. Our results suggest that the dissociated cell plating method induces differentiation more rapidly and more uniformly than EB seeding methods. All future differentiation experiments used the disaggregated seeding of ESCs before plating.

Defined medium versus conditioned medium for endoderm induction

Activin A is the most widely studied growth factor for endoderm induction of ESCs; however, early AEII differentiation studies showed efficient induction of endoderm and subsequent expression of lung endoderm markers using instead cocultures of lung mesenchyme. The mesenchymal tissue likely secretes a variety of factors that contribute to endoderm differentiation. We hypothesized that a cocktail of growth factors could efficiently specify endoderm from ESCs that is more suitable for further differentiation to lung endoderm. To investigate this, we cultured ESCs in conditioned medium from A549 cells, an AEII-like cell line, and monitored ES differentiation toward endoderm and subsequent lung lineage markers. Figure 3A–H shows immunostaining for endoderm markers in FBS (10%), 10% KO medium, 20 ng/mL activin, or 50:50% v/v A549-conditioned medium. To compare conditions, we quantified expression of Foxa2 and Sox17 as a percent of the total population of cells (Fig. 3I). ESCs cultured in activin-supplemented medium had a larger population of Foxa2-expressing cells ($38 \pm 5.7\%$) than cells maintained in KO medium alone ($23 \pm 6.1\%$) or FBS medium ($12.3 \pm 4.5\%$). ESCs cultured in A549-conditioned medium contained a significantly higher population of Foxa2-expressing ($66.2 \pm 7.7\%$) and Sox17-expressing ($64.5 \pm 7.5\%$) cells than those cultured in either activin or KO medium alone.

To confirm these results, we repeated our protocol with genetically engineered ES CD4-Foxa2/GFP-Bry cells in which the expression of the cell surface marker CD4 is under the control of the Foxa2 promoter and hence allows for easy quantification using flow cytometry. The dose response to activin for each cell line was similar to E14tG2a ESCs (data not shown). A549-conditioned medium induced over half (55%) of the cells to express Foxa2 (Fig. 4). Activin alone induced Foxa2 expression in 46% of cells analyzed. Taken together, we conclude that factors present in the conditioned medium of A549 cells provide potent signals for endoderm differentiation, at least equal to, if not more efficiently, than 20 ng/mL activin.

Previous studies have shown that EBs with upregulated expression of endoderm markers also had upregulated expression of Wnt3a and nodal. Wnt3a alone induced posterior primitive streak markers, though the combined effects with activin were not explored. A previous study revealed that A549 cells expressed high levels of Wnt3 mRNA, leading us to hypothesize that wnt3a may be one of the factors in the A549-conditioned medium that promotes endoderm formation. Comparing endoderm differentiation in ESCs treated with activin alone or activin with Wnt3a (10 ng/mL) (Fig. 5), we



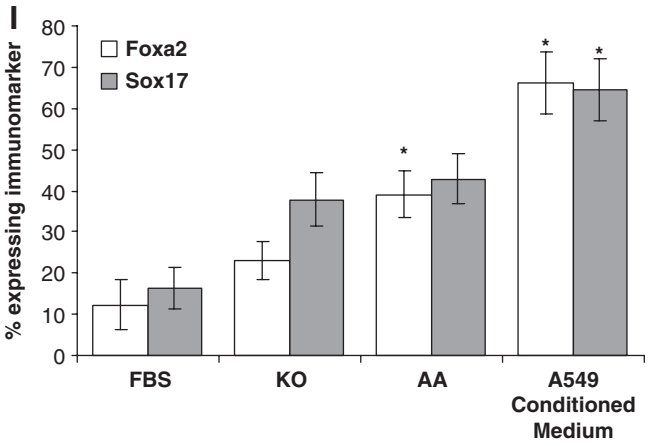


FIG. 3. Quantitative comparison of differentiation strategies to endoderm. Immunofluorescence images of ES cells dissociated and differentiated for 6 days in medium supplemented with 10% fetal bovine serum (FBS) (**A, E**), serum-free (**B, F**), serum-free + 20 ng/mL activin A (**C, G**), or serum-free + A549-conditioned medium (**D, H**). Cells were immunostained for Sox17 and Foxa2 (green). Fluorescent images were analyzed and quantified as percent positive population. (**I**) Error bars indicate standard deviation, and asterisks (*) indicate significant differences compared to KO conditions calculated using a *t*-test assuming unequal variance. Color images available online at www.liebertonline.com/ten.

found that wnt3a in combination with activin induced expression of foxa2 above that of activin alone. An analysis of extraembryonic endoderm markers revealed that both activin and wnt3a suppressed expression of hepatocyte nuclear factor 4α (HNF4α), a marker of visceral endoderm (Fig. 5). Interestingly, though activin promoted GATA6 expression, a marker for primitive endoderm, the addition of wnt3 suppressed GATA6 expression. This suggests that wnt3 may promote definitive endoderm differentiation through suppression of the primitive endoderm.

Characterization of ES-derived definitive endoderm

ESC cultures provide the strongest evidence of a population of mesendoderm that subsequently loses mesendoderm markers and commits to a definitive endoderm fate. Definitive endoderm gives rise to the epithelium of the ventral foregut. Visceral endoderm shares many of the same markers but is not thought to contribute to the lung *in vivo*. Because no single marker is sufficient to denote definitive endoderm, we used the combination of Brachyury and Foxa2 to characterize the population of ESC-derived cells that can give rise to definitive endoderm *in vivo*. Foxa2 expression is strongly associated with endoderm though Foxa2 is transiently expressed in axial mesoderm and in restricted patterns in nonendoderm tissue. The colocalization of these markers (shown in yellow in Fig. 6C, D) revealed that mesendoderm and definitive endoderm markers are induced concomitantly. Further, RT-PCR analysis showed that ESC-derived cells expressed many markers present in mesendoderm and definitive endoderm (Fig. 6E). Mixl1 and Goosecoid are homeodomain proteins that interact to orchestrate endoderm formation in the mice, though Goosecoid is found in mesoderm as well. All mesendoderm markers were also present in E17.5–18 murine FPCs. In subsequent experiments, activin-containing serum-free medium was used as our optimized medium before switching culture conditions to an FGF-supplemented medium for induction of lung differentiation.

Induction of lung lineages by FGF2 in ES-derived endoderm

In vivo, specification of lung from multipotent endoderm is determined largely by inductive cues from the cardiac mesoderm. Foregut endoderm explant studies provide evidence that high doses of FGF2 reprogram the default pancreatic cell fate into lung. Based on these studies, we hypothesized that high doses of FGF2 would specify the differentiation of ESC-

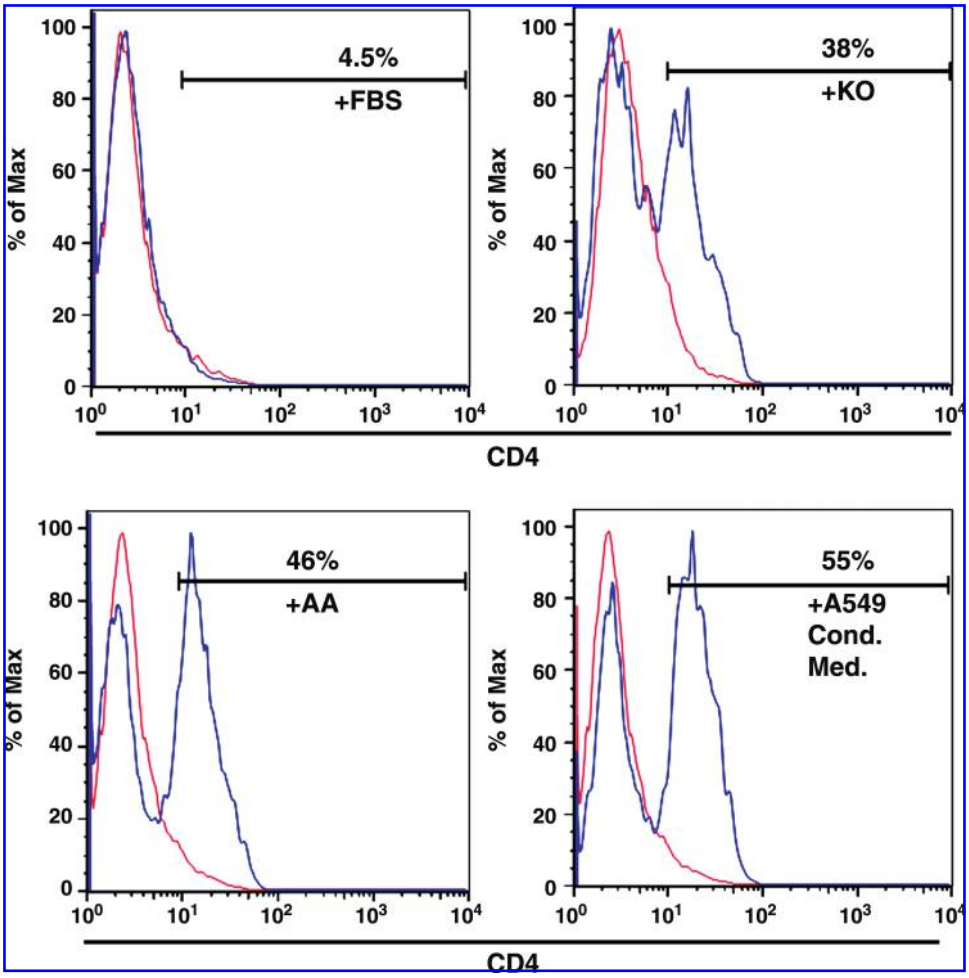


FIG. 4. FACS analysis of day 6 ES differentiation experiments. Flow cytometry experiments were carried out using ES CD4-Foxa2/GFP-Bry cells treated with 10% FBS (FBS), 10% KOSR (KO), activin (20 ng/mL), or A549-conditioned medium. The histograms represent CD-4 expression, corresponding to the expression of Foxa2. At least 10,000 events were collected for each condition. Color images available online at www.liebertonline.com/ten.

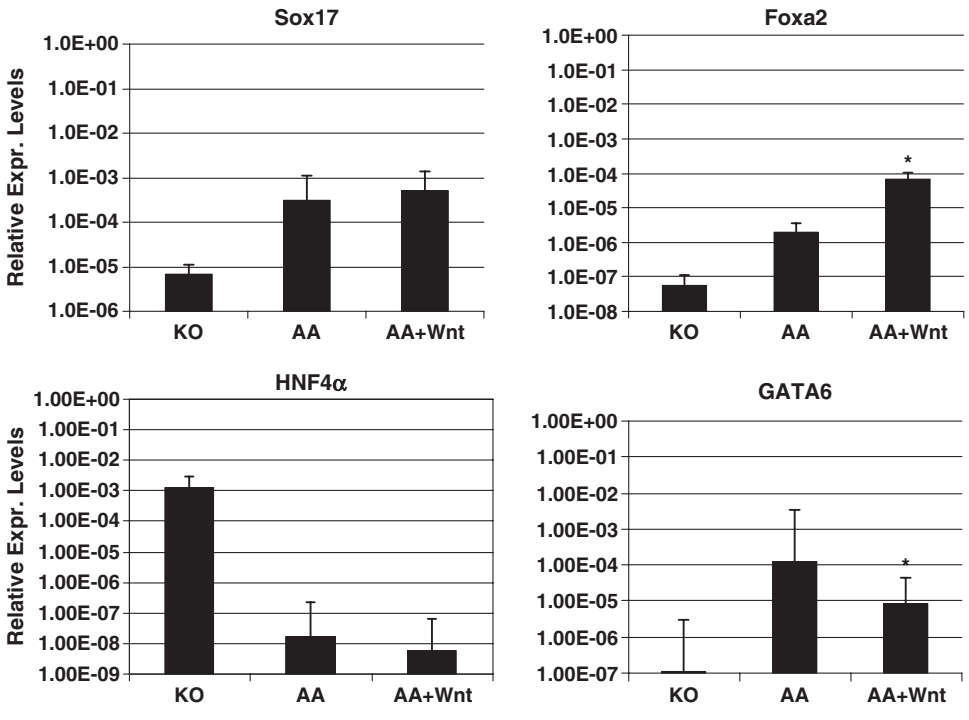


FIG. 5. Endoderm expression in activin- or Wnt-treated ESCs for 6 days. ES cells cultured in KO, AA, or AA + Wnt3A for 6 days in culture were analyzed using quantitative polymerase chain reaction (qPCR) for endoderm expression. Sox17 and Foxa2 are markers of endoderm. GATA6 is expressed in visceral endoderm. HNF4α and Keratin are expressed in visceral and primitive endoderm, respectively. All expression levels were normalized to β-actin expression for each condition. Bars indicate standard deviation and statistical significance was calculated using a *t*-test assuming unequal variance. Asterisk (*) indicates statistically significant difference compared to AA condition calculated using a *t*-test assuming unequal variance.

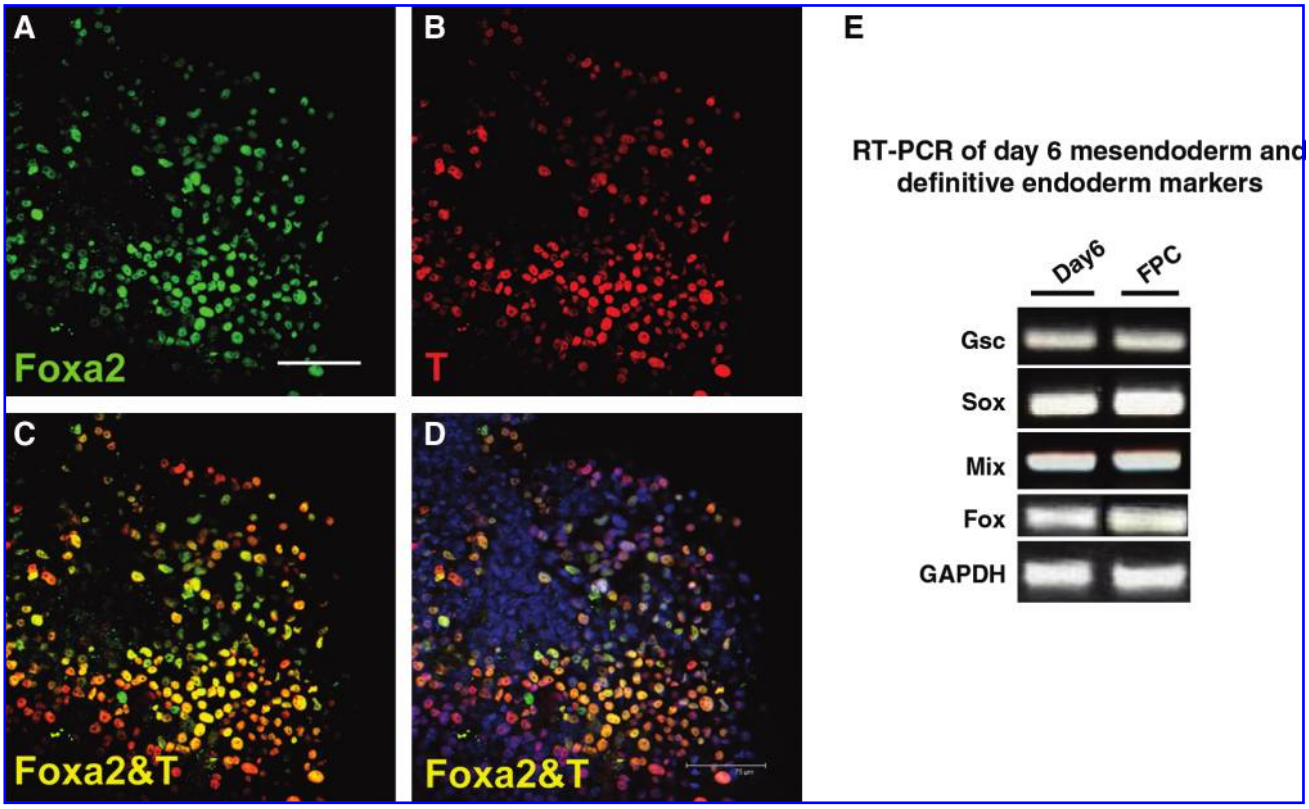


FIG. 6. Characterization of ES-derived endoderm progenitor cells. Confocal immunofluorescent images of ESCs cultured in activin A express both Foxa2 (**A**) and Brachyury T (**B**). Colocalization of these markers is shown in yellow (**C**). Nuclei were stained with DAPI (blue, **D**). Reverse transcriptase PCR analysis of mesendoderm and definitive endoderm markers compared to that of fetal pulmonary cells (E17.5–E18) (**E**). Scale bar represents 96 μm. Color images available online at www.liebertonline.com/ten.

derived definitive endoderm into lung epithelium. In our protocol, activin-primed (20 ng/mL) ESCs were switched after 6 days to a differentiation medium supplemented with different doses of FGF2.

Exposure for 5 days to both 50 and 500 ng/mL of FGF2 was sufficient to induce robust expression of SPC mRNA, a specific marker for AEII cells (Fig. 7A). Further, a quantitative comparison of the pancreatic marker Pdx-1 showed a significant decrease in expression in high (50 ng/mL) FGF2 compared to low (5 ng/mL) FGF2 (Fig. 7B). These data suggest that endoderm is specified to differentiate into lung with high doses of FGF2, and that low levels of FGF2 result in a default differentiation program of pancreas.

Characterization of FGF2 on lung differentiation

We next wanted to determine whether endoderm derived using different medium compositions could be induced to express SP-C in response to FGF2. To quantify AEII differentiation, we immunostained cells after exposure to 50 ng/mL

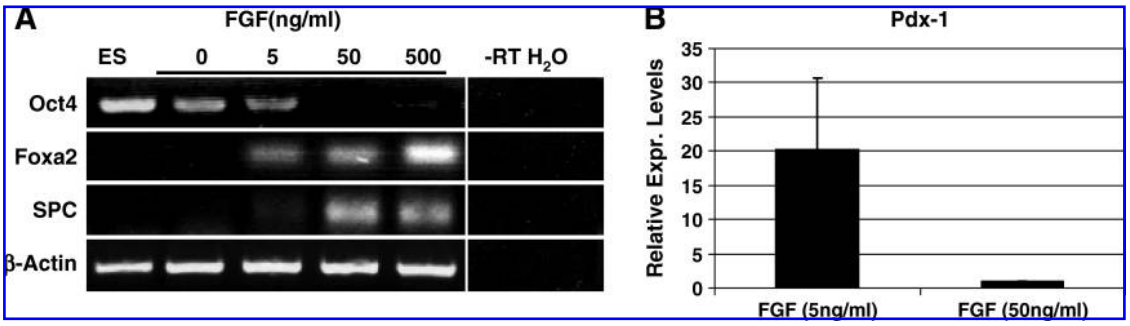


FIG. 7. High doses of FGF2 induce lung-specific markers in ES-derived definitive endoderm. ES cells primed with 20 ng/mL activin A for 6 days were switched to medium containing 0, 5, 50, and 500 ng/mL of FGF2 for an additional 5 days. Using reverse transcriptase PCR, each condition was screened for Oct4, Foxa2, SPC, and β-actin (**A**). qPCR analysis of pancreatic marker PDX-1 comparing 5 ng/mL and 50 ng/mL FGF2 conditions (**B**). Error bars indicate standard deviation.

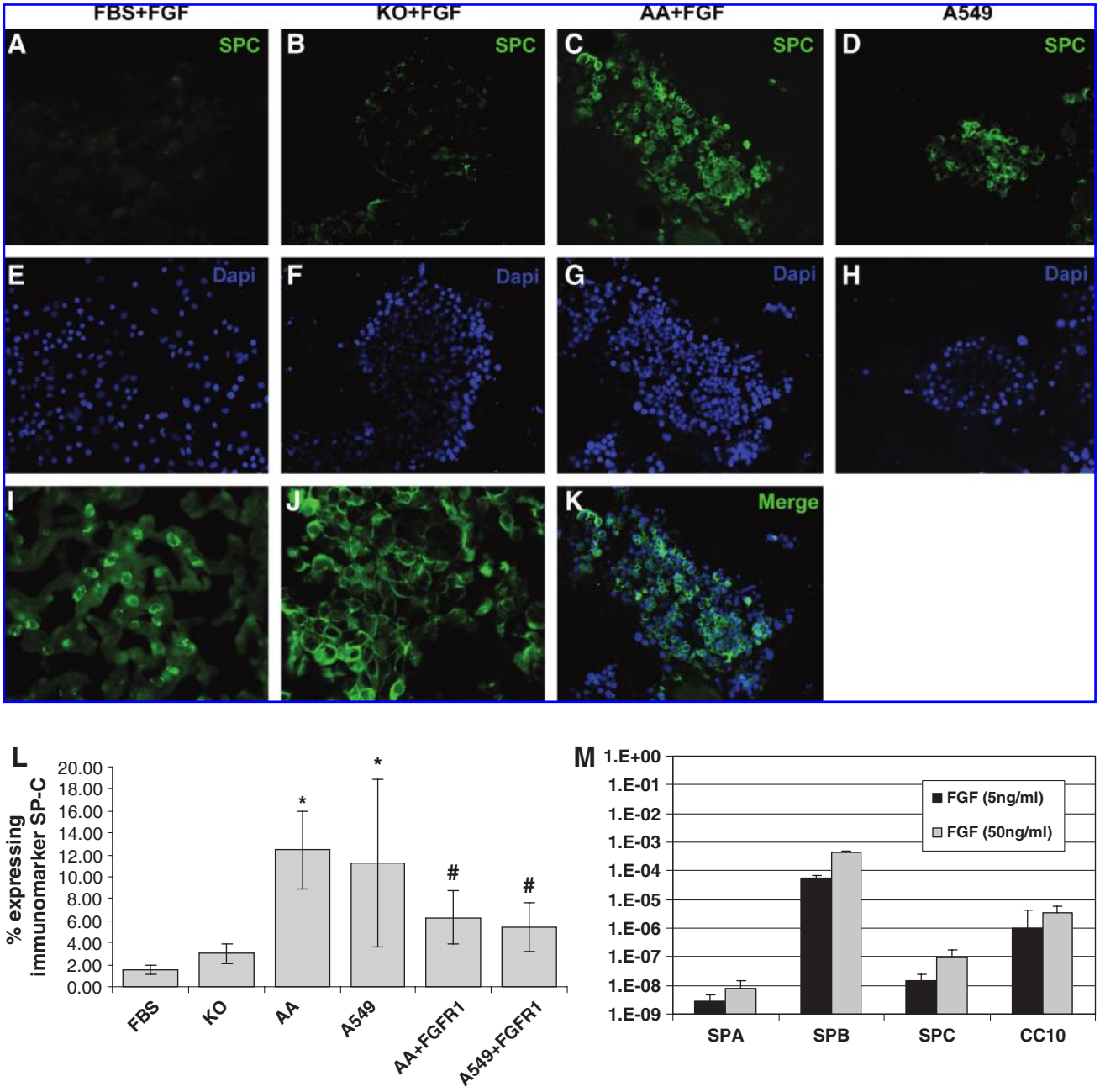


FIG. 8. Quantification of SP-C immunoreactivity in differentiated ES cells. ES cells cultured in FBS, KO, or activin for 6 days were switched to medium containing FGF2 (50 ng/mL) for an additional 5 days. Alternatively, ES cells were exposed to A549-conditioned medium for the entire 11 days. At day 11, cells were fixed and stained for SP-C (green) (**A–H, J, K**). Adult distal lung tissue stained for SP-C is shown in (**I**). A high-magnification image of representative SP-C staining of ES-derived alveolar epithelial type II cells is shown in (**J**). Fluorescent images were analyzed and percent positive cells were counted using ImageJ software. (**L**) ES cells primed with AA showed a significantly higher percent of SP-C expression compared to serum-free and serum-containing medium. (**L**) Asterisks (*) indicate significant difference compared to KO-treated conditions calculated using a *t*-test assuming unequal variance. (**L**) Pound signs (#) indicate significant difference compared to the corresponding condition in the absence of treatment with FGFR1. (**L**) qPCR analysis of lung markers comparing low (5 ng/mL) and high (50 ng/mL) FGF2 (**M**). Color images available online at www.liebertonline.com/ten.

FGF2 and counted numbers of SPC-positive cells as a percent of the total population (number of DAPI-stained nuclei). As a positive control, we also stained MLE-12 cells, which have been shown to express SPC, and adult lung tissue. When these results were quantified, we found $12.4 \pm 3.8\%$ of ESCs displayed immunoreactivity to SPC after exposure for 6 days to 20 ng/mL activin and subsequently for another 5 days to 50 ng/mL of FGF2 (Fig. 8D, K, L). Similarly, when cultured for 11 days in A549-conditioned medium $11.3 \pm 7.6\%$ of the ESCs were positive for SPC (Fig. 8H). Cultures of ESCs for 11 days

in serum-free or in serum-containing medium were significantly less effective in inducing SPC expression ($3.1 \pm 1.2\%$ and $1.5 \pm 1.6\%$, respectively.)

Although SP-C is one of the most faithful markers of AEII cells *in vivo*, previous studies showing SP-C immunostaining of ESCs demonstrate positive staining in cells with dendritic morphology, not typically associated with AEII cells. Although we did not observe these unusual cell morphologies in our culture, our cells exhibited a staining pattern that is consistently different from positive control adult lung sections. Although the SP-C immunostaining in ES cultures is punctate, the AEII cells in the context of the distal lung (adult lung sections; Fig. 8I) contain punctations with greater contrast and greater signal than most observed in ES cultures (Fig. 8J). Positive staining was not observed in sections incubated with rabbit IgG-FITC (data not shown), or serum and serum-free conditions (Fig. 8A, B) and is likely not background reactivity. Nevertheless, the localization of the staining pattern to the cytoplasm is consistent with control tissue, and can be easily quantified from many confocal images (Fig. 8L).

To test the possible receptor specificity of FGF signaling in inducing SP-C expression in ES cell cultures, FGF signaling was perturbed using a soluble FGFR1/IgG-Fc chimeric protein. FGF2 is a promiscuous growth factor that binds each of the four known high affinity FGF receptors, and inhibition of FGF2-mediated induction of Nkx2.1 expression has been demonstrated previously using this FGFR1/IgG-Fc chimeric protein. Culture of activin-induced or A549-conditioned medium–induced endoderm with both FGF2 (50 ng/mL) and FGFR1/IgG-Fc (500 ng/mL) for 5 days attenuated SP-C immunoreactivity in differentiated ES cells (Fig. 8L).

qPCR analysis of a panel of lung markers showed an increase in SP-A, SP-B, SP-C, and CC10 in response to high doses of FGF2 compared to low doses of FGF2 (Fig. 8I). The increase in SP-B and CC10 indicates the possibility of Clara cell differentiation, though SP-B is also found in alveolar type II cells. SP-A is an important surfactant for repression of inflammatory signaling, and is shown to be upregulated in the presence of high FGF2.

Electron microscopic analysis of ESC-derived lung epithelial cells

Ultrastructural analysis of differentiated ESCs was used to determine whether AEII specific organelle were present upon differentiation with FGF2. ESCs differentiated for 11 days in our optimized protocol (Activin and FGF2 containing medium) had fully formed mature lamellar bodies (Fig. 9A, B). Electron microscopic images of MLE-12 cells that are known to express SP-C contain few, but distinguishable lamellar bodies (Fig. 9C). For this reason, MLE-12 cells are thought to model alveolar epithelial progenitor cells of the pseudoglandular stage.

Endotracheal instillation of alveolar type II cells

To investigate whether ESC-derived pulmonary cells could be delivered *in vivo*, we harvested a population of type II–enriched cells derived from the E14tg2a cell line and labeled them with a fluorescent cell tracker dye for endotracheal instillation into preterm E18 mice. In line with our previous report, we observed uniform delivery of these cells throughout the distal alveoli after injection (Fig. 10A, B). We noted a significant amount of nonspecific staining, which is likely attributable to release of the dye by extrusion after cell death. To overcome this problem, we took advantage of the CD4 tag driven by the Foxa2 promoter in the ES CD4-Foxa2/GFP-Bry cells to identify *in situ* ES-derived cells that had differentiated into endoderm cells. As before, we instilled 1×10^5 type II–enriched ES-derived cells, this time without prior labeling with the cell tracker. As shown in Figure 10G and H, we detected some instances of double-positive CD4/SPC cells, indicating the engraftment of AEII cells that were derived from ESCs (arrow, Fig. 10G, H). Although these cells contain a GFP-Bry marker, GFP was not detectable in any of our *in vitro* cultures using fluorescence microscopy; still, we cannot rule out the possibility that the CD4-positive cells are unusually bright GFP fluorescing cells. We can, however, rule out the possibility that these double-positive cells were instead instilled cells ingested by macrophages, since a Mac-3

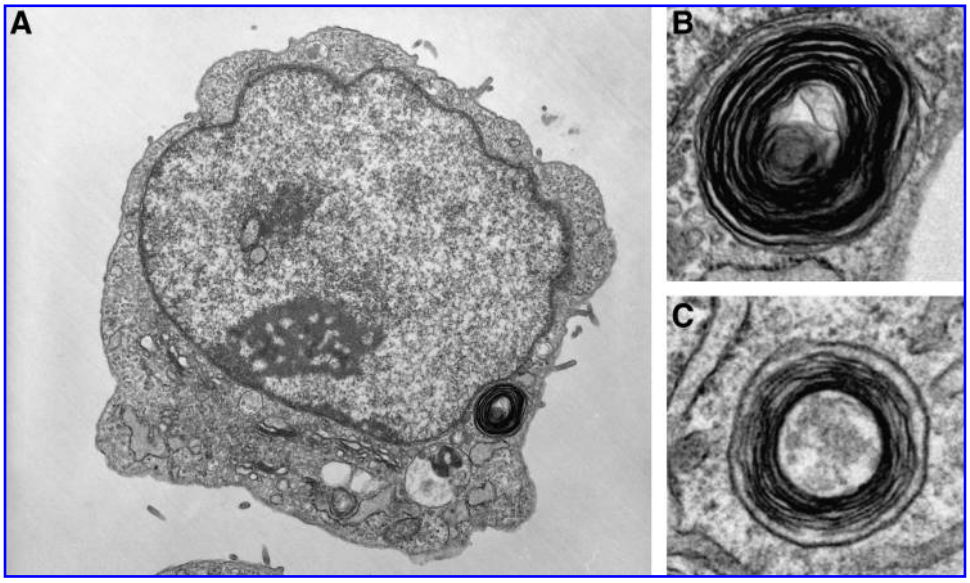


FIG. 9. Lamellar body formation in ESC-derived cells. (**A**) Ultrastructure of an ES cell differentiated with activin and FGF2 for 11 days. (**B**) Higher magnification of image (**A**) showing a well-developed lamellar body with electron-dense lamellae in a multivesicular body. In comparison, MLE-12 cells contained rare, but distinguishable lamellar body structures (**C**). Images were acquired at ×16,525 magnification.

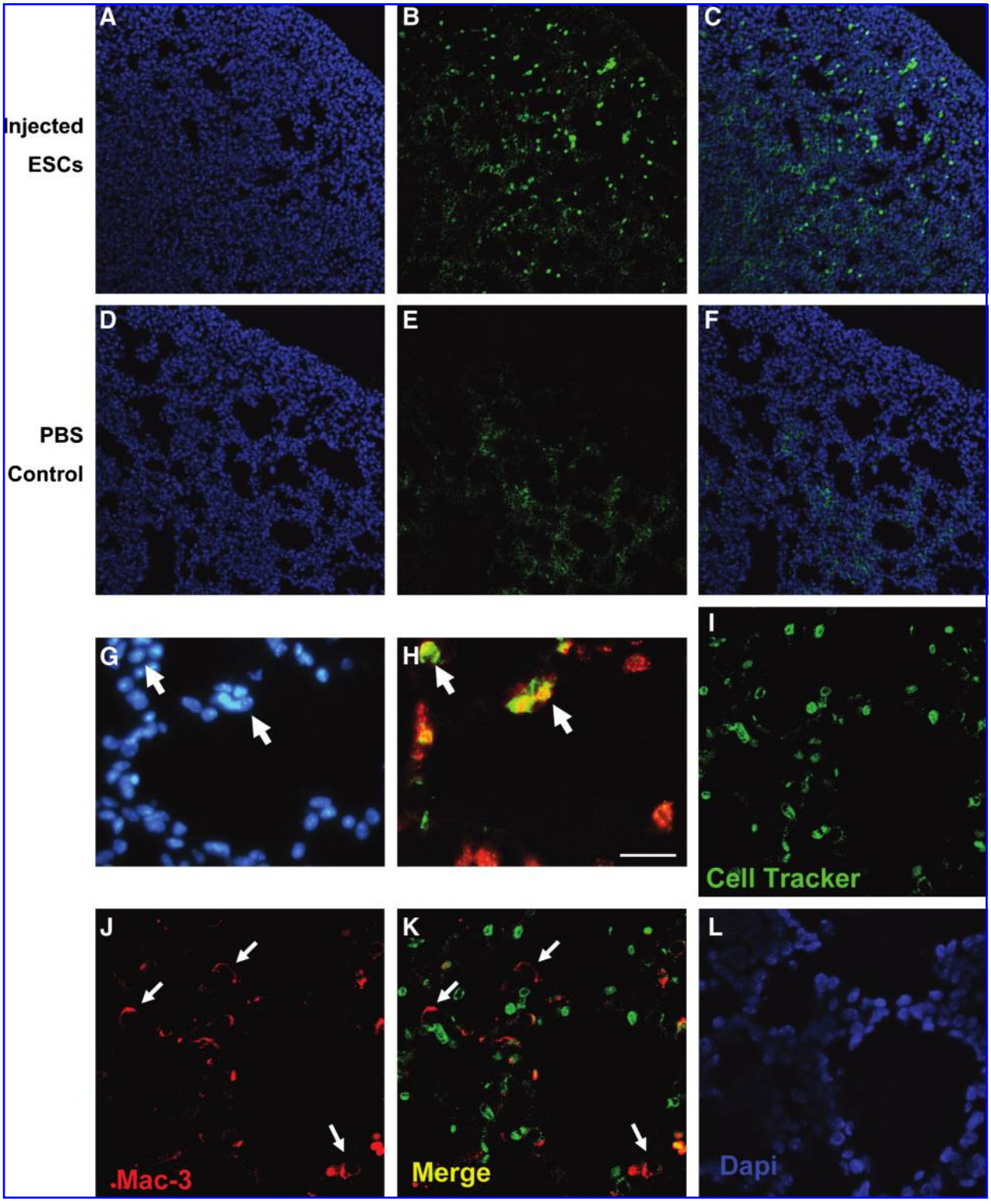


FIG. 10. Intratracheal delivery of ES-derived cells. An enriched population of type II cells (derived from E14tg2a cells) were labeled with CMTX cell tracker (green) and endotracheally instilled into preterm E18 mice. Twenty-four hours later, the mice were sacrificed and lung tissue was perfused, harvested, fixed, and counterstained with DAPI (blue) (**A–C**). As a control, PBS was injected and no labeled cells were detected (**D–F**). Next, ES CD-4Foxa2/Bry-GFP cells were instilled and detected by the CD-4 expressed under the Foxa2 promoter. (**C–I**) Colocalization of CD4 (green) and SPC (red) mark instilled cells that retain the alveolar epithelial type II phenotype. High-magnification images of CD4 (green) and SPC (red) coexpression (arrows) are shown in (**H**) along with the corresponding nuclear stain (BBZ; **G**). As a control, lung sections were also stained with Mac-3 (red, arrows) antibody and cell tracker (green) to exclude the possibility that double-positive cells mark macrophage phagocytosed ES-derived cells (**I–L**). Bars = 33 μm. Color images available online at www.liebertonline.com/ten.

staining revealed only rarely colocalized expression with Foxa-2/CD4-labeled cells (Fig. 10I–L). These results demonstrate the feasibility of endotracheal instillation of ES-derived cells for possible clinical applications.

Discussion

Cell replacement therapy to treat lung disease will require an abundant cell source for engraftment. AEII cells are attractive candidates for cell-based therapy, since these cells specialize in the production of surfactant in the distal alveoli. Additionally, AEII cells secrete high levels of vascular endothelial growth factor, a protein shown to extend life when injected endotracheally in a mouse model of respiratory distress. Still, generating large quantities of these cells remains a challenge. Here, we describe a protocol to derive an enriched population of lung-like cells based on a two-step differentiation protocol that recapitulates the development of lung epithelial cells *in vivo* and provides further evidence that FGF2 is a key factor for inducing differentiation of definitive endoderm to lung AEII cells. Importantly, serum-free protocols for AEII differentiation are being investigated by us and others so that these protocols can be applied without the need for genetic manipulation. In addition, we have demonstrated the feasibility of introducing these derived cells endotracheally as a preliminary step toward translating these findings into treatment options.

The first goal of this study was to find optimal culture conditions that promote efficient and rapid initial ESC differentiation into definitive endoderm. We compared aggregation of ESCs into EBs with a dissociated seeding technique since both have been previously used for endoderm or lung epithelial differentiation. Yasunaga *et al.* reported a protocol for efficient derivation of endoderm using a low-density seeding method together with activin that specifically inhibited visceral endoderm differentiation, and promoted definitive endoderm differentiation. Still, most studies that include an endoderm enrichment step to derive AEII cells have instead employed the more conventional ES differentiation strategies using EBs, but require culture periods as long as 35 days. Results from Wang *et al.* show that shorter culture times are required for detection of SP-C mRNA transcripts when using a disaggregated seeding method compared to an EB seeding method.

In the EB configuration, a population of ESCs in the core of the EBs consistently stained positive for Oct4, while the outer layer expressed differentiation markers GATA4 (Fig. 2G). By contrast, virtually no undifferentiated cells were detectable by day 4 using a single cell seeding and two-dimensional culture method, as inferred from the absence of Oct4 expression (Fig. 2H). Together, these data support our hypothesis that ESC differentiation is more rapid and effective using a disaggregated seeding method. One possible explanation is that a disaggregated seeding method allows for more uniform exposure of the cells to factors in the medium, thereby synchronizing the differentiation process to more efficiently direct cell fates.

Previous studies have shown that coculture with lung cells can promote ES differentiation into SP-C expressing cells. For example, Denham *et al.* showed that ESCs cocultured with E11.5 fetal lung cells displayed immunoreactivity to SPC and pan-keratin. Another study showed that cell extracts from MLE-12 cells, a murine AEII-derived cell line, could promote conditioned medium–induced pulmonary differentiation of ESCs. Rippon *et al.* have shown that an activin induction step increases lung-specific mRNA expression compared to serum-free medium alone. To determine whether the induction of ESCs from AEII-derived factors requires an endoderm intermediate, we cultured ESCs in A549-conditioned medium and assayed for expression of endoderm markers. Both immunostaining (Fig. 3) and FACS analysis (Fig. 4) showed that A549-conditioned medium promoted endoderm differentiation at least as efficiently as treatment with 20 ng/mL activin alone. Although activin has been shown to induce endoderm differentiation, these data suggest that additional factors besides activin may be capable of promoting more efficient endoderm differentiation.

We then attempted to identify likely factors that could potentially improve endoderm yield. In a recent study, EBs with upregulated expression of endoderm markers also had upregulated expression of Wnt3a and nodal. A549 cells express Wnt3a. We found that activin and Wnt3a efficiently induce endoderm differentiation. Further, our data suggest that Wnt3a likely improves endoderm differentiation by inhibition of activin-induced primitive endoderm differentiation, as assessed by GATA6 expression. Interestingly, later in development, GATA6 reappears in the lung epithelium and is required for proper differentiation of lung epithelial stem cells and acts to suppress the canonical Wnt pathway. In mice lacking GATA6 in the lung epithelium, an increase in the number of bronchoalveolar stem cells was observed, perhaps due to an inability of these stem cells to terminally differentiate into mature alveolar epithelial cell types.

Next, we tested the ability of the derived endoderm to further differentiate into AEII-like cells. In line with developmental considerations we hypothesized that FGF2 is a factor responsible for specifying lung from ES-derived endoderm *in vitro* and found (Figs. 7 and 8) that 50 and 500 ng/mL FGF2 was sufficient to promote SP-C expression and immunoreactivity. As a pleiotropic factor secreted from the cardiac mesoderm at the time of the lung specification, these results suggest that FGF2 is also capable of inducing SP-C in activin-primed ESCs, similar to its proposed role *in vivo*. Comparison of low (5 ng/mL) and high (50 ng/mL) doses of FGF2 also revealed that high doses of FGF2 induced a 20-fold downregulation of pancreatic marker PDX-1. Serls *et al.* showed that embryonic endoderm cultured in the absence of cardiac mesoderm or FGF2 defaulted to a pancreatic lineage, which is likely analogous to the situation with the ES-derived endoderm.

We observed the formation of SPC immunoreactive colonies (Fig. 8C, J, K) after differentiation with our optimized protocol. Interestingly, the conditioned medium method generated a larger standard deviation (7.6%) of SPC expression, whereas the developmentally similar route using activin plus FGF2 was a more reproducible method of generating SPC-expressing cells (standard deviation = 3.8%). Cell heterogeneity in A549 cultures and batch variability among conditioned medium may be responsible for its limited ability to be a consistent inducing factor in AEII differentiation. Addition of a chimeric FGFR1/IgG-Fc protein attenuated immunoreactivity to SP-C in differentiating cultures, suggesting that FGF signaling is required for AEII differentiation. Further studies are required to determine which specific receptors mediate AEII differen-

tiation, since FGF2 binds to each of the four FGFR isoforms. When assayed for ultrastructural evidence of AEII differentiation, FGF-stimulated cells were found to contain lamellar bodies, which resembled those found in MLE-12 cells.

It is unclear why current protocols using only soluble factors to promote AEII differentiation requires up to 31 days of culture time. Two lines of evidence suggest that this culture time can be reduced significantly. First, the report by Wang *et al.* demonstrate the detection of SP-C by RT-PCR and immunostaining in only 10 days of culture of human ESCs on matrigel in the absence of EB formation. Although it is difficult to determine which factors present in the matrigel may promote SPC expression, matrigel does contain FGF2 among other growth factors and extracellular matrix components. Second, our control cultures differentiated in the absence of FGF2 display colonies of beating cardiomyocytes by day 10 of differentiation (data not shown), an event known to coincide with initiation of lung-specific gene expression in ventral foregut explant assays.

Cell-based tissue engineering studies have shown promising results for lung regeneration. Recently, Carraro *et al.* have demonstrated that tail vein–injected amniotic stem cells preferentially home in on the lung and can contribute to both proximal and distal airway epithelial cell types in response to lung damage. Another approach to cell-based tissue engineering is the use of scaffolds that both direct cell differentiation and morphogenesis of lung precursors. As a caveat, however, these tissue-engineered constructs (especially synthetic polymers) have been shown to induce an immune reaction that is independent of the cell types used.

In previous studies, we refined a delivery technique for introducing cells into the lung, and show that these survive and maintain their phenotype *in vivo*. A refined method of delivery and a practical cell source, such as ESCs, creates an instant use for these cells for *in vivo* studies using lung disease models. For example, myofibroblasts have been used for cell-based endotracheal instillation to treat emphysema with evidence of engraftment. Also, studies have shown that AEII cells can engraft and reverse fibrosis in a bleomycin lung injury model. Here, we demonstrated the ability to endotracheally administer derived AEII-like cells into the lung with maintenance of differentiation. Further, these delivered cells appear to engraft into the lung parenchyma. In this study, we used CD4-labeled cells in lieu of cell tracker due to the observation that the dye may be released and yield nonspecific staining. Still, as a caveat, enriching for CD4-positive cells might result in enriching for T lymphocytes as well. As in some autoimmune diseases, overabundance of helper T cells can lead to cytotoxic granulocyte release. In all, our study demonstrates advancement toward a cell-based treatment of pulmonary hypoplasia.

In conclusion, differentiation to AEII cells is dose dependent on FGF2, requiring high doses of FGF2 for efficient induction. These results, in combination with other studies investigating medium composition on directed differentiation of AEII cells, will contribute to our ability to derive a large source of these cells for cell-based treatment of lung disease. In addition, we were able to reliably introduce derived AEII cells into the distal alveoli as a first step in translational research. Further characterization of the effects of AEII cells on pulmonary hypoplasia is needed to establish this as a viable treatment option.

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Disclosure Statement

No competing financial interests exist.

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Address correspondence to:
Christine M. Finck, M.D.
Connecticut Children's Medical Center
282 Washington St.
Hartford, CT 06106

E-mail: cfinck1234@hotmail.com

Peter I. Lelkes, Ph.D.
School of Biomedical Engineering, Sciences, and Health Systems
Drexel University
Bossone Research Enterprise, Room 707
3141 Chestnut St.
Philadelphia, PA 19104

E-mail: pilelkes@drexel.edu

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