

# Engineering Three-Dimensional Pulmonary Tissue Constructs

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## ABSTRACT

In this paper, we report on engineering 3-D pulmonary tissue constructs *in vitro*. Primary isolates of murine embryonic day 18 fetal pulmonary cells (FPC) were comprised of a mixed population of epithelial, mesenchymal, and endothelial cells as assessed by immunohistochemistry and RT-PCR of 2-D cultures. The alveolar type II (AE2) cell phenotype in 2-D and 3-D cultures was confirmed by detection of SpC gene expression and presence of the gene product prosurfactant protein C. Three-dimensional constructs of FPC were generated utilizing Matrigel hydrogel and synthetic polymer scaffolds of poly-lactic-co-glycolic acid (PLGA) and poly-L-lactic-acid (PLLA) fabricated into porous foams and nanofibrous matrices, respectively. Three-dimensional Matrigel constructs contained alveolar forming units (AFU) comprised of cells displaying AE2 cellular ultrastructure while expressing the SpC gene and gene product. The addition of tissue-specific growth factors induced formation of branching, sacculated epithelial structures reminiscent of the distal lung architecture. Importantly, 3-D culture was necessary for inducing expression of the morphogenesis-associated distal epithelial gene fibroblast growth factor receptor 2 (FGFr2). PLGA foams and PLLA nanofiber scaffolds facilitated ingrowth of FPC, as evidenced by histology. However, these matrices did not support the survival of distal lung epithelial cells, despite the presence of tissue-specific growth factors. Our results may provide the first step on the long road toward engineering distal pulmonary tissue for augmenting and/or replacing dysfunctional native lung in diseases, such as neonatal pulmonary hypoplasia.

## INTRODUCTION

**P**ULMONARY HYPOPLASIA (PH) is found in as many as 15 to 20% of all neonatal autopsies, which translates into more than 2800 deaths annually in the United States.<sup>1</sup> The pathology of PH includes reduced lung mass, insufficient surfactant production due to poorly differentiated alveolar epithelium, and reduction of alveolar gas exchange.<sup>2</sup> Augmentation of underdeveloped lungs may be achieved by engineered functional pulmonary tissue. The ability to engineer functional lung tissue *in vitro* would

have a substantial impact as it could provide therapeutic modalities for many other pediatric pulmonary disease states in addition to PH, such as bronchopulmonary dysplasia, hyaline membrane disease, congenital diaphragmatic hernia, and cystic fibrosis. In addition, engineered pulmonary-like tissue would provide a means of performing *in vitro* studies, such as pharmaceutical screening, models for lung development, and characterization of mechanical injury.

Alveoli are the terminal portions of the bronchial tree and are about 200  $\mu\text{m}$  in diameter in humans.<sup>3</sup> The alve-

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olus is composed of alveolar epithelium, endothelium, and interstitial cells. There are two types of alveolar epithelial cells: type I (AE1) and type II (AE2). The squamous AE1 cells are less numerous but their cytoplasm covers approximately 95% of the airspace walls.<sup>3</sup> The cuboidal AE2 cells cover significantly less surface area; however, they comprise approximately 30% of the total cells in the entire lung, with the majority lying basal to the alveolar surface. AE2 cells synthesize and store surfactant in lamellar bodies and secrete it into the alveolar space. In the alveolus, surfactant transforms into tubular myelin, which unfolds; surfactant lipids are inserted into the lipid monolayer at the air-liquid interface.<sup>4</sup> This surfactant is responsible for stabilizing alveoli and decreasing surface tension, thus facilitating gas exchange.<sup>3,4</sup> While recognizing the importance of the AE1 cell in generating an alveolar structure, in this study we focused on the functionality of AE2 cells cultured in 3-D scaffolds.

In conventional 2-D culture, AE2 cells lose many of their specialized features, such as their ability to produce surfactant.<sup>5,6</sup> Morphological change and lamellar body loss can be demonstrated after several days of primary culture and suggest that AE2 cells rapidly de-differentiate.<sup>7</sup> In extending previous work by others,<sup>8</sup> we hypothesize that 3-D biomimetic scaffolds, in combination with tissue-specific growth factors, will induce organotypic differentiation. In testing our hypothesis, we successfully established primary cultures of murine mixed fetal pulmonary cells containing epithelial, mesenchymal, and endothelial cells. Furthermore, we were able to maintain the differentiation state of AE2 cells for extended periods *in vitro* utilizing diverse 3-D scaffolds. In addition, our results demonstrate that the use of tissue-specific growth factors enhances epithelial branching morphogenesis in 3-D Matrigel hydrogels *in vitro*, while also demonstrating the necessity of 3-D culture to induce expression of epithelial genes related to the morphogenetic process, specifically FGFR2.

## MATERIALS AND METHODS

### *Fetal pulmonary cell isolation and in vitro culture*

All animal procedures were carried out in accordance with a protocol approved by the Institutional Animal Care and Usage Committee (IACUC #30511).

Murine fetal pulmonary cells were obtained from the lungs of timed-pregnant Swiss Webster mouse fetuses at gestational day 18 (Charles River Laboratories, Wilmington, VA), essentially following the protocol described by Bates *et al.*<sup>9</sup> Briefly, fetal lungs were surgically removed, rinsed in Hanks Balanced Salt Solution (HBSS; Cellgro, Herndon, VA), minced, triturated, and digested with 0.5% trypsin in PBS for 5 and 20 min, respectively.

Following quenching of the trypsin with Dulbecco's modified Eagle medium containing 10% FBS (Cambrex; East Rutherford, NJ) and filtration through a 70  $\mu\text{m}$  filter (BD Falcon, San Jose, CA), the cell suspension was pelleted for 5 min at 800 rpm. The pellet was resuspended for 30 s in distilled water to remove red blood cells by hypotonic lysis,<sup>9</sup> followed by the addition of 100  $\mu\text{L}$   $10 \times$  PBS (Cellgro). The cells were washed once more in  $1 \times$   $\text{Ca}^{2+}/\text{Mg}^{2+}$  containing PBS, resuspended in complete medium (DMEM + 10% fetal bovine serum + antibiotics) and counted. Cell viability was assessed in a fluorescent microscope using the live/dead assay (Molecular Probes, Eugene, OR), according to the manufacturer's instructions. For the initial 24 hours, primary isolates were cultured in DMEM medium (Cambrex), supplemented with 10% fetal bovine serum (Hyclone, Logan, UT), L-glutamine, penicillin-streptomycin antibiotics, and 1% insulin-transferrin-selenium (ITS) supplement containing linoleic acid and BSA (BD Biosciences, San Jose, CA). After 24 hours, the culture media was switched to one of two serum-free media formulations: serum-free DMEM with the same supplements noted above (SF-ITS) was used in early experiments, whereas in later series of experiments, a serum-free, tissue-specific, growth factor-defined medium (SF-GF) containing FGF-7 (12.5 ng/mL), FGF-10 (25 ng/mL), and bFGF (12.5 ng/mL) in an 8:2 mixture of DMEM:F12 with L-glutamine and penicillin-streptomycin antibiotics was used to enhance epithelial cytodifferentiation and tissue construct morphogenesis. All cell culture was carried out at 37°C in a 5%  $\text{CO}_2$  humidified incubator.

### *Immunohistochemistry*

Identification of AE2 cells and epithelial cells in our cultures was carried out by indirect immunohistochemistry (IHC), as previously described.<sup>10</sup> In brief, fetal pulmonary cells were cultured in tissue culture chamber slides (Fisher) on Matrigel (BD Biosciences), type I collagen (BD Biosciences), and poly-D-lysine (Sigma, St. Louis, MO) matrices as described. At various time points (1–4 days post plating), the cultures were fixed with 10% neutral buffered formalin (Formalde-Fresh, Fisher) for 15 min at room temperature, washed with tris buffered saline (TBS, pH 7.4) and permeabilized with 25  $\mu\text{M}$  digitonin (in TBS) for 10 min. For visualization with peroxidase-labeled secondary antibodies, the samples were treated for 5 min with 0.3%  $\text{H}_2\text{O}_2$  in methanol to block endogenous peroxidase activity. For paraffin-embedded samples, sections were deparaffinized and rehydrated according to standard protocol, washed with TBS containing 1% BSA, and exposed to the same peroxidase blocking steps. All subsequent washing and incubation procedures were carried out in TBS containing 0.1% BSA to block nonspecific staining. In the case of paraffin-embedded samples, an additional blocking step with 3% BSA

in TBS pH 7.4 for 30 min was implemented. In the case of vimentin immunostaining, the use of a monoclonal mouse antibody necessitated the use of a Mouse2Mouse kit (Scytek, Logan, UT) containing specific blocking agents for mouse tissue and cells. The slides were incubated for 45–90 minutes at room temperature with polyclonal rabbit primary antibodies against prosurfactant protein C (1:500, Chemicon, Temecula, CA), cytokeratin (1:500, Dako, Carpinteria, CA), and vimentin (1:100, Santa Cruz Biotechnology, Santa Cruz, CA). Negative controls were processed by omission of the primary antibody and/or utilizing preimmune serum of the animal where the primary antibody was raised. After rinsing each with TBS three times for 1 min, the slides were incubated for 30 min with either fluorescent (1:1000, Alexa 488 conjugated, goat-anti rabbit IgG for cytokeratin staining; Molecular Probes) or immunoperoxidase conjugated secondary antibody (Dako AEC+ kit, prosurfactant C and cytokeratin staining or Scytek M2M kit, vimentin). If desired, samples stained with fluorescent conjugated secondary antibody were counterstained with the fluorescent DNA stain bisbenzimidazole (2  $\mu\text{g}/\text{mL}$ ; Hoechst 33258, Sigma) and/or rhodamine-phalloidin (1  $\mu\text{g}/\text{mL}$ ; Sigma) and viewed on a fluorescent microscope (Leica DMRX, Wetzlar, Germany). Peroxidase-stained samples were counterstained for 30 s with hematoxylin (Fisher). Endothelial cells were stained with a murine endothelial specific marker GSL I isolectin B<sub>4</sub> (GSL Iso B<sub>4</sub>) (FITC-conjugate from the African legume, *Griffonia simplicifolia*; Vector Labs, Burlingame, CA). In brief, cells were fixed in formalin and incubated in a 5  $\mu\text{g}/\text{mL}$  FITC-conjugated GSL Iso B<sub>4</sub> lectin solution, containing bisbenzimidazole and rhodamine-phalloidin for counterstaining, prepared in 1  $\times$  PBS for 10 min, washed three times for 1 min in 1  $\times$  PBS, and viewed with a Leica fluorescent microscope. Percentages for individual cell counts were obtained by means of automated counting of DAPI stained nuclei and manual counting of GSL Iso B<sub>4</sub> and cytokeratin-positive cells in 45 individual fields across three independent experiments (15 fields per experiment).

#### Reverse transcriptase polymerase chain reaction

Reverse transcriptase polymerase chain reaction (RT-PCR) was utilized to detect steady-state mRNA expres-

sion of relevant genes: surfactant protein C (SpC) for AE2 cells and vimentin for cells of mesenchymal origin. RT-PCR was carried out as previously described<sup>11,12</sup> with some modifications. Briefly, total RNA was isolated from the 2-D cell cultures growing on Matrigel, collagen, and poly-D-lysine substrates using the RNeasy column method (Quiagen, Valencia, CA) with an additional DNA digestion step to remove contaminating genomic DNA. Total RNA was isolated from 3-D Matrigel hydrogels and synthetic polymer scaffolds by digestion with TriReagent (Sigma) and subsequent purification of the RNA-containing aqueous extraction phase on an RNeasy column (Quiagen), according to the manufacturers' protocols. The quality of isolated RNA was assessed by measuring the ratio of OD<sub>260</sub>/OD<sub>280</sub> and by electrophoresis in 1% agarose/formamide gels with ethidium bromide containing loading buffer. The isolated RNA was reverse transcribed using a commercial RT kit (Promega, Madison, WI), according to the manufacturer's instructions; the resultant complementary DNA was used for PCR amplification. Briefly, the cDNA was added to a reaction mixture containing 1.5 mM magnesium chloride, 10 mM dNTP, 2% v/v Taq enzyme, and 15  $\mu\text{M}$  forward and reverse primers optimized for each gene of interest in preliminary experiments. For all genes, a 35 cycle 2-step PCR routine with a 45 s denaturation step at 94°C and an 80 s combined annealing and extension step at 68°C was used (conditions specified by Clontech, Mountain View, CA, for their Atlas gene arrays). Negative controls run for all PCR reactions included no reverse transcription samples to check for genomic DNA, as well as reactions without the addition of the cDNA templates. The primer sequences used in characterization of FPC populations are shown in Table 1 (Clontech Atlas, Mouse 1.2 Array II, Cat. # 7857-1, BD Biosciences).

#### Fabrication of PLLA nanofiber scaffolds

The electrospinning solution was prepared by adding 1.25 g poly-L-lactic acid (PLLA, a gift from Dr. Frank K. Ko, Dept. of Materials Science and Engineering, Drexel University) to 40.625 g chloroform (Sigma) while stirring and heating the solution to 50°C. After 1 h, 8.125 g dimethyl formamide (Sigma) was added under continued stirring. The final concentration of PLLA used for

TABLE 1. CDNA PRIMER SEQUENCES USED FOR REVERSE-TRANSCRIPTASE POLYMERASE CHAIN REACTION

| Gene     | Forward primer              | Reverse primer            | Product length |
|----------|-----------------------------|---------------------------|----------------|
| SpC      | AGCGAGCAGACACCATCGCTACC     | CTCGGAACCAGTATCATGCCCTTC  | 242            |
| Vimentin | GCTCGCTCGGCGGCTAGGATG       | CTGGTAGACATGGCTTCGAAGGTG  | 218            |
| FGF10    | AGATAACATCAGTGGAAATCGGAGTTG | GTACATTTGCCTGCCATTGTGCTGC | 197            |
| FGFr2    | GAGAGCACCGTACTGGACCAACAC    | GACCACACTTTCATAATAAGGCTCC | 214            |

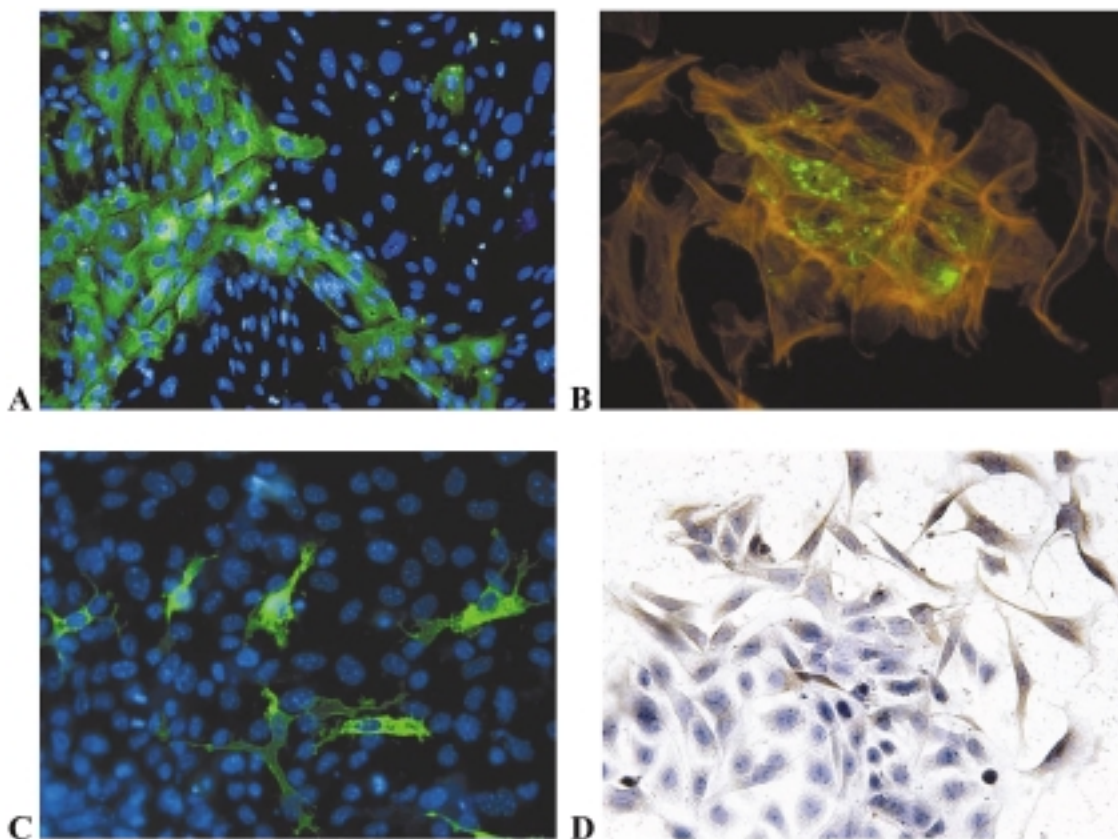
electrospinning was 2.5 wt %. The PLLA solution was loaded into a calibrated syringe pump (KD Scientific Single-Syringe Infusion Pump, Fisher, 14-831-1). Electrospinning was carried out using the following system parameters: electrical field strength of 25 kV (1.25 kV/cm), spin distance of  $\sim 12$  cm, with a spinning time of  $\sim 6$  h.<sup>13</sup> The effective thickness of PLLA nanofiber scaffolds was 1–2 mm.

#### *Poly L-lactic-co-glycolic acid sponges*

Porous sponges, composed of 90% polylactic acid–10% polyglycolic acid (90:10 PLGA) used for 3-D culture of fetal pulmonary cells were a generous gift from Professor Dr. Xuesi Chen (Chinese Academy of Sciences, Changchun Institute of Applied Chemistry, Key National Polymer Laboratories, Changchun, China). These scaffolds were fabricated in Dr. Chen's laboratory using NaCl as a porogen. The scaffolds were trimmed to approximately  $5 \times 5$  mm cubes and sterilized with 70% ethanol prior to use.

#### *Scaffold seeding*

Matrigel hydrogels were created by admixing either 100,000 FPC/mL in early experiments or 1,000,000 FPC/mL in later experiments into liquid Matrigel at a 1:9 volume ratio (cell suspension–Matrigel) under aseptic conditions and inducing gelation by incubation at 37°C. Prior to seeding, PLGA porous foams and PLLA nanofiber matrices were sterilized by soaking in 70% ethanol for 1 h, followed by air drying and prewetting in serum-free DMEM. Murine fetal pulmonary cells were seeded onto the electrospun PLLA nanofiber matrix and PLGA porous sponges by dynamic seeding of scaffolds with high-density cell suspension overnight in an orbital shaker (Belly Dancer, Stovall, Greensboro, NC). A cell suspension of 500,000–1,000,000 cell/mL was found to be optimal (unpublished observations). The total time period for dynamic seeding was  $\sim 24$  hours. Following dynamic seeding, scaffolds were cultured for up to 4 weeks in static conditions and for select experiments in 55 mL rotating wall vessel bioreactors (RWV, Synthecon, Hous-



**FIG. 1.** Immunohistochemical analysis of primary fetal pulmonary isolates following 4 days of *in vitro* culture on Matrigel-coated tissue culture chamber slides with SF-ITS medium (except **C** with SF-GF medium). **(A)** Cytokeratin (green) staining of epithelial cells, bisbenzimidazole nuclear counterstain ( $200\times$ ). **(B)** Prosurfactant protein C (green) staining of AE2 cells, rhodamine-phalloidin cytoskeletal counterstaining (original magnification  $\times 400$ ). **(C)** GSL Iso B<sub>4</sub> (green) staining of endothelial cells, bisbenzimidazole nuclear counterstaining ( $400\times$ ). **(D)** Vimentin (brown, DAB-horse radish peroxidase) staining of mesenchymal cells, hematoxylin nuclear counterstaining ( $400\times$ ).

ton, TX) for up to 14 days.<sup>14</sup> All RWV experiments were carried out with SF-ITS medium. For economical reasons, all experiments investigating the effect of growth factors on cultures in 3-D polymer scaffolds were carried out under static conditions in 12-well plates.

### Routine histology

All 3-D assemblies were processed for routine histology (H&E staining). Briefly, formalin fixed samples were dehydrated through a series of graded alcohols, cleared with xylene, and embedded with paraffin wax according to standard protocol.<sup>12,15,16</sup> Ten micrometer sections were cut using a rotary microtome (Leitz 1512), deparaffinized, rehydrated, and stained with H&E.

### Transmission electron microscopy

The ultrastructure of 3-D cultures maintained *in vitro* for 4 weeks in Matrigel gel and electrospun fibrous scaffolds, respectively, were examined as previously described.<sup>17</sup> Briefly, the samples were washed with cold 0.2 M Na cacodylate buffer (pH 7.4) and fixed overnight with 2.5% glutaraldehyde in cacodylate buffer. Following an additional wash in buffer, the samples were post-fixed with 2% OsO<sub>4</sub> in cacodylate buffer (1 h, 4°C), dehydrated in graded concentrations of cold ethyl alcohol, and embedded in epoxy resin. Ultrathin sections were examined with a Zeiss EM 109 microscope operated at 80 kV. At the time of sectioning for TEM, semi-thick sections were prepared for toluidine staining to examine overall cellular morphology within the constructs.<sup>18</sup>

## RESULTS

### Two-dimensional cultures

Immunohistochemical analysis of primary isolates of fetal pulmonary cells cultured in 2-D on a thin layer of Matrigel for 4 days with SF-ITS medium provided evidence of

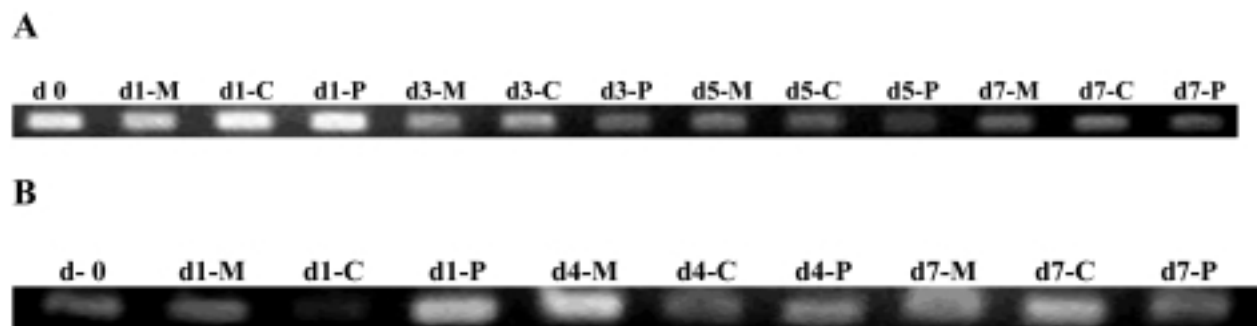
the presence of mixed cultures comprised of epithelial, mesenchymal, and endothelial cells (Fig. 1). The presence of epithelial and AE2 cells was assessed from the expression of cytokeratin and prosurfactant protein C, respectively (Fig. 1A and B). Pulmonary endothelial cells were detected with the endothelial specific lectin GSL Iso B<sub>4</sub> (Fig. 1C). Expression of vimentin confirms the presence of mesenchymal cells surrounding epithelial clusters in 2-D culture (Fig. 1D). Similar mixed populations were also observed in SF-GF medium, with noticeable increases in the number of epithelial and endothelial cells relative to mesenchymal cells (unpublished observations). Using the latter medium, we have quantified phenotypic distributions in 2-D on Matrigel-coated chamber slides. On the average, our primary isolates grown for 2 days on Matrigel comprised  $\sim 49 \pm 15\%$  epithelial cells and  $\sim 14 \pm 3\%$  endothelial cells.

RT-PCR analysis of cultured fetal pulmonary cells over 7 days on Matrigel, type I collagen, and poly-D-lysine substrates with SF-ITS medium demonstrated the continued expression of mRNA for the AE2 specific marker surfactant protein C (Fig. 2A) and the mesenchymal marker vimentin (Fig. 2B). Similar results were obtained with SF-GF medium.

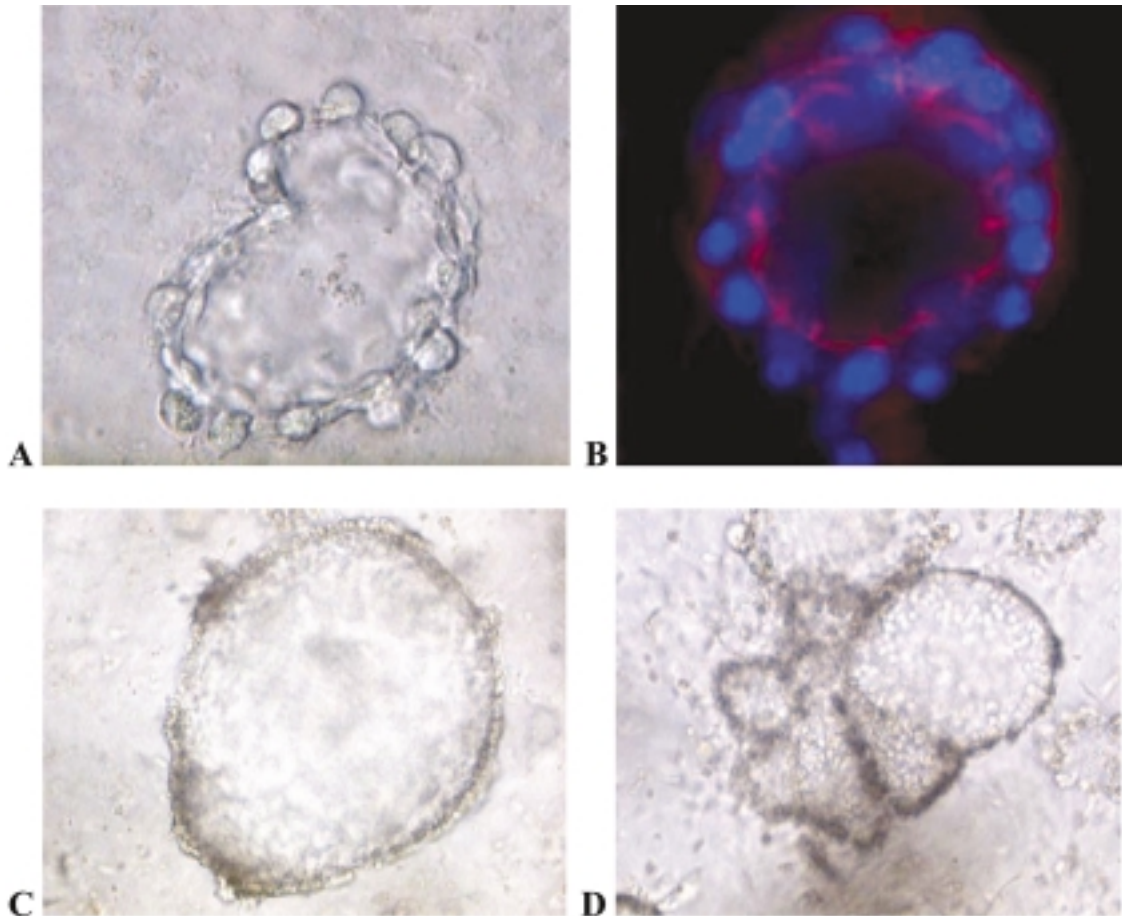
### Low seeding density, long-term 3-D hydrogel cultures with SF-ITS medium

When fetal pulmonary cells were cultured inside a 3-D Matrigel hydrogel, we observed, by phase contrast microscopy, the formation of ring like structures, resembling alveolar forming units (AFU) (Fig. 3A). These structures are seen as early as 24 h post-isolation and increase in size for up to 6 weeks in culture (unpublished observations). Nuclear staining illustrates the ring structure and indicates the presence of cells lining these structures (Fig. 3B). Staining of microfilaments with rhodamine-phalloidin outlines the cells in the AFU (Fig. 3B).

Evaluation of semi-thin sections of Matrigel constructs with toluidine blue staining demonstrates numerous



**FIG. 2.** Expression of type II alveolar and mesenchymal marker genes in 2-D cultures with SF-ITS medium. Steady-state mRNA expression was assessed by RT-PCR. (A) Surfactant protein C gene expression over 7 days *in vitro* on Matrigel (M), Collagen (C), and poly D lysine (P) coated slides. (B) Vimentin gene expression over 7 days on Matrigel (M), Collagen (C), and Poly D lysine (P) coated slides.



**FIG. 3.** Assembly of alveolar forming units *in vitro* in 3-D Matrigel hydrogel. **(A)** Phase-contrast micrograph of alveolar-like ring structure forming in Matrigel after 4 weeks in culture with SF-ITS medium (original magnification  $\times 400$ ). **(B)** Fluorescent micrograph of bisbenzamide (nuclei) and rhodamine-phalloidin (actin cytoskeleton) staining of alveolar-like ring structure in Matrigel after 4 weeks in culture in SF-ITS medium (original magnification  $\times 400$ ). **(C)** Phase-contrast micrograph of ring-shaped AFU following 14 days of culture in SF-ITS ( $100\times$ ). **(D)** Phase contrast micrograph of branching AFU following 14 days of culture of in SF-GF medium ( $100\times$ ).

AFUs at 4 weeks *in vitro* (Fig. 4). The histiotypic structure is reminiscent of the morphology of adult mouse lung tissue (Fig. 4A and B).

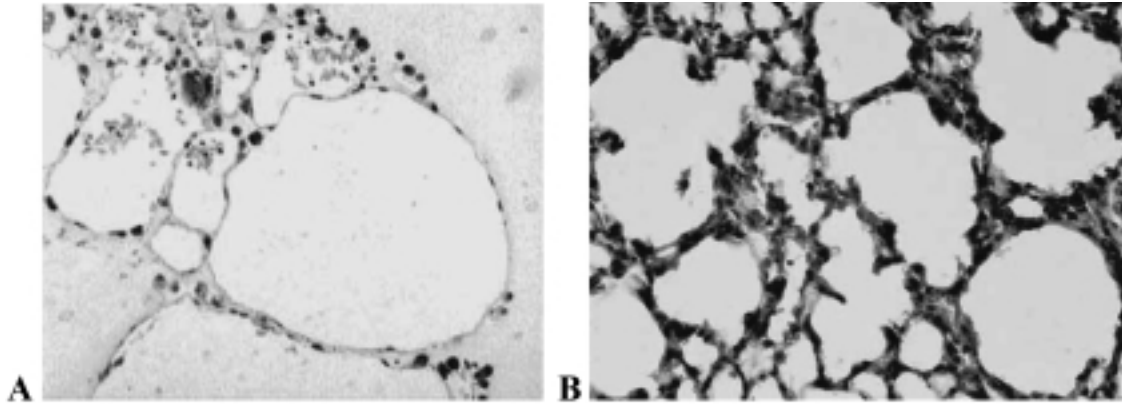
Ultrastructural analysis by TEM of FPC cultured in Matrigel hydrogel for 4 weeks with SF-ITS medium demonstrated the presence of gap junctional complexes (arrows), indicating cell-cell communication of the epithelial cells within the alveolar forming units (Fig. 5A) and microvilli commensurate with differentiated epithelial cells<sup>9</sup> (Fig. 5A and 5B). In line with similar reports in the literature,<sup>9</sup> lamellar bodies and tubular myelin indicative of differentiated AE2 cells were also detected (Fig. 5B).

#### *High seeding density, short(er)-term 3-D hydrogel cultures with SF-GF medium*

In SF-ITS media the AFUs expanded in size for up to 6 weeks (data not shown) indicating growth; however sacculation and formation of stabilized clefts were not

observed (Fig. 3C). However, with the use of high seeding densities and SF-GF medium, the induction of branching morphogenesis and sacculation were observed (Figs. 3D, 6A and C). Hematoxylin & eosin staining of pulmonary tissue constructs following 1 week of culture revealed branching lumen structures (Fig. 6A). These lumen structures, reminiscent of sacculation, were similar in appearance to the histology of native fetal lung (Fig. 6B). The epithelial phenotype of the cells lining these branching structures was confirmed with cytokeratin immunostaining (Fig. 6C). In addition, AE2 cells were localized in Matrigel hydrogel constructs, as illustrated by prosurfactant protein C immunostaining (Fig. 6D).

In order to demonstrate the importance of 3-D culture for maintaining distal epithelial gene expression associated with induction of branching morphogenesis and sacculation (Fig. 7), we compared expression of surfactant protein C (AE2 marker), FGF 10 (mesenchymal-derived morphogenetic inducer of the epithelium), and FGFr 2



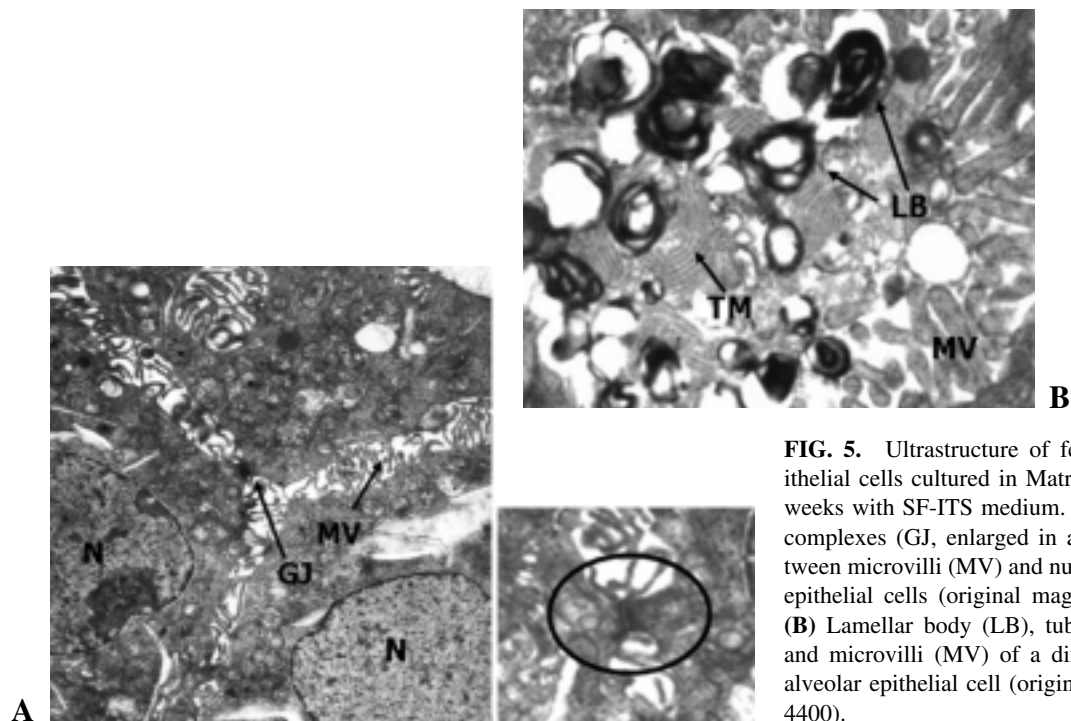
**FIG. 4.** Generation of histotypic lung structures *in vitro* in 3-D Matrigel hydrogel. (A) Toluidine Blue staining of mixed fetal pulmonary cells after 4 weeks in culture in 3-D Matrigel hydrogel with SF-ITS medium (200 ×). (B) Toluidine Blue staining of whole adult mouse lung (200 ×).

(epithelial morphogenetic receptor) in 3-D and 2-D cultures over 14 days *in vitro* (Fig. 7). Importantly, expression of the branching morphogenesis associated receptor FGFR2 was only detected in 3-D culture, despite the use of Matrigel substrate and tissue-specific growth factors in both 2-D and 3-D cultures.

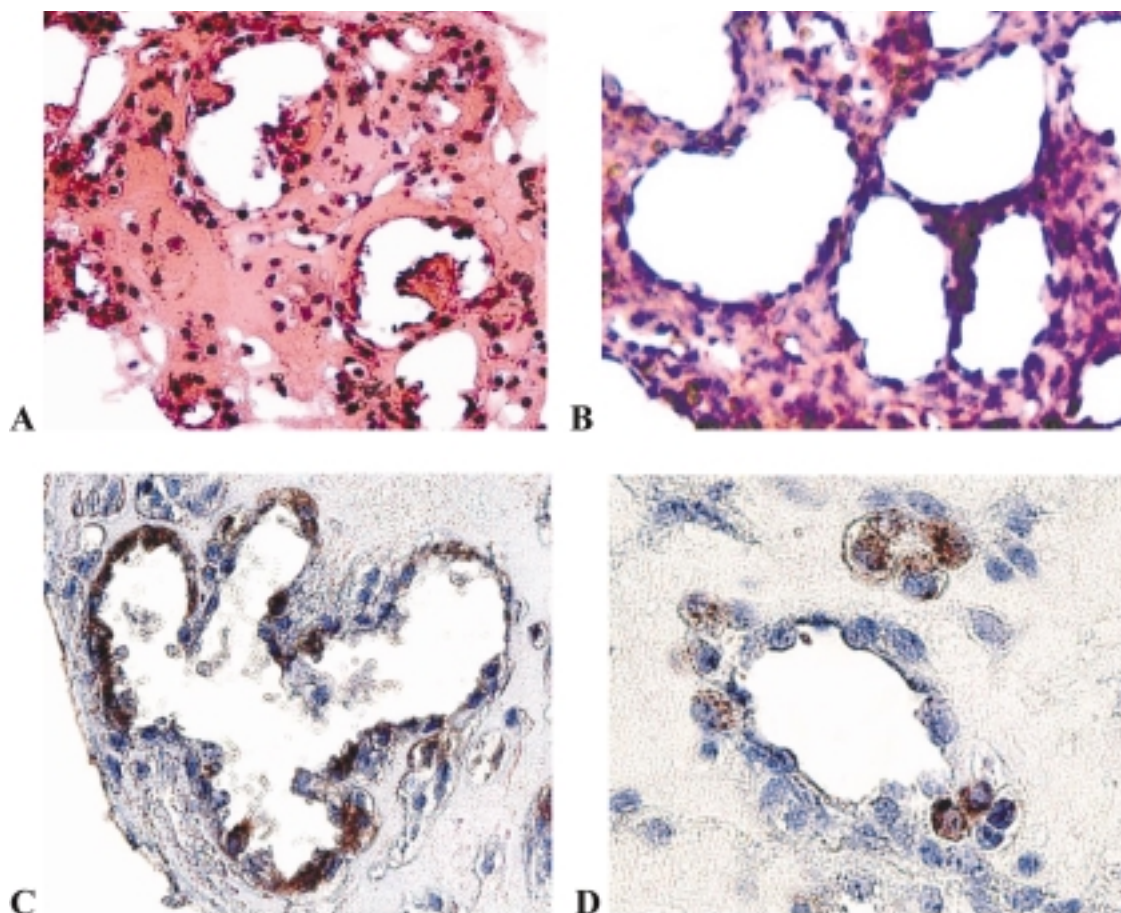
#### *Synthetic polymer scaffolds*

In extending these studies beyond natural hydrogels, we investigated the use of synthetic scaffolds following the polymer construct paradigm for engineering tissue constructs.<sup>19</sup> The architecture of the porous PLGA

sponges and PLLA electrospun submicron fibers, as analyzed with scanning electron microscopy, is shown in Figure 8A and B. PLGA sponges displayed a highly interconnected, randomly oriented porous structure. Fibrous electrospun PLLA scaffolds had nonwoven mesh morphologies with fiber diameters ranging from 100 nm to 1 μm. Hematoxylin & Eosin staining of fetal pulmonary cells cultured for up to 4 weeks on these synthetic scaffolds demonstrated cellular infiltration of both the fibrous matrices and porous sponges, albeit to different degrees. After 2 weeks in culture, the engineered constructs generated on the porous sponges developed along the sides of the porous architecture but did not fill the



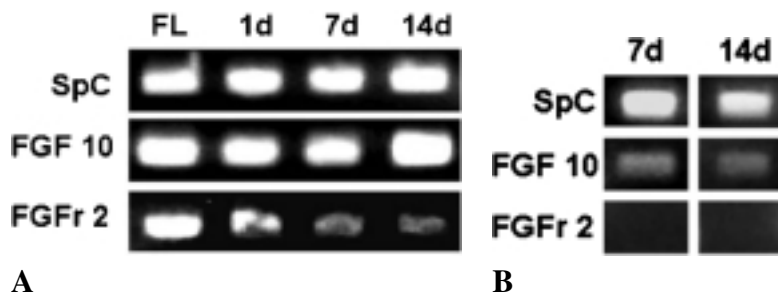
**FIG. 5.** Ultrastructure of fetal pulmonary epithelial cells cultured in Matrigel hydrogel for 4 weeks with SF-ITS medium. (A) Gap junctional complexes (GJ, enlarged in adjacent photo) between microvilli (MV) and nuclei (N) of adjacent epithelial cells (original magnification × 4400). (B) Lamellar body (LB), tubular myelin (TM), and microvilli (MV) of a differentiated type II alveolar epithelial cell (original magnification × 4400).



**FIG. 6.** Histological and phenotypic analysis of fetal pulmonary cells cultured in Matrigel hydrogel for 1 week with SF-GF medium. **(A)** H&E staining of fetal pulmonary cell constructs (400  $\times$ ). **(B)** H&E staining of embryonic day 17 mouse lung for histological comparison (400  $\times$ ). **(C)** Cytokeratin (red, AEC horse radish peroxidase) immunostaining of fetal pulmonary cell constructs demonstrating epithelial lining of branching AFU structures (original magnification  $\times$  400). **(D)** Prosurfactant protein C (red, AEC horse radish peroxidase) staining of fetal pulmonary constructs demonstrating presence of type II alveolar epithelial cells (original magnification  $\times$  400).

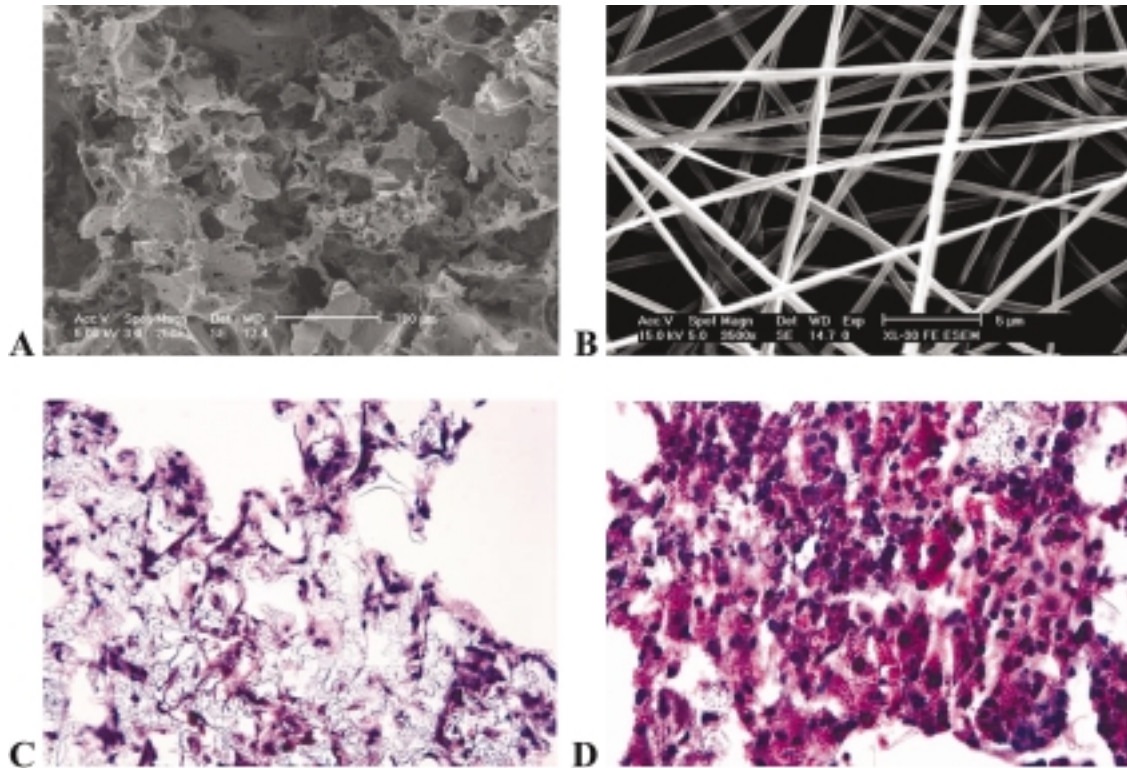
pores (Fig. 8C). In contrast, constructs generated using the nanofiber scaffolds displayed much higher cell density and more intimate cell-cell contact (Fig. 8D). AE2 differentiation in the synthetic scaffolds was assessed by ultrastructural analysis with TEM and RT-PCR of the

SpC gene. TEM analysis of the cells populating electrospun fibrous scaffolds after 4 weeks of static culture in SF-ITS medium demonstrated the presence of sporadic lamellar bodies. However, we could not detect tubular myelin, junctional complexes, or microvilli (Fig. 9A).



**FIG. 7.** Effect of 3-D culture on expression of the type II alveolar marker surfactant protein C (SpC) and the branching morphogenesis associated genes fibroblast growth factor 10 (FGF 10) and fibroblast growth factor receptor 2 (FGFr 2) *in vitro* with Matrigel and SF-GF medium. **(A)** Gene expression in 3-D Matrigel hydrogel at 1, 7, and 14 days of *in vitro* culture. RNA isolated from embryonic day 17 mouse lungs (FL) serves as a positive control. **(B)** Gene expression on 2-D on Matrigel-coated chamber slides with SF-GF medium.

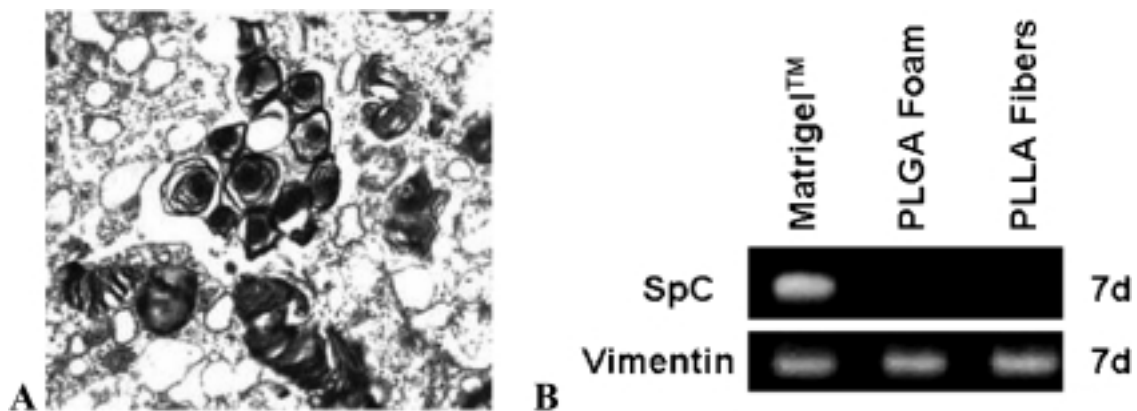




**FIG. 8.** Scanning electron micrographs of synthetic polymer scaffolds. (A) Porous PLGA sponge (original magnification  $\times 250$ ). (B) PLLA nanofiber scaffold (original magnification  $\times 3500$ ). (C and D) H&E staining of fetal pulmonary cells cultured for 14 days in rotating wall vessel bioreactors with SF-ITS medium on porous PLGA sponges (400  $\times$ ) (C) and electrospun PLLA nanofiber scaffolds (original magnification  $\times 400$ ) (D).

RT-PCR analysis for expression of the surfactant protein C gene in 4 week static cultures in PLGA foams and PLLA fiber scaffolds with SF-ITS medium yielded no positive results (unpublished observations), indicating

possible AE2 de-differentiation within these matrices. Similarly, following 1 week of *in vitro* static culture with SF-GF medium, SpC mRNA was not detected in PLGA foam or PLLA fiber scaffolds despite the presence of tis-



**FIG. 9.** Ultrastructure and gene expression of fetal pulmonary cells cultured in synthetic polymer scaffolds. (A) Transmission electron micrograph of lamellar bodies of a fetal pulmonary epithelial cell cultured on an electrospun fibrous PLLA scaffold under static conditions for 4 weeks with SF-ITS medium. (B) Expression of the type II alveolar epithelial marker surfactant protein C (SpC) and the mesenchymal marker vimentin following 7 days of *in vitro* static culture in Matrigel hydrogels, porous PLGA foams, and PLLA fiber scaffolds cultured with SF-GF medium.

sue-specific growth factors (Fig. 9B). However, at the same time, we found persistent expression of the vimentin gene, indicating the survival of mesenchymal cells (Fig. 9B).

## DISCUSSION

In this report we describe for the first time the generation of branching sacculated 3-D pulmonary tissue constructs using mixed populations of murine fetal pulmonary isolates, cultured in 3-D hydrogels in the presence of tissue-specific growth factors. Conversely, when grown on synthetic scaffolds, neither branching morphogenesis nor maintenance of epithelial differentiation was observed. Our studies are an extension of previous reports on 3-D growth of alveolar epithelial cells using a variety of matrices.<sup>8,20–24</sup> Douglas *et al.*<sup>20,21</sup> cultured dissociated pieces of rat fetal lung on top of a gelatin disc and demonstrated the presence of alveolar forming units *in vitro*. However, in these early studies, the authors noted a clear change in morphology of the cells by day 15, suggesting cellular de-differentiation. Most closely related to our work is the pioneering study by Sugihara *et al.*,<sup>8</sup> who reported that adult rat AE2 cells cultured in growth factor enriched media in a 3-D collagen gel matrix formed structures similar to alveolar forming units. In contrast to our observations, Sugihara did not report branching morphogenesis in their cultures. We believe that the enriched media contributed to organotypic growth, as in our findings, collagen gels alone, in the absence of the supplements used by Sugihara *et al.*, did not support AE2 cell differentiation (data not shown). In contrast to the studies by Sugihara and co-workers, who report the loss of cytokeratin 18 and surfactant protein A, we demonstrate the maintenance of surfactant protein C and cytokeratin over time in our 3-D cultures. Although surfactant protein A has a different biosynthetic pathway than surfactant protein C, we believe that prolonged maintenance of AE2 cell differentiation requires specific growth-factors present in Matrigel or added exogenously in concert with 3-D scaffolds.

Recapitulation of the cellular organization during development of the distal airway is necessary as a first step in generating functional distal lung tissue constructs. When cultured in appropriate scaffolds and media, the 3-D organization of FPC will lead to the induction and maintenance of a differentiated phenotype (Fig. 6). AE2 differentiation can be assessed based on cellular morphology, presence of lamellar bodies, and presence of surfactant phospholipids and proteins.<sup>5–9</sup> In conventional 2-D cultures, AE2 cells rapidly de-differentiate. Past attempts to prolong the differentiation state in 2-D cultures included altering the physical environment, such as providing an air-liquid interface<sup>22</sup> or plating the cells on

physiologic substrates such as Matrigel or collagen.<sup>23</sup> In our experiments, we utilized both 3-D scaffolds as well as a co-culture system comprised of a mixed population of primary isolates of fetal lung cells that contain mesenchymal and AE2 cells. This “co-culture” system was able to maintain AE2 cell differentiation as evidenced by the robust presence of lamellar bodies and the continued expression of the surfactant protein C gene and gene product in 2-D cultures with SF-ITS medium, which does not provide differentiative cues, only base nutrients required for survival (Figs. 1B and 2A). We believe the differentiation of the cells is most likely attributable to paracrine interactions between epithelial and mesenchymal cells. Furthermore, addition of exogenous growth factors to the medium prolonged the maintenance of differentiation, as well as induction of histiotypic morphology in 3-D cultures. This was only observed in 3-D cultures, stressing the pivotal role of three dimensionality in tissue-specific morphogenesis. Of particular importance is the expression of the branching morphogenesis related marker, FGFr2 in 3-D culture but not in 2-D culture (Fig. 7).

Previous studies have shown that cell-cell interaction, such as between the distal lung mesenchymal cell and AE2 cell, is essential<sup>5,25,26</sup> for maintenance of epithelial differentiation state. Epithelial-mesenchymal interactions provide the necessary differentiative cues for lung development<sup>27</sup> and have been shown to promote organotypic differentiation of functional engineered proximal airway constructs.<sup>28</sup> Furthermore, during lung development, there is a dynamic interplay between the diverse cell types that will form the distal airways.<sup>25</sup> For example, interactions with fibroblasts are necessary to increase production of mRNA for surfactant protein A (SP-A).<sup>26</sup> Conversely, AE2 cells secrete a factor(s) that inhibits the proliferation of lung fibroblasts *in vitro*.<sup>29</sup> The key event leading to the distal lung architecture is induction of epithelial branching morphogenesis (sacculatation in the alveoli) by the surrounding mesenchyme.<sup>27</sup> Numerous studies in developmental biology have identified mesenchymal cell secreted fibroblast growth factor 10 (FGF 10) and its associated receptor, fibroblast growth factor receptor 2 (FGFr 2), expressed exclusively in the epithelium, as key molecular players in the temporally and spatially controlled morphogenetic process.<sup>30,31</sup> These data show cell-cell interaction is pivotal to the formation of airways, and recapitulation of this is necessary in culture conditions. In line with these findings, our data indicate that use of a co-culture system, as well as 3-D Matrigel scaffolds, maintained epithelial differentiation.

In addition to cell-cell interactions, the interaction of the cells with the surrounding matrix is critical in lung development. In the developing lung the complex basal lamina, on which the epithelium resides, is composed mainly of type IV collagen, laminin, and a tissue-specific mix of glycosaminoglycans.<sup>32</sup> The importance of the ex-

tracellular matrix composition, specifically laminin, for modulating AE2 cell structure and function has been well documented.<sup>33,34</sup> The closest available product to a laminin-rich ECM is Matrigel, a reconstituted basement membrane derived from the Engelbroth-Holm-Swarm (EHS) mouse sarcoma. Studies by others,<sup>23</sup> as well as our data (Figs. 1B and 2A), have shown that AE2 cells grown in 2-D on Matrigel retain their differentiated form when compared with cells cultured on plastic surfaces. Moreover, in contrast to Matrigel, nonspecific ECM produced by endothelial cells failed to maintain AE2 cell phenotypic differentiation.<sup>35</sup> Similarly our results demonstrated that FPC grown on synthetic scaffolds did not maintain AE2 cell differentiation.

In using synthetic scaffolds as matrices, we compared porous and fibrous scaffolds composed of the paradigmatic biodegradable polymers PLLA and PLGA, respectively.<sup>36</sup> The ability to engineer a scaffold that provides architecture and the possibility of controlled release of morphogenetic signals in a spatially and temporally controlled manner would be valuable for recapitulating lung development. The natural ECM of the lung displays a fibrous morphology and the small diameters of the electrospun fibers have a large surface area–volume ratio, which enables absorption of liquids while facilitating cellular adhesion and cell-cell contact.<sup>37,38</sup> Recent results suggest that nano-topography is beneficial for cell function and differentiation.<sup>13,37,38</sup> Therefore, it is reasonable to assume that tissues with complex morphology such as lung would benefit from the “guidance” which can be provided with engineered fibrous scaffolds. As a first step toward this goal, we seeded our primary fetal pulmonary isolates into porous and randomly oriented scaffolds comprised of fibers smaller than 1  $\mu\text{m}$ . As seen in Figure 8C and 8D, penetration of the cells is evident into both scaffolds; however, the cells in the fibrous scaffold demonstrate improved cell assembly without exhibiting tissue-specific differentiation. In both our scaffolds, while there are no epithelial cells, there is robust mesenchymal cell proliferation. These results indicate that unmodified synthetic polymers provide mechanical support and allow for mesenchymal cell proliferation but lack the differentiative cues necessary for organotypic assembly of pulmonary tissue. In the case of synthetic scaffolds, even the addition of tissue-specific growth factors to the medium did not overcome the lack of epithelial differentiation. Therefore, we conclude that three dimensionality, in concert with tissue-specific cues provided by natural scaffolds, and the addition of tissue-specific growth factors to the culture medium are all necessary for inducing both distal lung epithelial cytodifferentiation and tissue morphogenesis. These results suggest that surface modification of synthetic scaffolds will be essential for supporting distal lung epithelial differentiation.

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## REFERENCES

- Centers for Disease Control and Prevention. Deaths: final data for 1998. *Natl. Vital Stat. Rep.* **48** (11), 2000.
- Leinwand, M.J., Tefft, J.D., Zhao, J., Coleman, C., Anderson, K.D., and Warburton, D. Nitrofen inhibition of pulmonary growth and development occurs in the early embryonic mouse. *J. Pediatr. Surg.* **37**, 1263, 2002.
- Junqueira, L.C., Carneiro, J., and Kelley, R.O. *Basic Histology*. Danbury, CT: Appleton and Lange, 1989, pp. 342–348.
- Poelma, D.L., Zimmermann, L.J., Scholten, H.H., Lachmann, B., and van Iwaarden, J.F. In vivo and in vitro uptake of surfactant lipids by alveolar type II cells and macrophages. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **283**, L648, 2002.
- Nandkumar, M., Yamato, M., Kushida, A., Konno, C., Hirose, M., Kikuchi, A., and Okano, T. Two-dimensional cell sheet manipulation of heterotypically co-cultured lung cells utilizing temperature-responsive culture dishes results in long-term maintenance of differentiated epithelial cell functions. *Biomaterials* **23**, 1121, 2002.
- Dobbs, L.G. Isolation and culture of alveolar type II cells. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **258**, L134, 1990.
- Eckenhoff, R., Rannels, S., and Fisher, A. Secretory granule calcium loss after isolation of rat alveolar type II cells. *J. Physiol.* **260**, L129, 1991.
- Sugihara, H., Miyabara, S., Fujiyama, C., and Yonemitsu, N. Reconstruction of alveolus like structure from alveolar type II epithelial cells in three dimensional collagen gel matrix culture. *Am. J. Pathol.* **142**, 783, 1993.
- Bates, S.R., Gonzales, L.W., Tao, J.Q., Rueckert, P., Ballard, P.L., and Fisher, A.B. Recovery of rat type II cell surfactant components during primary cell culture. *Am. J. Physiol. Lung Cell. Mol. Physiol.* **282**, L267, 2002.
- Yuli, I., and Lelkes, P.I. Evaluation of an exoplasmic neutral endopeptidase activity in the interaction of human polymorphonuclear leukocytes with the chemoattractant N-formyl-L-methionyl-L-leucyl-L-phenylalanine. *Eur. J. Biochem.* **177**, 1, 1991.
- Papadimitriou, E., Unsworth, B.R., Maragoudakis, M.E., and Lelkes, P.I. Time course and quantitation of extracellular matrix maturation in the chick chorioallantoic membrane and in cultured endothelial cells. *Endothelium* **1**, 207, 1993.

12. Kanda, K., Hayman, G.T., Silverman, M.D., and Lelkes, P.I. Comparison of ICAM-1 and VCAM-1 expression in various human endothelial cell types and smooth muscle cells. *Endothelium* **6**, 33, 1998.
13. Li, M., Mondrinos, M.J., Gandhi, M.R., Ko, F.K., Weiss, A.S., and Lelkes, P.I. Electrospun protein fibers as matrices for tissue engineering. *Biomaterials* **26**, 5999, 2005.
14. Lelkes, P.I., and Unsworth, B.R. Neuroectodermal cell culture: endocrine cells. In: Atala, A., and Lanza, R.P., eds., *Methods of Tissue Engineering*. New York: Academic Press, 2002, pp. 375–381.
15. Silverman, M.D. Hayman, G.T., Waters, C.R., Wigboldus, J., Samet, M.M., and Lelkes, P.I. Tissue factor activity is increased in human endothelial cells cultured under elevated static pressure. *Am. J. Physiol.* **277**, C233, 1999.
16. Papadimitriou, E., Unsworth, B.R., Maragoudakis, M.E., and Lelkes, P.I. Time course and quantitation of extracellular matrix maturation in the chick chorioallantoic membrane and in cultured endothelial cells. *Endothelium* **1**, 207, 1993.
17. Lazarovici, P., Fujita, K., Contreras, M.L., DiOrio, J.P., and Lelkes, P.I. Affinity-purified tetanus toxin binds to isolated chromaffin granules and inhibits catecholamine release in digitonin permeabilized chromaffin cells. *FEBS Lett.* **253**, 121, 1989.
18. Lelkes, P.I., and Unsworth, B.R. The role of heterotypic interactions between endothelial cells and parenchymal cells in organ specific differentiation: a possible trigger for vasculogenesis. In: Maragoudakis, M.E., Gullino, P., and Lelkes, P.I., eds., *Angiogenesis in Health and Diseases*. Plenum Press, New York, London, NATO ASI Series, 1992, vol. 227, pp. 27–41.
19. Kim, S.S., Utsunomiya, H., Koski, J.A., Wu, B.M., Cima, M.J., Sohn, J., Mukai, K., Griffith, L.G., and Vacanti, J.P. Survival and function of hepatocytes on a novel three-dimensional synthetic biodegradable polymer scaffold with an intrinsic network of channels. *Ann Surg.* **228**, 8, 1998.
20. Douglas, W., and Teel, R. An organotypic in vitro model system for studying pulmonary surfactant production by type II alveolar pneumocytes. *Am. Rev. Resp. Dis.* **113**, 17, 1976.
21. Douglas, W., Moorman, G., and Teel, R. The formation of histotypic structures from monodisperse fetal rat lung cells cultured on a three dimensional substrate. *In Vitro* **12**, 373, 1976.
22. Geppert, E.F., Williams, M.C., and Mason, R.J. Primary culture of rat alveolar type II cells on floating collagen membranes. *Exp. Cell Res.* **128**, 363, 1980.
23. Blau, H., Guzowski, D.E., Siddiqi, Z.A., Scarpelli, E.M., and Bienkowski, R.S. Fetal type 2 pneumocytes form alveolar like structure and maintain long-term differentiation on extracellular matrix. *J. Cell. Physiol.* **136**, 203, 1988.
24. Lwebuga-Mukasa, J.S., Ingbar, D.H., and Madri, J.A. Repopulation of a human alveolar matrix by adult rat type II pneumocytes in vitro. A novel system for type II pneumocyte culture. *Exp. Cell. Res.* **162**, 423, 1986.
25. Mollard, R., and Dziadek, M. A correlation between epithelial proliferation rates, basement membrane component localization patterns, and morphogenetic potential in the embryonic mouse lung. *Am. J. Respir. Cell. Mol. Biol.* **19**, 71, 1998.
26. Griffin, M., Bhandari, R., Hamilton, G., Chan, Y.C., and Powell, J.T. Alveolar type II cell-fibroblast interactions, synthesis and secretion of surfactant and type I collagen. *J. Cell Sci.* **105**, 423, 1993.
27. Shannon, J.M., and Hyatt, B.A. Epithelial-mesenchymal interactions in the developing lung. *Ann. Rev. Physiol.* **66**, 625, 2004.
28. Paquette, J.S., Tremblay, P., Bernier, V., Auger, F.A., Lavolette, M., Germain, L., Boutet, M., Boulet, L.P., and Goulet, F. Production of tissue-engineered three-dimensional human bronchial models. *In Vitro Cell Dev. Biol.-Anim.* **39**, 213, 2003.
29. Pan, T., Mason, R.J., Westcott, J.Y., and Shannon, J.M. Rat alveolar type II cells inhibit lung fibroblast proliferation in vitro. *Am. J. Respir. Cell Mol. Biol.* **25**, 353, 2001.
30. Hyatt, B.A., Shangguan, X., and Shannon, J.M. FGF-10 induces SP-C and Bmp4 and regulates proximal-distal patterning in embryonic tracheal epithelium. *Am. J. Physiol. Lung Cell Mol. Physiol.* **287**, L1116, 2004.
31. Arman, E., Haffner-Krausz, R., Gorivodsky, M., and Lonai, P. Fgfr2 is required for limb outgrowth and lung-branching morphogenesis. *Proc Natl. Acad. Sci. USA* **96**, 11895, 1999.
32. Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K., and Watson J. *Molecular biology of the cell*. New York: Garland, 1983.
33. Woodcock-Mitchell, J., Rannels, S.R., Mitchell, J., Rannels, D.E., and Low, R.B. Modulation of keratin expression in type II pneumocytes by the extracellular matrix. *Am. Rev. Respir. Dis.* **139**, 343, 1989.
34. Furuyama, A., and Mochitate, K. Assembly of the exogenous extracellular matrix during basement membrane formation by alveolar epithelial cells in vitro. *J. Cell Sci.* **113**, 859, 2000.
35. Adamson, J., and Young, L. Alveolar type II cell growth on a pulmonary endothelial extracellular matrix. *Am. J. Physiol.* **270**, L1017, 1996.
36. Athanasiou, K.A., Niederauer, G.G., and Agrawal, C.M. Sterilization, toxicity, biocompatibility and clinical applications of polylactic acid/polyglycolic acid copolymers. *Biomaterials* **17**, 93, 1996.
37. Li, W., Laurencin, C., Caterson, E., Tuan, R., and Ko, F. Electrospun nanofibrous structure: a novel scaffold for tissue engineering. *J. Biomed. Mater. Res.* **60**, 613, 2002.
38. Thapa, A., Miller, D., Webster, T., and Haberstroh, K. Nano-structured polymers enhance bladder smooth muscle cell function. *Biomaterials* **24**, 2915, 2003.

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