A Novel *In Vitro* Model to Study Alveologenesis

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Abstract

Many pediatric pulmonary diseases are associated with significant morbidity and mortality due to impairment of alveolar development. The lack of an appropriate *in vitro* model system limits the identification of therapies aimed at improving alveolarization.

Herein, we characterize an *ex vivo* lung culture model that facilitates investigation of signaling pathways that influence alveolar septation. Postnatal Day 4 (P4) mouse pup lungs were inflated with 0.4% agarose, sliced, and cultured within a collagen matrix in medium that was optimized to support cell proliferation and promote septation. Lung slices were grown with and without 1D11, an active transforming growth factor- β -neutralizing antibody. After 4 days, the lung sections (designated P4 + 4) and noncultured lung sections were examined using quantitative morphometry to assess alveolar septation and immunohistochemistry to evaluate cell proliferation and differentiation. We observed that the P4 + 4 lung sections exhibited *ex vivo* alveolarization, as evidenced by an increase in septal density, thinning of septal walls, and a decrease in mean linear intercept comparable to P8, age-matched, uncultured lungs. Moreover, immunostaining showed ongoing cell proliferation and differentiation in cultured lungs that were similar to P8 controls. Cultured lungs exposed to 1D11 had a distinct phenotype of

decreased septal density when compared with untreated P4 + 4 lungs, indicating the utility of investigating signaling in these lung slices. These results indicate that this novel lung culture system is optimized to permit the investigation of pathways involved in septation, and potentially the identification of therapeutic targets that enhance alveolarization.

Keywords: alveolarization; secondary septation; postnatal lung growth; organ culture

Clinical Relevance

Many pediatric pulmonary diseases are associated with significant morbidity and mortality due to impairment of alveolar development. The lack of an appropriate *in vitro* model system limits the identification of therapies aimed at improving alveolarization. Herein, we characterize an *ex vivo* lung culture model that facilitates investigation of signaling pathways that influence alveolar septation and the identification of therapeutic targets that enhance alveolarization.

Several important pulmonary diseases in newborns and infants are associated with significant morbidity and mortality due to impaired alveolar development. These diseases include bronchopulmonary dysplasia, congenital pulmonary airway malformations, and the lung hypoplasia associated with congenital diaphragmatic hernia (1–3). Nevertheless, there is a deficit of model systems that can be used to

examine mechanisms that control this last stage of lung development.

Alveolarization occurs in humans from 36 weeks gestational age until adolescence. A human neonate is born with roughly 50 million alveoli, and the number of these gas exchange units increases nearly 6-fold by adolescence. The majority of alveoli, however, form in the first 6 months after birth during a period referred to as “bulk

alveolarization” (4). In mice, bulk alveolarization occurs from Postnatal Day 4 (P4) to P14 (5). However, recent studies indicate that lung septation continues in the mouse at a slower rate through 40 days of age, corresponding to young adulthood (6). This massive expansion in alveolar number is accomplished by a phenomenon called “secondary septation,” during which evaginations arise from within the saccular

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walls of peripheral airways, dividing the distal airspace into alveoli, and thereby lead to a substantial increase in the gas exchange surface of the lung (7).

Although many techniques have been developed to investigate lung development and disease, few of these methods provide a means through which alveolarization can be directly studied. Previously, the study of alveolar development has been indirect, because it examined airway structure in pathologic specimens during lung development in animals with lung injury, genetic manipulation, or exposure to systemic agents. Certainly, *ex vivo* culture of fetal lung buds has been successfully employed to study early stages of lung organogenesis (8, 9). However, this model is limited by oxygen and other substrate diffusion, and can only be used to investigate branching morphogenesis, as the fetal lung tissues used in this model are not developmentally ready to undergo alveologenes. To overcome the limitations of substrate diffusion and potentially examine developmental processes in the more mature lung, *ex vivo* culture of tissue fragments has been used. For example, minced late-gestation fetal rat lungs have been grown semisubmerged in culture media, and this model has provided much information about mechanisms regulating surfactant production in response to hormonal stimulation (10). However, the cultured fetal lung fragments employed in this model did not demonstrate alveolar septation, and the distal airspaces collapsed during the 72 hours in culture. Although, in other models, the lungs of adult animals were inflated with agarose before the generation of tissue slices in an attempt to preserve distal airway structure, the effect of this approach on alveolarization is unknown. That is because these tissues were used for studies of airway reactivity and injury, and the adult lungs were fully developed and unlikely to exhibit alveolar development (11, 12). Moreover, the *ex vivo* culture conditions for the sections of agarose-infused lung sections were not optimized to support cell proliferation and differentiation.

The goal of the studies described in this report was to characterize a model that is specifically optimized to support the investigation of alveologenes. This *ex vivo* system was designed to permit the direct examination of dynamic mechanisms that regulate late lung development. Moreover,

the model system was designed to allow easy access to signaling pathways, the manipulation of which likely will enhance our understanding of the basic mechanisms regulating secondary septation, and provide a setting in which therapeutic strategies could be identified and tested without the confounding influence of systemic manifestations of treatment.

Materials and Methods

Animal protocols used in these investigations were approved by the MGH Subcommittee on Research Animal Care.

Postnatal Lung Slice Cultures

C57BL/6 mouse pups were killed, the neck and chest carefully opened, avoiding injury to the lungs, a catheter was secured in the right atrium, and the pulmonary vessels were perfused with 0.1 M sodium citrate at 15 cm H₂O pressure for 5 minutes. After the trachea was cannulated, the lungs were inflated at 20 cm H₂O pressure for 10 minutes with culture media containing 0.4% low-melt agarose, which was maintained liquefied by radiant warming (13). Subsequently, the lungs were excised from the body and immersed in ice-cold PBS to solidify the agarose. Isolated left lungs were sliced by hand into six to eight 1-mm-thick transverse slices using a number 10 scalpel under a dissecting microscope using a ruler as a template to generate uniform slice thickness (Figure 1). One slice from the left lung was placed directly into fixative to be used as the P4 control, and the remaining slices placed into organ cultures. The lungs from P8 and P12 control pups were similarly prepared with heart perfusion, lung inflation with agarose, and sectioned into 1-mm-thick transverse slices.

Collagen Matrix Preparation

Collagen matrices were prepared by combining 1 mg/ml type 1 rat tail collagen (BD Biosciences, Franklin Lakes, NJ) in modified M199 media containing 0.6 N NaOH supplemented with 1× insulin/transferrin/selenium (Gibco, Life Technologies, Grand Island, NY), 2 µg/ml vitamin C (Sigma-Aldrich, St. Louis, MO), 0.1 µg/ml vitamin A (Sigma-Aldrich), 0.1 µg/ml hydrocortisone (Sigma-Aldrich) and penicillin/streptomycin/fungizone (Gibco) (14). The lung slices were then floated on

top of an aliquot of the mixture that had gelled at 37°C. After the slices adhered to the matrix for 10 minutes, they were covered with an additional aliquot of collagen solution and then maintained for 4 days in a humidified 37°C incubator containing 5% CO₂. Additional modified M199 media were added to the matrices every 48 hours.

To investigate the effect of transforming growth factor (TGF)-β signal inhibition on *ex vivo* alveolarization, some slices were exposed to 1D11, a panspecific active TGF-β-neutralizing monoclonal antibody (R&D Systems, Minneapolis, MN) that was diluted to 1.25 µg/ml in modified M199 media in the initial and subsequent lung slice feedings. This level of 1D11 had been observed to inhibit TGF-β signaling in cells in culture (15). An IgG isotype control antibody, IgG isotype control antibody (G3A1; Cell Signaling, Danvers, MA), as well as antibody diluent (PBS), were used as controls.

Hart's Elastin and Immunohistochemical Staining

After fixing with 0.1% glutaraldehyde and 4% paraformaldehyde, and imbedding the lung slices in paraffin, 5-µm slices were obtained, dewaxed, and stained with Hart's reagents using standard techniques (16). After sodium citrate antigen retrieval and 10% BSA in PBS blocking, additional sections were reacted with antibodies that detect calponin (clone EP789Y; Abcam, Cambridge, MA), α-smooth muscle actin (clone 14A, Thermo Scientific, Fremont, CA), prosurfactant-C (rabbit polyclonal, Abcam), Ki67 (clone SP6; Abcam), aquaporin-5 (rabbit polyclonal; Abcam), and *Griffonia simplicifolia* isolectin IB₄ (Invitrogen, Paisley, PA) overnight at 4°C, followed by biotinylated secondary antibodies (Vector Labs, Burlingame, CA), avidin-biotin complexed peroxidase and 3,3'-diaminobenzidine (Vector Labs).

Western Blot Analysis

Total protein lysates were extracted from the lung slices, after the removal of hylar components under a dissecting microscope, in RIPA lysis buffer (Cell Signaling), and concentrations determined using the Pierce BCA Protein Assay Kit (Thermo Scientific). Equivalent amounts of protein were diluted in SDS-protein loading buffer, resolved using Tris-HCl gels and electrophoresis, and transferred onto nitrocellulose

Figure 1. Schematic representation of organ culture model and tissue handling. Mouse pups of the indicated postnatal age were killed and the heart and lungs were perfused with sodium citrate. The lungs were inflated with 0.4% low-melt agarose in modified M199 media at 20 mm water for 10 minutes, and then placed in ice-cold PBS. The left lung was isolated and sectioned by hand into 1-mm-thick slices. One slice of the Postnatal Day 4 (P4) lung was fixed immediately as P4 control; the remainders were placed into culture in 1-mg/ml type 1 collagen matrices. Similarly processed lung slices from P8 and P12 pups were fixed immediately as controls. After cultured P4 lung slices had grown for 4 days, the slices were fixed and processed for structural work. For morphometric analysis, Hart's stained sections were imaged in a nonbiased fashion by taking three sequential, nonoverlapping images starting at the left upper portion of the section along the pleura and moving counter-clockwise.

membranes. Membranes were incubated with primary antibodies overnight at 4°C, and signal was detected using the Immun-Star ECL detection system (Bio-Rad, Hercules, CA). The same primary antibodies were used for immunohistochemistry and Western blot analysis, with the exception of CD31 (rabbit polyclonal; Abcam).

Image Capture and Stereology

Lung images were obtained using microscopes with attached digital image capture systems. For structural analysis of each cultured lung specimen, three images encompassing 0.25 mm² of lung tissue were analyzed. The alveolar septal density was determined by investigators masked with

respect to the specimen source by dividing the numbers of elastin-containing septa by the parenchymal area using standard methods (17). The mean linear intercept (Lm) was determined using methods detailed in previous work (17–20). As described in that work, chords less than 8 μm or greater than 250 μm, which represent capillaries and bronchioles, as well as cords that included image margins, were removed from the analysis. Wall thickness measurements were obtained by direct measurement of 30 alveolar walls for each specimen using ImageJ. The lung slice areas were determined using 2×-magnified lung images and digital planimetry (NSI Elements; Nikon, Melville, NY). Areas from the same slice on Day 0 and 4 were

compared with calculated percent change over time.

Statistical Analysis

The mean and SEM for nonparametric data (Lm) were calculated after a log transformation, which normalized the skewed data distribution (21). Subsequently, an exponential transformation was used to convert these values back to the linear scale. Moreover, for the Lm data, a Wilcoxon rank-sum test was performed to compute the *P* values. Parametric data (septal density) was analyzed using a *t* test with Bonferroni corrections. The data means and SEM are reported; a *P* value less than 0.05 was considered significant.

Results

Agarose Inflation Maintains Cultured Lung Explant Alveolar Architecture

In pilot studies, we observed that peripheral lung architecture was not maintained in standard mouse lung slice cultures. Guided by approaches used by others to generate adult lung slices, we tested whether initially distending the airways with agarose allowed preservation of mouse pup lung saccular and alveolar structure in culture (22, 23). As shown in Figure 2, the delicate alveolar architecture of the distal airspaces was well maintained when low-melt agarose in tissue culture media was used to inflate the lungs before *ex vivo* culturing. Because the cultured lung slices did not exhibit necrosis during later examinations, this method also appears to permit the diffusion of oxygen and nutrients and the elimination of waste that is necessary to sustain the tissue during culture.

Media and Scaffold Choice Was Critical for Optimal Explant Growth

The optimization of media formulation and the scaffold on which the lung explants were maintained was observed to be essential to the success of the culture system. As indicated in Tables 1 and 2, several media formulations and scaffold types were tested before we arrived at a system that permitted optimal growth characteristics of lung slices maintained for 4 days in culture. The lung slices maintained within a type 1 collagen matrix containing modified M199 media were observed to have an optimal peripheral lung architecture without hypercellularity, and, therefore, this combination was used for the subsequent experiments detailed subsequently here.

Histology and Morphometric Analyses Showed Alveolar Septation in Culture

If the conditions are optimal, lung slices grown in culture should continue to exhibit

secondary septation and cell proliferation and differentiation similar to the *in vivo* lungs obtained at a comparable age. Postnatal lungs processed and cultured as detailed previously here appeared histologically similar to lungs obtained from mouse pups of a comparable age. Lung slices obtained from P4 mouse pups that were cultured for 4 days (P4 + 4) had similar parenchymal complexity, accumulation of elastin in secondary septae, and alveolar wall thickness as lungs obtained from P8 mouse pups maintained *in vivo* (Figure 2).

To determine whether the lung slices septated while in culture, the morphometric parameters, septal density and Lm, were assessed. Septal density increases as the lung undergoes alveolarization (Figure 3A). The septal density of P4 + 4 lungs was significantly greater than noncultured (P4) lungs (550 septa/mm² vs. 439 septa/mm²; $n = 5$; $P = 0.0035$), and was equivalent to *in vivo* P8 (550 septa/mm² vs. 576 septa/

Figure 2. Hart's stained images of lungs grown *in vivo* and *in vitro*. P4, P8, and P12 lungs show the expected increase in complexity of the parenchyma that accompanies alveolarization that has occurred in lungs growing *in vivo*. After 4 days, P4 (P4 + 4) *in vitro*-grown lungs show an increased complexity when compared with P4 lungs, and a similar level compared with P8. Higher-power images reveal increased parenchymal complexity and more septa (arrows) and accumulation of elastin in the septae (arrowheads) as alveolarization progresses from P4 through P12. P4 + 4 lungs also show increased complexity, septal formation, and elastin organization when compared with P4 lungs, and appear similar to P8 lungs. Original magnifications of image capture are indicated.

Table 1: Media Formulations Tested

	BGJb*	BGJb+	EBM-2MV†	M199‡
FBS*	—	0.5%	5%	—
Insulin/transferin/selenium	—	1×	—	1×
Vitamin C	0.2 mg/ml	0.2 mg/ml	Yes [§]	0.2 mg/ml
Vitamin A	—	0.1 µg/ml	—	0.1 µg/ml
Hydrocortisone	—	0.1 µg/ml	Yes [§]	0.1 µg/ml
hFGF†	—	—	Yes [§]	—
VEGF†	—	—	Yes [§]	—
Inulin-like growth factor-1†	—	—	Yes [§]	—
Gentamycin/amphotericin-B†	—	—	Yes [§]	—
Penicillin/streptomycin/fungizone	2%	2%	—	2%

Definition of abbreviations: EBM-2MV, endothelial growth medium-2 microvascular; hFGF, human fibroblast growth factor; VEGF, vascular endothelial growth factor.

*Gibco (Grand Island, NY).

†Lonza (Hopkinton, MA).

‡Optimal growth was seen with this medium.

§Final media concentration of additives not provided by vendor.

mm²; $n = 8$; $P =$ nonsignificant [NS]), indicating that the cultured lungs undergo secondary septation during time in culture (Figure 3A).

The Lm, which is the average distance between alveolar walls, decreases as the lung undergoes secondary septation and the parenchymal complexity increases (Figure 3B). As shrinkage of the lung slices in culture could falsely lower the Lm, total lung slice area was measured to assess shrinkage. From Day 0 to Day 4 in culture, total slice area decreased by 9.8% (range, 8–11%; $n = 4$). This shrinkage was accounted for in the data analysis of the P4 + 4 lungs by increasing the Lm in this group by 9.8%. Similar to *in vivo* growth, we observed that the corrected Lm decreased in P4 + 4 cultured lungs when compared with the P4 lungs (32.7 µm vs. 34.7 µm; $n = 5$; $P = 0.0253$). Importantly, the change in Lm in the cultured lung slices was similar to that *in vivo* as P4 + 4 lungs had a Lm that was not different

from that observed in the P8 lungs (32.7 µm vs. 32.8 µm; $n = 8$; $P =$ NS; Figure 3B).

Alveolar wall thickness decreases as pulmonary interstitial cells differentiate and organize, the lung matures, and septation proceeds. Many pathological states in the lung include the presence of thickened alveolar walls and septae. We ascertained the thickness of the alveolar and septal walls in the lungs grown in culture and control lungs. As expected, the wall thickness of the control lungs decreased with advancing postnatal age, Days 4–12 (Figure 3C). Importantly, the cultured lungs, P4 + 4, had wall thickness that was similar to P8 *in vivo* lungs (15.2 µm vs. 15.0 µm; $P =$ NS) and that was thinner than P4 wall thickness (15.2 µm vs. 23.1 µm; $P < 0001$; Figure 3C).

Cell Proliferation and Differentiation Continue in Cultured Lung Slices

Lung parenchymal cell proliferation and differentiation are essential to secondary

septation. Evaluation of lung slices using immunohistochemistry revealed that cell proliferation and differentiation in cultured slices were similar to those observed in *in vivo* lungs of an equivalent postnatal age (P8; Figure 4A). Documentation of protein expression levels was obtained by Western blotting (Figure 4B). Ki67 immunoreactivity showed robust proliferation in the cultured lung slices. Calponin and α -smooth muscle actin (α -SMA) immunoreactivity were used to evaluate smooth muscle and myofibroblast differentiation. Calponin, which is thought to characterize cells undergoing differentiation toward a smooth muscle cell fate, was similarly expressed in P4 + 4 and P8 lungs when compared with P4. α -SMA staining appeared to be similar at all time points as well (24, 25). α -SMA is found in many contractile cell types, and was observed to be localized to myofibroblasts in the tips of growing alveolar septa in the cultured and uncultured lungs (arrows, Figure 4A) (26). Myofibroblast protein expression was found in the cultured lungs by immunoblot (Figure 4B). Pro-surfactant C, which is made by type 2 pneumocytes, was present in the P4 + 4 cultured lung throughout the alveolar walls and at expression levels similar to those seen in the P8 lungs (Figures 4A and B). Aquaporin-5, a transmembrane protein found in type 1 pneumocytes, showed increased reactivity in cells lining alveolar walls in P4 + 4 and P8 lungs when compared with P4 lungs (Figure 4A). Expression of aquaporin-5 was increased in P4 + 4 cultured lungs and P8 lungs when compared with P4 lungs (asterisks, Figure 4B). IB₄ lectin immunoreactivity, a marker of vascular endothelial cells, was observed in the

Table 2: Scaffold Used and Resulting Histology

	Polycarbonate*	Polyester*	Gel Foam*	1% Agarose Sheet on Wire Mesh*	Type 1 Collagen Matrix†
BGjB	Often hypercellular/ submerged	Hypercellular/ submerged	Hypercellular	Hypercellular	NT
EBM-2MV	NT	Submerged	NT	Hypercellular	NT
M199	Often hypercellular/ submerged	Submerged	Hypercellular	Occasionally hypercellular	Normal morphology

Definition of abbreviations: EBM-2MV, endothelial growth medium-2 microvascular; NT, not tested; Submerged, media flooded air chamber submerging lung slices.

*Grown at the air-liquid interface.

†Grown imbedded in collagen matrix.

Figure 3. Septal density and mean linear intercept (Lm) of lungs grown *in vivo* and *in vitro*. (A) Septal density increases with advancing age (P4–P12) in lungs growing *in vivo*. Lungs grown in culture (P4 + 4) show a significant increase in septal density compared with P4 lungs (* $P = 0.0035$) and a similar density to lungs grown *in vivo* (P8). (B) Lm decreases as lung complexity and septation advance with increased age. Cultured (P4 + 4) lungs show a significant decrease in Lm when compared with P4 lungs (* $P = 0.0253$), but no difference when compared with P8 lungs. (C) Alveolar wall thickness decreases as postnatal age increases. Cultured lungs have significantly thinner walls than P4 uncultured controls (* $P < 0.001$), but are of similar thickness as P8 *in vivo* control lungs. WT, wild type.

cultured lung slices and the age-matched postnatal lung (arrowheads, Figure 4A). The protein expression of the vascular

marker, CD31, however, seemed to be lower in the cultured lungs than in the control lungs (Figure 4B).

TGF- β Contributes to Alveolar Development in Cultured Lungs

TGF- β signaling plays an important role in normal alveologenesis and is increased in the lungs of newborns with bronchopulmonary dysplasia (18, 27, 28). Given the role of TGF- β in alveologenesis, we sought to test whether the model system could be used to study pathways involved in secondary septation by introducing neutralizing TGF- β antibody into the organ culture system. When comparing the TGF- β -neutralizing antibody-treated lung (P4 + 4 TGF- β Ab) with the untreated P4 + 4 lung, we observed decreased septation and increased alveolar wall thickness (Figure 5A). Morphometric analysis confirmed that exposure to 1D11 decreased septal density in the cultured lung slices (P4 + 4 lungs with 1D11, 357 septa/mm² vs. P4 + 4 control lungs, 550 septa/mm²; $n = 4$; $P = 0.0002$; Figure 5B). However, the Lm in the P4 + 4 TGF- β Ab-treated lungs was not significantly different from either untreated P4 + 4 lungs (33.7 μ m vs. 32.8 μ m; $P = \text{NS}$) or from P4 lungs (33.7 μ m vs. 34.7 μ m; $P = \text{NS}$). Wall thickness in cultured lungs treated with 1D11 was significantly increased when compared with untreated lungs (22.2 μ m vs. 15.2 μ m; $P < 0.001$; Figure 5D). The somewhat smaller Lm in the P4 + 4 TGF- β Ab-treated lungs compared with that measured in P4 lungs likely resulted from the thickening of the alveolar walls, but was not significantly lowered because of a lack of secondary septation (Figure 5C). There was no difference in any morphometric parameters in lungs grown with G3A1, nor with the antibody diluent (PBS), when compared with P4 + 4 control lungs.

Discussion

Although the postnatal period might be one of the optimal times during which therapies could be used to improve the alveolarization of hypoplastic lungs, little is understood about the complex nature of lung development at this time, and how agents might promote secondary septation of peripheral lung segments. To address this deficiency, we developed and characterized a postnatal, *ex vivo* lung culture model that recapitulates many important aspects of *in vivo* lung growth.

The study of postnatal alveolarization *ex vivo* demands the initial maintenance of

Figure 4. Protein expression in lungs compared with *in vivo* grown lungs. (A) Immunohistochemical staining for proliferating cells (Ki67), myofibroblast differentiating marker (calponin), differentiated myofibroblasts (α -smooth muscle actin [α -SMA]), type 2 pneumocytes (pro-surfactant C [Pro-Surf C]), type 1

the delicate distal airway architecture to provide the scaffolding onto which secondary septae later form. Such a requirement does not appear to be important during the investigation of branching morphogenesis, which occurs during earlier embryonic stages of lung development. To improve distal airway maintenance during initial lung slice culturing, previous groups have used low-melt agarose to inflate adult lungs to open the distal airways (23). We observed that application of this method to postnatal lungs allowed the cultured lung slices to maintain airway architecture while, at the same time, have adequate diffusion of media and gas throughout the tissue in culture. Introduction of this critical step to the organ culture model also allowed septation, proliferation, and differentiation to proceed, and facilitated immunohistochemical localization of lung-specific cell types, as well as objective morphometric measurements of septation.

There are significant metabolic demands on early postnatal lungs that are undergoing rapid cell proliferation and differentiation during the time of alveolar septation. We therefore compared the integrity of postnatal lung cultured in different media and scaffold types to obtain the optimal combination to support alveologenesis. Although embryonic lung buds have been grown supported on membranes or on thin agarose sheets supported on stainless steel mesh at the air-liquid interface, neither of these methods, nor growth on gel foam squares, provided the necessary environment to allow postnatal lung slices to septate (29, 30). We observed that septation of the airways and cellular proliferation and differentiation was best recapitulated during *ex vivo* growth when the cultured lungs were grown within a type 1 rat tail collagen matrix, made with modified M199

media. A culture system in which the tissue slices were maintained at an air-liquid interface did not support lung alveolarization, whereas the collagen matrix allows both the internal and external surfaces to be in contact with the growth media. Given the high metabolic demands of the early postnatal lung, our investigations showed that a semiliquid collagen matrix provides the best diffusion capacity for nutrients and waste.

The experiments described here show that P4 lungs inflated with agarose and grown for 4 days embedded within a collagen matrix appear to septate in a manner similar to lungs that grow *in vivo*. When compared with P8 lungs, cultured lungs demonstrated increased elastin production and organization in septal tips and septal densities, and Lms that were nearly identical to the P8 lungs. Furthermore, cell proliferation and differentiation appeared similar in P4 + 4 and P8 lungs. Evidence of ongoing smooth muscle cell differentiation while the lungs are in culture is suggested by the expression of calponin in cells of the alveolar walls. SMA, present in myofibroblasts that are known to play a critical role in septation, was also found in levels similar to that seen in P8 lungs in alveolar walls and in the appropriate location at septal tips (31, 32). Importantly, cells expressing markers of type-1 pneumocytes, aquaporin-5, and type-2 pneumocytes, pro-surfactant C, also stained positively in the cultured lungs and at levels and locations similar to P8 control lungs. Identification of endothelial cells by IB₄ lectin reactivity revealed double capillary layers, which are observed typically in newly formed, immature alveolar septa. Moreover, the overall protein expression of another endothelial cell protein, CD31, was slightly lower in the cultured lung than the control. Although vascular development occurs in the lung

without pulsatile blood flow, the result of growth in a system where there is neither pulsatile blood flow nor rhythmic breathing may lead to some diminution in endothelial cell differentiation and protein expression in the cultured lung slices when compared with *in vivo* growth (33, 34).

The utility of this model system is to facilitate the *ex vivo* study of mechanisms that might influence alveolar septation. We have shown that the addition of neutralizing TGF- β antibodies to the culture system leads to a phenotypic change in the cultured lungs when compared with untreated controls. TGF- β 1 and -2 null mice have normal lung anatomy, but manifest postnatal lung inflammation or collapsed distal airspaces after birth, respectively (35, 36). TGF- β 3 null mice, however, have a striking lung phenotype characterized by mesenchymal thickening and delayed septation (37). Therefore, the lungs cultured with pan-TGF- β -neutralizing antibody would be expected to have decreased septation and thickened alveolar walls, consistent with loss of TGF- β 3 signaling. The lungs grown in culture with the neutralizing TGF- β antibody reproduce the phenotype of TGF- β 3 knockout lungs, with a significant decrease in alveolar septation and increased alveolar wall thickness when compared with the cultured lungs without antibody. The Lm in the treated lungs does not change significantly from uncultured lungs, due to lack of secondary septation. The lack of phenotype in cultures treated with G3A1 or PBS confirms that the alterations in phenotype were due to the 1D11 effects on the growing lung in culture. These findings confirm the feasibility of the model for studying signaling pathways involved in septation.

Although this *ex vivo* model recapitulates many important components of *in vivo* alveologenesis, there remain

Figure 4. (Continued). pneumocytes (popoplanin, podo), and endothelial cells (aquaporin-5) in P4, P4 + 4 cultured lungs, and P8 lungs. P4 + 4 lungs show ongoing proliferation comparable to P8 lungs and markers of cell differentiation similar to that seen in P8 lungs. Calponin staining appears similar in the P4 + 4 and P8 lungs when compared with P4. SMA localizes to septal tips in cultured and uncultured lungs (arrows). Type 2 pneumocytes are seen scattered throughout the parenchyma in P4, P4 + 4, and P8 lungs. Aquaporin-5 in the cell membrane of type 1 pneumocytes is seen in cells of the alveolar walls in P4 lungs with increased reactivity in P4 + 4 and P8 lungs (asterisks). IB₄ lectin identifying capillary endothelial cells is seen in cells of alveolar walls in P4 lungs (arrowheads). In P4 + 4 lungs, the lectin identifies the double capillary network in secondary septa, but appears in a more lacy and diffuse pattern in the parenchyma than in the P8 lungs. Original magnification, $\times 20$, except IB₄ lectin at $\times 100$. Within each antibody group, images were color matched to match the P4 image. (B) Western blot analysis reveals ongoing expression of cell type-specific proteins in cultured and uncultured lungs. Myofibroblast proteins, calponin and α -SMA, are expressed similarly in the cultured and uncultured controls. Pro-Surf C, a protein expressed in type two pneumocytes, is also found in P4 + 4 cultured lungs, as well as control *in vivo* lungs. The type 1 pneumocyte-specific protein, aquaporin-5, shows increased expression in P4 + 4 cultured lungs and the P8 *in vivo* control when compared with P4 uncultured lungs. Expression of CD31 in endothelial cells is diminished when compared with P4 and P8 controls.

Figure 5. Histology and morphometrics of neutralizing transforming growth factor (TGF)- β antibody (TGF- β Ab)-treated lungs. (A) Hart's stained sections from P4 *in vivo* lungs and P4 + 4 cultured lungs compared with P4 + 4 lungs cultured with neutralizing TGF- β antibody. The antibody-treated lungs have fewer septa and thickened alveolar walls (indicated by "H") when compared with the P4 and P4 + 4 controls. (B) Septal density measurements show decreased septation in lungs treated with the TGF- β -neutralizing antibody, 1D11, when compared with the P4 + 4 control cultured lung (* P = 0.0002). (C) Lm in the lungs treated with neutralizing TGF- β antibody is not significantly different than the Lm of P4 lungs or P4 + 4 lungs. (D) Wall thickness is increased in lungs grown in the presence of neutralizing TGF- β antibody when compared with P4 + 4 cultured lungs (* P < 0.001). No differences in morphometric parameters were identified between P4 + 4 lungs and controls (IgG isotype control antibody [G3A1] or antibody diluent [PBS]).

a number of potential limitations to the system. Although we have characterized the alveolar structural and cellular proliferation and differentiation of the cultured lung slices, a more complete characterization of lung slice metabolism, cell migration, and gene expression will be necessary to characterize the developmental processes in this *ex vivo* organ culture system to address specific hypotheses. In addition, the septation in this system has not been examined for longer than 4 days in culture. Should investigators be interested in examining later lung developmental

processes, then additional work will be necessary to characterize the lung slice differentiation at later time points. Furthermore, this is a nonperfused organ culture system, and therefore may not be an appropriate model for the investigation of microvascular maturation beyond that which is associated with the formation of the secondary septa. Moreover, slicing of adult rat lungs for culture has been shown to release a mitogenic factor that increases cell proliferation (38). Although not specifically investigated, it is possible that the release of such factors during the

production of the organ culture might have played a role in modulating the lung slice structure. We have successfully introduced a neutralizing antibody to the system to demonstrate the ability of the system to examine a signaling pathway known to play a role in lung development, TGF- β ; however, the introduction of other regulators of signaling, such as RNA interference or signaling peptides, was not tested. It is likely that additional optimization of the model system may, however, allow its use for high-throughput small-molecule or RNA interference screens

in the future. Similarly, we have not yet explored whether lungs from transgenic or knockout animals can be cultured using this method. However, the success of such lung culturing might ultimately be influenced by the nature of the genes modified in the lungs themselves. Perhaps one of the greatest deficits of this *ex vivo* model is the lack of physiologic blood flow and cyclical stretch from ventilation. Until further refinements to the *ex vivo* lung culture system are developed, the effects of

these factors on alveologenesis would likely be better studied using an *in vivo* system.

In summary, we have designed and tested an *in vitro*, postnatal lung slice organ culture model that demonstrates *in vivo* lung growth with ongoing secondary septation, cell proliferation, and differentiation. The system can now be employed to identify pathways involved in normal septation and, furthermore, can be used to identify

and evaluate new therapeutic targets aimed at enhancing alveolarization and treating lung disease in children. ■

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