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RESEARCH ARTICLE

Pulmonary surfactant expression analysis—Role of cell–cell interactions and 3-D tissue-like architecture

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Abstract

Surfactant production is important in maintaining alveolar function both in vivo and in vitro, but surfactant expression is the primary property lost by alveolar Type II Pneumocytes in culture and its maintenance is a functional requirement. To develop a functional tissue-like model, the in vivo cell–cell interactions and three dimensional architecture has to be reproduced. To this end, 3D button-shaped synthetic gelatin vinyl acetate (GeVAc) co-polymer scaffold was seeded with different types of lung cells. Functionality of the construct was studied under both static and dynamic conditions. The construct was characterized by Environmental Scanning Electron and fluorescent microscopy, and functionality of the system was analyzed by studying mRNA modulations of all four surfactant genes A, B, C, and D by real time-PCR and varying culture conditions. The scaffold supports alveolar cell adhesion and maintenance of cuboidal morphology, and the alveolar-specific property of surfactant synthesis, which would otherwise be rapidly lost in culture. This is a novel 3D system that expresses all 4 surfactants for a culture duration of 3 weeks.

Keywords: alveolar cell; co-cultures; pulmonary surfactants; three-dimensional dynamic culture

Introduction

Pulmonary surfactant is a surface active material which prevents alveolar collapse and preserves bronchiolar patency during respiration. Many diseases like respiratory distress syndrome of newborns (hyaline membrane disease) asthma, bronchiolitis, chronic obstructive pulmonary disease, etc., are caused by surfactant abnormalities (Griese, 1999). It is a complex mixture of proteins and lipids. Surfactant protein B and C are required for correct biophysical functions of the lung, whereas A and D are important in immune response (Mason and Crystal, 1998; Bourdos et al., 2000). Of the major cell types in the lung, alveolar type II pneumocytes synthesize and secrete pulmonary surfactants. Type II cells in culture on polystyrene dishes spread rapidly and lose both their cuboidal morphology and surfactant expression (Shannon et al., 1987; Nandkumar et al., 2002). This problem led us to hypothesize that a three-dimensional frame work and interactions of different lung cell types would mimic the in vivo situation in vitro and provide the necessary structural and signaling cues for maintenance of tissue specific functions.

Understanding several lung diseases and providing suitable therapeutic modalities is lagging due to lack of suitable experimental models. Engineering a functional lung tissue would provide a suitable system for such studies. Such three-dimensional constructs would provide suitable in vitro systems for pharmaceutical screening of potential therapeutics, aerosol toxicology, and studies on lung cell interactions in health and disease. The alveoli are terminal respiratory units in the lung with an approximate diameter of 200 μm in humans. To recreate this structure, a suitable scaffold with agreeable porosity is necessary. Scaffolds are also essential for providing the necessary three-dimensional frame work and specific cues for cellular adhesion, proliferation and functionality. Gelatin Venyl Acetate (GeVAc) with porosity of 70–100 μm was selected as a suitable scaffold for maintenance of the complex three-dimensional architecture of the alveolar tissue.

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Abbreviations: GeVAc, gelatin vinyl acetate; SPA, surfactant protein A, B, C, D

Type I pneumocytes, Type II pneumocytes, and the fibroblasts are the major alveolar cell types. Alveolar type I cells are large columnar cells responsible for gas exchange and it covers about 95% of the alveolar surface (Gonzalez et al., 2005). The alveolar type II pneumocytes cover about 5% of the alveolar surface area, acts as progenitor for alveolar type I cells, and secrete the alveolar surfactants. Fibroblasts are the major group involved in ECM synthesis, repair mechanism, and contribute towards maintenance of type II cell functionality.

In vivo, the tissues are constantly supplied with nutrients by the capillaries that remove metabolic waste. In case of engineered tissues, such a circulatory system is absent. To overcome this limitation, we established a dynamic culture condition to mimic the in vivo situation. This system provides continuous supply of fresh medium and removes secreted metabolic waste products (Minuth et al., 2000). The perfusion system allows long-term culture of cells as well as the use of various cell-support materials, as scaffolds for three-dimensional cultures.

The current study hypothesizes that a 3-D architecture, cell–cell interactions offered by multiple cell types, and a dynamic culture condition would recreate the tissue like architecture and promote maintenance of surfactant synthesis in vitro. To achieve this, we have used GeVAc scaffold with porosity of 70–100 μm, multiple cell types of the lung—the pneumocytes and fibroblasts, and a dynamic culture condition. Further, an effort has also been made to recreate this construct using human lung cell lines, and characterized it for possible use as in vitro test system for analyzing effect of pollutants, and drugs on alveolar epithelium.

Materials and methods

Synthesis of GeVAc (Gelatin vinyl acetate) scaffold for lung tissue engineering

In brief, GeVAc scaffold (Figure 1A) was synthesized by co-polymerizing gelatin and vinyl acetate monomer in the presence of AIBN (*N*,*N*′-azobisisobutyronitrile, 0.1% (w/w); Spectrochem) as the initiator and subjected to physico-chemical characterization. A porous three-dimensional scaffold with open inter connected pores was then produced from GeVAc. The porous GeVAc scaffold used has 0.8 mm diameter, 2 mm thickness, and pore size ranging from 70 to 100 μm. The scaffold was characterized by FTIR, XRD, and microCT. FTIR showed that grafting of vinyl acetate to gelatin was achieved by the presence of ester carbonyl group of vinyl acetate moiety. It further confirmed co-polymerization of vinyl acetate on to gelatin, and XRD showed destruction of crystalline phase of gelatin molecule during co-polymerization. MicroCT established the highly interconnected uniform porosity of size 90.2 μm of scaffold (Thomas and Nair, 2012).

Cell isolation and in vitro culture

All animal procedures were carried out by the approval of IAEC (Institutional Animal Ethics Committee) following the guidelines of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals).

The alveolar cells were obtained from the lung tissue isolated from Wister rats having body weight in the range of 150–200 g. Wistar rats used in the experiment were withdrawn from feed 16–18 h prior to the experiment, with water ad libidum. The rats were sacrificed by overdose of intra-peritoneal injection of thiopentone. The lungs were excised and cut free of bronchioles. It was finely minced and subjected to dissociation with 0.5% collagenase type I (Himedia) and elastase pancreatic solution type I (Sigma) in RPMI medium at 37°C in an incubator with gentle shaking at 80 rpm. The cells were monitored under a phase contrast microscope for optimal stromal dissociation. A few drops of DNAase (Sigma) were added to prevent cell clumping. The cells were washed off collagenase, and alveolar epithelial cells were enriched by density gradient separation on preformed percoll gradient. Gradient was prepared by adjusting percoll

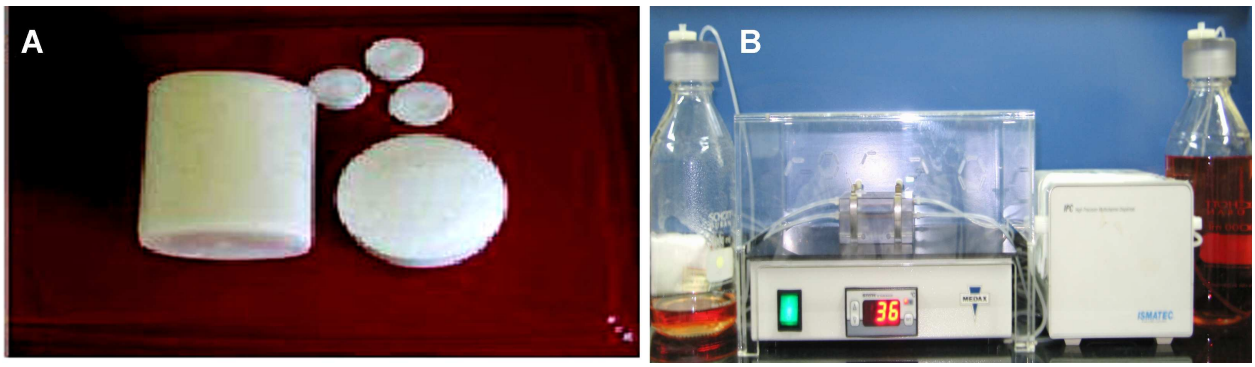

Figure 1 (A) Gelatin venyl acetate scaffold (GeVAc) scaffolds of different shapes. Of this the button-shaped scaffold was used for 3D lung constructs. (B) Minu cell perfusion system which was used for setting up the dynamic culture system for tissue engineered constructs.

to physiological salt and pH condition with RPMI-1640 and spinning at 12,000 rpm for 60 min in a fixed angle rotor. The epithelial band was collected, washed off percoll, and plated in Tissue Culture Polystyrene (TCPS) dishes and on GeVAc scaffolds. The culture dishes were maintained at 37°C and 5% CO_2. The culture medium used was DMEM/F12 (1:1, v/v) containing 10% FBS and insulin (10 μg/mL), cholera toxin (10 ng/mL), hydrocortisone (0.5 μg/mL) along with antibiotics amphotericin (Sigma) and gentamycin (Himedia)(Maya et al., 1997).

Cell lines and co-culture

A549 (ATCC) human lung adenocarcinoma representing Type II pulmonary alveolar epithelial cells and MRC5 (ATCC)—a human lung fibroblast cell line were used for co-culture. F12K (Sigma) and EMEM (Sigma) were used as the culture medium for A549 and MRC5, respectively. Both the cells were seeded on TCPS dishes and scaffolds at 2:1 ratio and medium used was F12K and EMEM at (1:1, v/v). The cultures were maintained at 37°C and 5% CO_2.

Scaffold seeding

The ETO sterilized GeVAc scaffold was conditioned with growth medium for 1 h prior to seeding. The alveolar cells isolated from Wistar rats and the co-culture of A549–MRC5 were seeded over the scaffolds with cell suspension having 1–2×10^6 cells/mL. Following seeding, scaffolds were cultured up to 3 weeks under both static and dynamic culture conditions.

Establishment of dynamic culture

Dynamic culture (Figure 1B) was established with the perfusion system (Minucells & Minutissue, Bad Abbach, Germany). GeVAc scaffolds were seeded with either primary cells isolated from lungs of Wister rats or A549 and MRC-5 cells and fixed onto minucell support rings. They were transferred into the perfusion chamber. The dynamic system was set up by perfusing the chamber continuously with fresh nutrient medium at a rate of 2 mL/h using a peristaltic pump. The perfusion chamber was maintained at 37°C with a thermoplate. The growth medium used for perfusion culture was supplemented with 20 mM HEPES to maintain constant pH 7.4 in the perfusion system.

Environmental scanning electron microscopic analysis

The lung cells were seeded on scaffolds and after 3 weeks of dynamic culture, fixed with 3% gluteraldehyde in Dulbecco's phosphate buffered saline (Himedia) for 30 min at room temperature. The material was then observed with Environmental Scanning Electron Microscope (ESEM, Quanta 200 FEI, Netherlands) to evaluate the ability of the polymer to support cell adhesion and growth of the different alveolar cells and also to assess the distribution of cells.

Immunofluorescence staining

After culturing for 3 weeks, the scaffold seeded with lung cells were fixed with 4% paraformaldehyde, permeablized with 0.5% triton X-100 (Merck) in PBS followed by blocking using 0.1% BSA (Himedia) in PBS. The samples were washed with PBS and stained with rhodamine–phalloidin (Sigma) for actin, Heochst 33342 (Sigma) for DNA and were visualized by confocal microscope (Zeiss 510 Meta). For surfactant protein expression analysis, SPC antibody (Santa Cruz Biotechnology, Inc.) was used with counter stain for DNA and visualized by Zeiss Axio Observer Z1M.

Viability

Viability of alveolar cells was assessed by the Live-dead assay kit (Molecular probes L-3224). The assay was done according to the kit protocol supplied by the manufacturer. Briefly, after 3 weeks of culture, the constructs were stained for 1 h with a standard growth medium containing 1 μL/mL live/dead staining (Calcein AM/ethidium homodimer). Then, the cell viability was assessed using confocal laser scanning microscopy (Carl Ziess LSM META 510).

Histological evaluation

In order to evaluate the cell distribution, a histological evaluation was performed. The statically and dynamically cultured cell constructs were dehydrated through a series of graded alcohols, cleared with xylene, and embedded with paraffin wax. Four millimeter thick sections were cut, de-paraffinized, rehydrated, and stained with Hematoxylin/eosin (H&E) viewed under 40X magnification in Leica DMR microscope.

Real time polymerase chain reaction (PCR)

Total RNA was extracted from cells with TRIzol® reagent (Invitrogen) following manufacturer's instructions. The precipitated RNA was resuspended in sterile water and quantified by absorbance at 260 nm using Nanovue spectrophotometer (GE Health Care, Amersham). One microgram of RNA was reverse transcribed to cDNA using Eurogentec RT-PCR kit containing random primers. Surfactant mRNA gene expression was analyzed by RT-PCR using Chromo4™, real-time PCR system, and SYBR green chemistry. The Syber green Master Mix from Bio-Rad was used as per manufacturer's instructions. qRT-PCR

experimental conditions were: 10 min at 95°C, 15 s at 95°C, 20 s at 53°C, 40 s at 72°C, and 40 cycles and finally 5 min at 72°C.

Gene expression was evaluated for surfactant protein A, surfactant protein B, surfactant protein C, and surfactant protein D. The expression levels were analyzed versus the house-keeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The forward and reverse primer sequences for each gene are listed in Table 1. Relative mRNA expression was quantified using the comparative CT (ΔCT) method and expressed as $2^{-\Delta\Delta ct}$ relative to the corresponding expression in cultures on TCPS dishes.

Statistical analysis

Statistical analysis was performed with SPSS-16 Software. Differences among groups were assessed by one-way analysis of variance. Results were presented as means ± standard error of the mean (SEM). *P* values less than 0.05 were considered significant.

Table 1 Primer pairs used for the amplification of surfactant genes.

Gene	Primer sequence
SP-A	FP: 5′TTTCTTGGAGCCTGAAAAGAA3′
	RP: 5′CAGGCCTACATAGGCATATG3′
SP-B	FP: 5′GAACACCAGTGAACAGGCTATG3′
	RP: 5′AAACTGTTCACACTTTTGCCTGTCTA3′
SP-C	FP: 5′ACCCTGTGTGGAGAGCTACCA3′
	RP: 5′TTTGCGGAGGGTCTTTCCT3′
SP-D	FP: 5′CAAGAAGGAAGCAATCTGACAT3′
	RP: 5′AAGACAAGCATGGAGAGAAAGG3′
GAPDH	FP: 5′TGTGTCCGTGGTGGATCTGA3′
	RP: 5′CCTGCTTCACCACCTTCTGA3′

GAPGH was used as the housekeeping gene.

Results

Three-dimensional tissue-like construct and characterization

Under conventional two dimensional culture conditions, Type II pneumocytes rapidly lose their cuboidal morphology dedifferentiate become flattened and lose their ability to secrete surfactants. A three-dimensional culture and cell–cell interactions as seen in tissues and organs would facilitate maintenance of alveolar functions in vitro. One of the methods of developing three-dimensional architecture is with the use of scaffolds. Various scaffolds like collagen gel, matrigel, hydrogel, decellularised tissue, sponges have all been used with varying degrees of success (Mondrinos et al., 2007). Here we describe using porous gelatin vinyl acetate (GeVAc) as a three-dimensional scaffold and a co-culture system of major alveolar cell types for development of tissue-like architecture of the lung.

GeVAc is a bio-hybrid co-polymer which can be cast into any desirable shape and size. To improve the mechanical strength, stability and maneurability GeVAc co-polymer was developed. A button-shaped scaffold (Figure 1A) was used for this 3-D lung tissue-like construct. The cell source was primary alveolar cells isolated from Wister rats, human lung adenocarcinoma cell line A549 which resembles the Type II pneumocytes, and MRC5 which is human lung fibroblast cell line.

Initially, the ability of the scaffold to promote alveolar epithelial cell adhesion was investigated using A549 cells. Figure 2 shows the scaffold seeded with A549 cells and stained with hematoxylin and eosin showing adhesion and distribution of A549 cells (arrows Figure 2) in the scaffold. Figure 2A represents A549 cells cultured under static condition and Figure 2B under dynamic condition using the minucell system. Figure 3 is ESEM of A549 cells at 3 weeks of

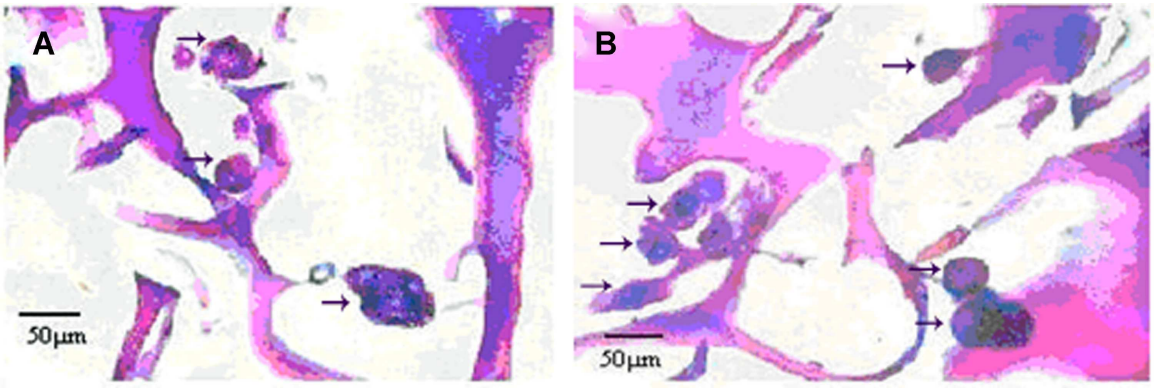

Figure 2 A549 cells were seeded on three-dimensional button-shaped scaffold and cultured under (A) static and (B) dynamic culture conditions. These constructs were processed, embedded, sectioned, and stained with Hemotoxylin and Eosin for histological evaluation. The cuboidal lung cells are found distributed through the pores attached to the porous walls of the scaffold (indicated by arrows).

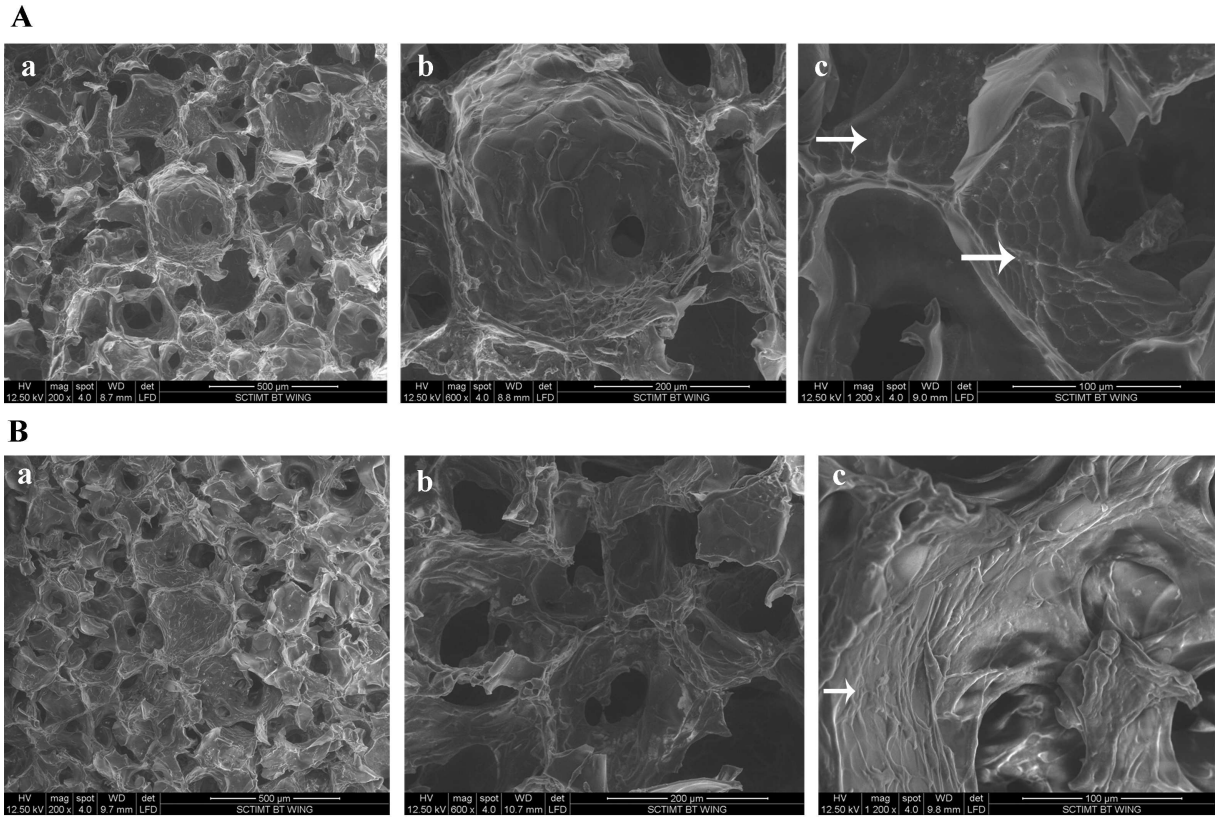

Figure 3 ESEM analysis of 3D construct using button-shaped GeVAc scaffold and A549 cells for a period of 3 weeks. (A) Culture construct under static conditions (a) at 200× magnification, (b) at 600× magnification, and (c) at 1200× magnification. (B) Culture of construct under dynamic conditions (a) at 200× (b) at 600× (c) at 1200× the streaming of cells along direction of flow.

culture with Figure 3A showing cells under static condition and Figure 3B showing cells under dynamic condition. A549 cells adhered along the walls of the porous scaffold and proliferated to cover it as in Figure 3A-a, b, c. In Figure 3A-c, the hexagonal morphology of the A549 cells as it has arranged itself along the scaffold walls is observed. Figure 3B-a, b, c are A549 cells on GeVAc scaffold cultured under dynamic condition using the minucell system. In Figure 3B-c, the cells have arranged themselves in line with flow.

The ability of this scaffold to promote adhesion, viability, and penetration of primary alveolar cells was investigated subsequently. The GeVAc scaffolds were seeded with a heterotypic mixture of primary alveolar cells isolated from lungs of Wister rats. The isolated cells were enriched for alveolar epithelial cells by percoll gradient centrifugation and cultured under static condition using DMEM: F12 medium with I, CT, F. Figure 4A is the bare scaffold which is highly porous with pore size ranging from 70 to 100 μm which is similar to the size of human alveoli which is about 200 μm in diameter. Figures 4B and 4C are primary alveolar cells adhering and spreading across the scaffold. The cuboidal architecture as in Figure 4B of Type II pneumocytes was also maintained at day 9. Figure 5 shows that by day 9 of culture, the pneumocytes and fibroblasts had penetrated the scaffold up to a depth of 96 μm and remained viable as shown in Figure 5B. Figures 6 and 7 shows the cell seeded scaffold at day 9 of culture stained for nuclei by Hoechst and for actin by rhodamine–phalloidin, clearly demonstrating that the cells adhered along the walls of the pores and spread.

Long term 3D cultures

The next step in building a 3D tissue like architecture was to keep these cells in culture for extended period of time. For this 3 weeks was chosen and heterotypic primary alveolar cells were seeded on to the scaffold and cultured under static and dynamic conditions. Ultra-structural analysis by environmental scanning electron microscopy at 3 weeks revealed that cells had proliferated and populated the scaffold. Figure 8A-a, b, c shows the heterotypic primary alveolar cells under static culture. The Type II pneumocytes with cuboidal morphology and Type I pneumocytes which had spread thin over the scaffold assuming squamous morphology and fibroblast cells

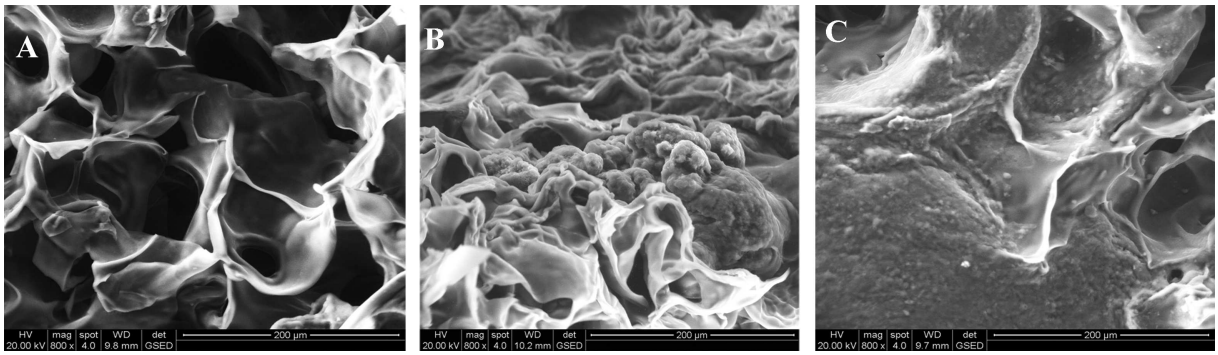

Figure 4 ESEM analysis of 3D construct of primary alveolar cell types isolated from Wister rats using button-shaped GeVAc scaffolds. (a) The bare GeVAc scaffold, (b) the primary cells showing cuboidal morphology, and (c) the primary cells covering the scaffold.

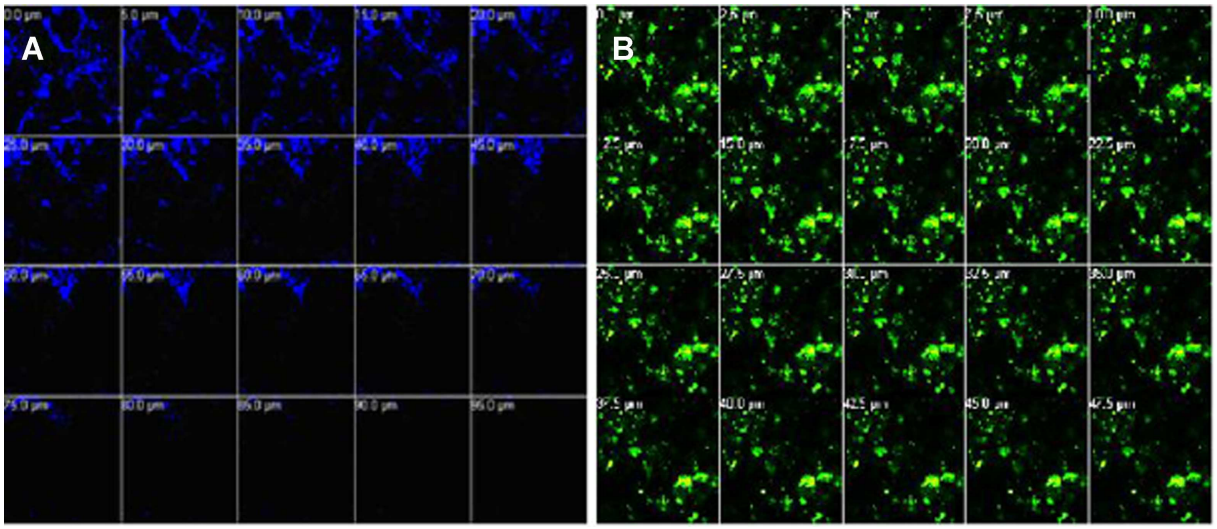

Figure 5 Confocal laser scanning microscopic analysis of adhesion and penetration of alveolar cells into GeVAc scaffold. (A) Depth of penetration where the construct has being stained with nuclear dye Hoechst 33342. (B) Viability analysis where the construct has been stained with the viability kit.

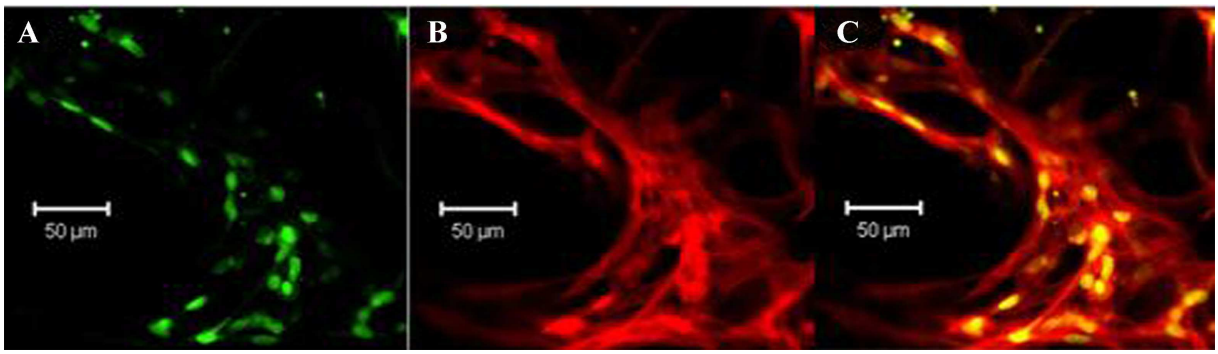

Figure 6 Confocal laser scanning microscopic analysis of 3D construct using primary lung cell types and GeVAc scaffold. (A) Nucleus stained with Hoechst 33342 (B) Actin filament stained with rhodamine-phalloidin (C) The merged image of both nucleus and actin filaments.

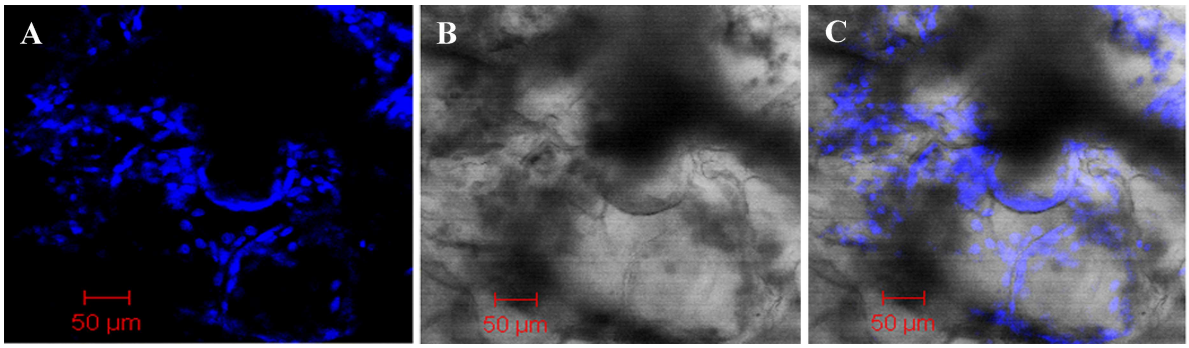

Figure 7 Confocal laser scanning microscopy of adhesion and spread of alveolar cells on GeVAc scaffold. (A) Nucleus stained with Hoechst 33342 (B) Phase contrast image (C) Merged image of both (A) and (B).

with spindle shaped morphology was observed. This was observed under dynamic conditions of culture also as seen in Figure 8B-a–c.

Development of system using human cells

In extending these studies to the human beyond rat primary alveolar cells, human cell lines were used. A549 representing Type II cells and MRC-5 representing alveolar fibroblast were chosen. They were seeded at an optimal ratio of 2:1 (Mereena et al., 2013). The typical A549 hexagonal morphology was visible in Figure 3A-c, but when co-cultured with fibroblasts, the cells assumed cuboidal morphology as in Figure 9A-a–c. This is typical of Type II pneumocytes under static conditions. Under dynamic conditions by 3 weeks of culture, the cells were observed

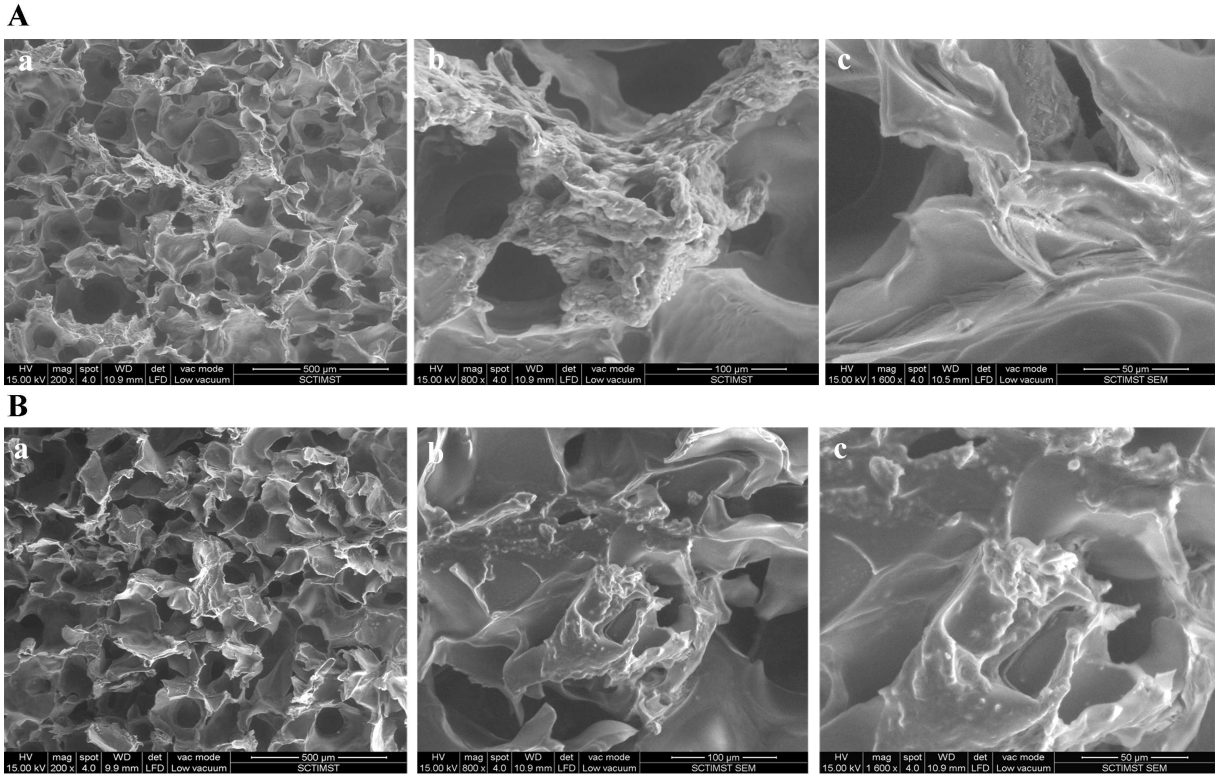

Figure 8 ESEM analysis of 3D construct using button shaped GeVAc scaffold and primary alveolar cell types from Wister rats for a period of three weeks. (A) Culture construct under static conditions (a) at 200× magnification, (b) at 600× magnification, (c) at 1200× magnification. (B) Culture of construct under dynamic conditions (a) at 200× magnification, (b) at 600× magnification, (c) at 1200× magnification.

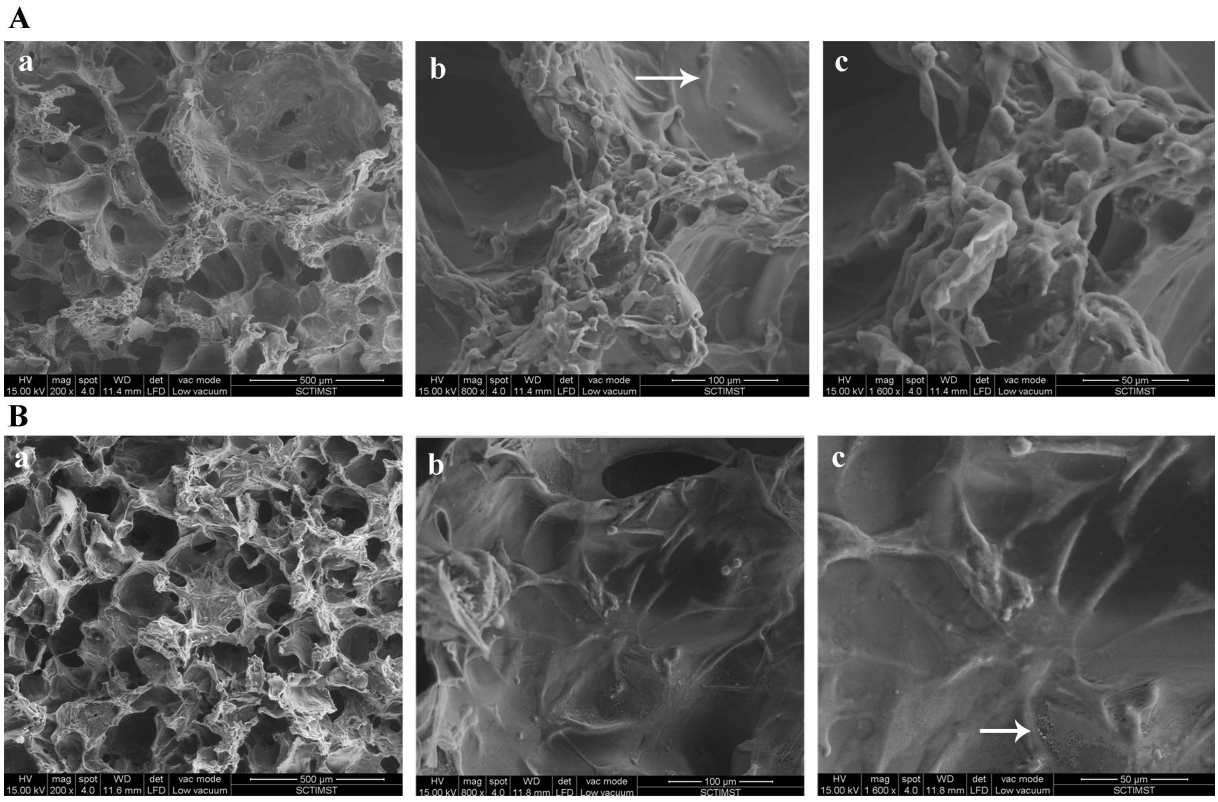

Figure 9 Environmental scanning electron microscopy of 3D construct using button shaped GeVAc scaffolds with A549-MRC5 cells for 3 weeks. (A) Under static conditions (a) A549–MRC-5 co-culture covering the scaffold, (b) A549–MRC-5 spreading along the wall with surfactant (indicated by white arrow), (c) cuboidal morphology is seen. (B) Under dynamic conditions (a) A549–MRC-5 co-culture covering the scaffold, (b) A549–MRC-5 proliferating on the scaffold, (c) surfactant secretion (indicated by white arrow).

to fill a large portion of the interconnecting porous space within the scaffold structure as seen in Figure 9B-a–c.

Functional analysis of the tissue engineered constructs

The Type II pneumocytes synthesize and store surfactants in lamellar bodies. These surfactants are responsible for lowering surface tension and facilitating respiration. In conventional 2D culture, this property is rapidly lost along with morphological changes in Type II pneumocytes leading to de-differentiation. The functionality of the construct was analyzed by its ability to express surfactants and surfactant associated protein mRNA was estimated under different cell combinations and culture conditions. The time points of analysis were 0 day, 2 day, 9 day, and 3 week of static and dynamic culture. The four different surfactant proteins SP-A, SP-B, SP-C, and SP-D were analyzed. Figure 10A was SPA mRNA m odulation over 3 weeks, Figure 10B was of SPB, Figure 10C was of SPC, and Figure 10D was of SPD. SPC which is specifically expressed by Alveolar type II pneumocytes was the surfactant that was maximally expressed at 3 weeks and it is responsible for lowering of surface tension along with SPB. SPA and SPD are responsible for immune response. SPD was minimally expressed vis-a-vis the other three surfactant proteins. However, when compared to day zero of culture for primary and cell-line co-cultures, surfactant protein mRNA was significantly increased at 2nd and 9th days and 3 weeks of culture. This analysis points towards the fact that the dynamic co-culture of cell lines on 3D GeVAc scaffold promoted maintenance of alveolar specific functions. Figure 11 is immunofluorescent microscopic analysis of the SPC in 3D tissue engineered construct of A549 and MRC 5 under dynamic culture condition and was counter-stained with Hoechst for nucleus. Figure 11c and Figure 9B-c showed granular structures under ESEM which could be surfactant secretion. Such granular secretions are present in construct of primary alveolar cells also although to a lesser degree as seen in Figure 8B-c.

Discussion

The challenges faced in development of functional three-dimensional pulmonary tissue-like structure are due to the complexity of the lung, the number of different cell types in the lung, and the inability to maintain surfactant synthesis in vitro. For this a careful selection of scaffold, cell types, cell

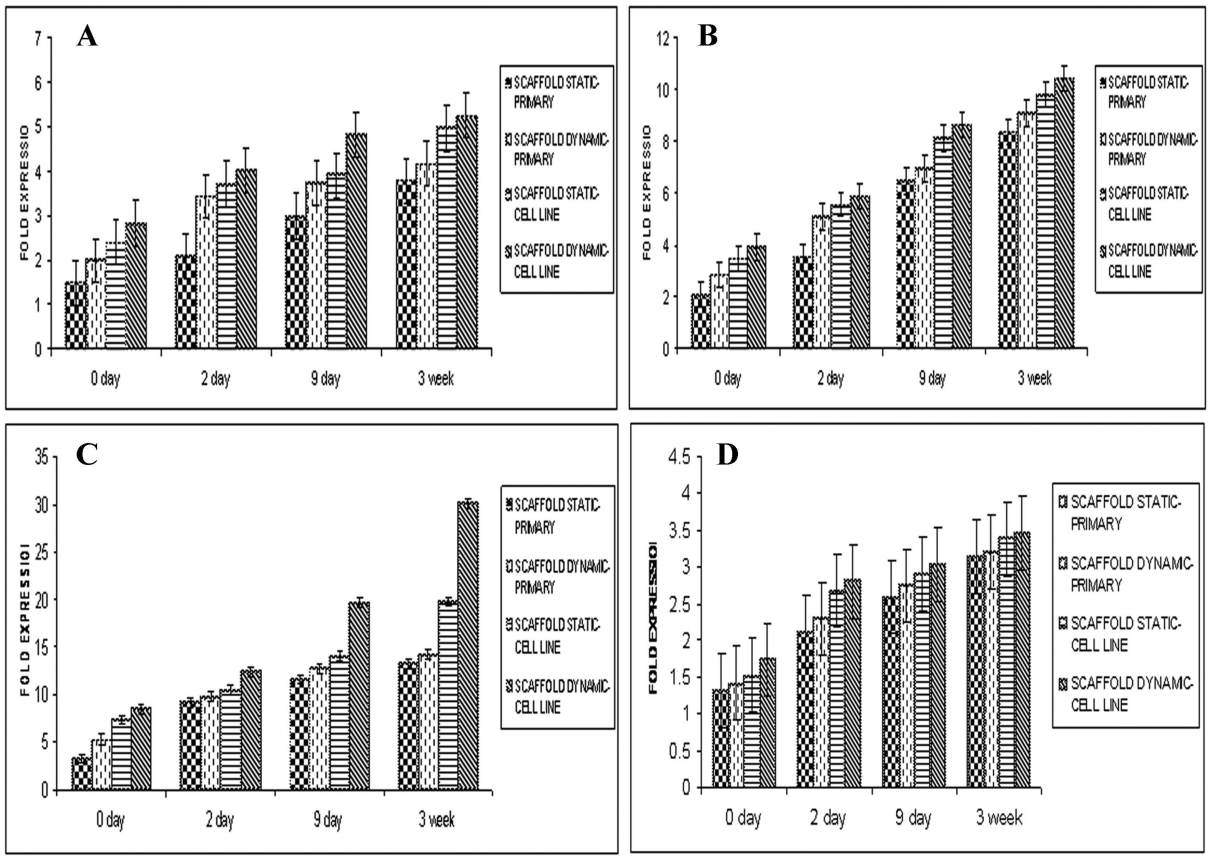

Figure 10 Real time qRT-PCR analysis of the four different surfactant mRNAs in the different 3D constructs. (A) SP-A, (B) SP-B, (C) SP-C, (D) SP-D.

source, and culture conditions are essential. In our work, we have used alveolar pneumocytes and fibroblasts which are the major lung cell types. Epithelial cells are the functional cells in an organ and alveolar epithelial cells are the Type I and Type II pneumocytes. Type I pneumocytes are terminally differentiated and concerned with gas exchange. It is the Type II pneumocytes which divide and form Type I pneumocytes on injury, secrete the various surfactants for performance of different alveolar functions, and mount an immune response as first line of defense. So in culture they are the cells that will be proliferative and also have functionality like expression of the four surfactants. The

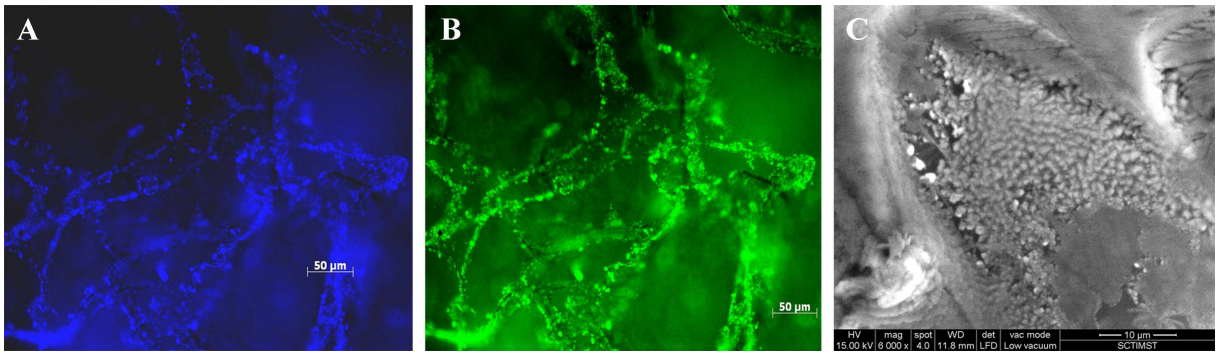

Figure 11 Surfactant protein C expression evaluation in the 3D construct on GeVAc scaffolds of A 549–MRC-5 combination by fluorescent microscopy. (A) Construct stained with Hoechst 33342 showing cell distribution in the construct. (B) SPC staining showing cells expressing surfactant protein C. (C) ESEM micrograph showing surfactant globule secretion in the construct under dynamic co-culture of A549–MRC-5.

other proliferative cells in the alveoli are the fibroblasts, which play a major role in maintaining Type II pneumocytes function. Wister rat lungs were the source for primary cells, while human cell lines were the source for human cells. Here we report the development of 3D construct using different alveolar cells and maintenance of functionality over maximum culture period of three weeks. Functionality of the system was determined by its ability to synthesize the different surfactant mRNAs which is one of the first features lost in culture (Nandkumar et al., 2002). In conventional 2-D culture, alveolar type II cells lose many of their specialized features, especially their ability to produce surfactant (Dobbs, 1990; Nandkumar et al., 2002). Mondrinos et al. (2006) showed only SPC mRNA expression with matrigel, but with synthetic nanofiber scaffold poly-L-lactic Acid (PLLA) and poly-L-lactic-co-glycolic acid (PLGA) sponges failed to show even this. They also recorded the absence of epithelial differentiation in synthetic scaffold. But here, we have shown the expression of all four surfactant mRNA when using synthetic GeVAc scaffold till 3 weeks of culture. Thus our results provide another step towards distal pulmonary tissue constructs which could be used as an in vitro test system.

Alveolar type II cells rapidly de-differentiate in conventional 2D cultures. Previous attempts to prolong the differentiation state in 2D cultures included altering the physical environment such as providing air-liquid interface or seeding cells on matrigel or collagen substrates (Blau et al., 1988). It was also shown that maintenance of cuboidal morphology was essential for preserving surfactant synthesis by type II cells in vitro (Nandkumar et al., 2002). Here, cuboidal morphology was maintained using GeVAc scaffold which is a synthetic hybrid scaffold of gelatin and vinyl acetate. Gelatin is a partial hydrolytic product of collagen and has many of the factors like RGD peptide which promote cell adhesion. Vinyl acetate contributes the necessary mechanical strength to the scaffold. The critical components in matrix selection for lung tissue construct are biocompatibility, elasticity, adsorption kinetics, and the necessary environmental cues both structural and chemical. The GeVAc scaffold reported here satisfies all these requirements except elasticity although some of our recent studies suggest that it is amenable to being spun into nano fibers by electro spinning.

In our experiments, we utilized both 3D scaffolds and a co-culture system comprised of mixed population of lung cells that contained mesenchymal and epithelial cells. This 3D co-culture system was able to maintain type II cell functionality evidenced by the expression of surfactant proteins. We believe that the maintenance of surfactant mRNA expression in the cells was most likely attributable to cell–cell and cell–matrix interactions between epithelial and mesenchymal cells possibly through juxtacrine and paracrine mechanisms.

Recapitulation of cellular organization and interactions during alveolar development is essential in generating functional constructs. Use of suitable scaffold and cell types would facilitate such interactions. Previous studies have shown that cell–cell interactions between the distal lung mesenchymal cells and type II cells provide the necessary differentiation cues (Griffin et al., 1993; Mollard and Dziadek, 1998; Shannon and Hyatt, 2004) for maintenance of epithelial function. Interactions with fibroblasts were necessary to increase production of mRNA of surfactant proteins (Shannon and Hyatt, 2004). Conversely, type II cells secrete factors that inhibit multiplication of lung fibroblasts in vitro (Woodcock et al., 1989). Our results suggest that within the novel gelatin vinyl acetate scaffold, the cells have found an optimal anchoring site where they begin to construct their own tissue-specific matrix for typical structural and functional features to develop. It provided mechanical support, promoted lung cell adhesion, and maintenance of alveolar specific properties like surfactant gene expression. Electron microscopic analysis showed spreading and uniform distribution of cells throughout the scaffolds which proved the fact that the scaffold was cytocompatible. Confocal microscopic observations revealed the uniform growth of cells into the pores of scaffolds.

One of the limiting factors in any tissue engineered construct is mass transport. Under in vivo conditions, the tissues are constantly supplied with nutrients by the capillaries which also remove metabolic waste. In case of engineered tissues, such a circulatory system is absent and hence the nutrient supply and metabolic waste removal will be a big problem. This may lead to loss of function. In order to get rid of this limitation, we have established a dynamic culture condition with the help of Minucell system. The cell seeded scaffolds were placed inside the tissue carriers and fresh culture medium was supplied which enters at the basal side, while the metabolized medium is drained at the upper side of the container. By this method, we can sufficiently mimic the physiological system, guaranteeing constant nutrition supply, prevention, and accumulation of metabolic wastes.

Most primary cell cultures have limited life span. After a certain number of population doublings, the cells undergo senescence. Use of such an in vitro test system specifically to delineate human responses to chemical stimuli may be challenging. So, human cell lines were considered. These immortalized cell lines have acquired the ability to proliferate faster and indefinitely. Thus they can be grown for prolonged periods in vitro. To develop such a system, A549 representative of Type II pneumocytes and MRC-5 for fibroblast were used at an optimal ratio and assayed for surfactant mRNA expression under static and dynamic culture conditions and compared with primary constructs. The results obtained clearly reveal that dynamism of

surfactant expression using primary cells and cell lines was comparable although construct with cell line under dynamic condition expressed all four surfactant mRNAs maximally.

Conclusion

Lung tissue engineering is in its nascent stage. Preservation and maintenance of functionality of alveolar pneumocytes in vitro is a challenge as the cuboidal morphology is rapidly lost along with the ability to synthesize surfactants. This is the first report showing expression of all four surfactant mRNAs in vitro by primary alveolar pneumocytes when synthetic scaffolds were used to develop the three-dimensional construct of lung cells. Use of three-dimensional synthetic scaffold helped in maintaining the cuboidal architecture of the Type II pneumocytes and co-culture with fibroblasts helped in preserving specific property of synthesis of all four surfactants. This clearly demonstrates that cuboidal architecture and cell–cell interactions facilitated by the use of scaffold, co-culture of different lung cell types, and dynamic culture conditions helped in maintaining functionality in vitro. In three-dimensional culture, one of the limiting factors of mass transport for removal of metabolic waste and transport of nutrients were overcome using minucell perfusion system and this enhanced functionality for up to 3 weeks of culture period. When cell lines A549 and MRC-5 were used for the three-dimensional construct, there was increased expression of all four surfactant mRNAs.

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Conflicts of interest

The authors declare no conflict of interest including any financial interest.

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