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Tissue Engineering

Pulmonary tissue engineering using dual-compartment polymer scaffolds with integrated vascular tree

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ABSTRACT: Objectives: *The persistent shortage of donor organs for lung transplantation illustrates the need for new strategies in organ replacement therapy. Pulmonary tissue engineering aims at developing viable hybrid tissue for patients with chronic respiratory failure.*
Methods: *Dual-chamber polymer constructs that mimic the characteristics of the pulmonary air-blood interface were fabricated by microfabrication techniques using the biocompatible polymer polydimethylsiloxane. One compartment ("vascular chamber") was designed as a capillary network to mimic the pulmonary microvasculature. The other compartment ("parenchymal chamber") was designed to permit gas exchange. Immortalized mouse lung epithelium cells (MLE-12) were cultured on the surface of polystyrene microcarrier beads. These beads were subsequently injected into the parenchymal chamber of the dual-chamber microsystems. The vascular compartment was perfused with cell culture medium in a bioreactor and the construct was maintained in culture for 1 week.*
Results: *The microcarriers evenly distributed MLE-12 cells on the parenchymal compartment surface. Confluent cell layers were confirmed by fluorescent and electron microscopy. Adequate proliferation of MLE-12 cells within the construct was monitored via the DNA content. Viability of the cells was maintained over 1 week. Finally, cellular specificity and functional capacity in situ were demonstrated by immunostaining for proSP-B and proSP-C (alveolar epithelium), and by using MLE-12 cells transfected to overexpress green fluorescent protein.*
Conclusion: *We conclude that functional hybrid microsystems mimicking the basic building plan of alveolar tissue can be engineered in vitro. (Int J Artif Organs 2009; 32: 701-10)*

KEY WORDS: Tissue engineering, Lung, Microvascular network, MEMS, Alveolocapillary membrane

INTRODUCTION

Lung transplantation represents the only long-term treatment for end-stage pulmonary failure. The waiting list for lung transplantation in the Eurotransplant area has continuously lengthened over the last decade, currently comprising over 900 candidates. The supply of organs for transplantation has steadily been outgrown by the demand (1-3). The use of mechanical lung assist devices such as extracorporeal membrane oxygenation has shown occasional success as a bridge to transplantation, but can only be used for a limited period of time and is subject to numerous complications (4). Tissue engineering represents an innovative approach to help address the issue of donor organ shortage by creating viable and functional tissue substitutes for replacement therapy (5). New understand-

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ing in cell biology and advances in biomedical engineering and biomaterial technology have contributed to clinical applications for tissue engineering in various fields (6), but little research has concentrated on tissue engineering of the lung. This might be due to the fact that the lung is a large organ that consists of multiple, different cell lineages and features a complex three-dimensional structure. Satisfactory vascularization to meet the metabolic needs for oxygen, nutrients and waste product removal remains the critical obstacle for the fabrication of large tissues or complex organs.

One approach to address the issue of vascularization in tissue-engineered constructs is to provide a pre-established vascular circulation and develop viable tissue around it *in vitro* (7). The field of Micro-Electro-Mechanical Systems (MEMS) is rapidly emerging and provides novel manufacturing technologies and materials for tissue engineering at a resolution of one micron (8). Utilizing techniques such as silicon microfabrication and soft lithography, the fabrication of scaffolds with precise vascular patterns that mimic the vascular tree of an organ has become feasible (9). This paper describes the development of a bioartificial pulmonary microsystem based on the basic building plan of the alveolar wall.

Fig. 1 - *Dual-layer microsystem in PDMS for tissue engineering of alveolar tissue. The close-up displays details of the microvascular network adjacent to the parenchymal compartment. Scale bar = 1 cm.*

METHODS

Cells

For this study we used MLE-12 cells, an alveolar epithelial cell line derived from murine pulmonary adenocarcinoma (CRL-2110; ATCC, Manassas, VA, USA), that represent a model of type II pneumocytes (10). The cells were cultured using HITES cell culture medium, composed of Dulbecco's Modified Eagle Medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) and Ham's F-12 medium (Gibco, Invitrogen Corp., Carlsbad, CA, USA) in equal parts, 5 µg/mL insulin, 10 µg/mL apo-transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM beta-estradiole, 10 nM Hepes buffer (all Sigma, St. Louis, MO, USA) and 1 nM L-glutamine (Gibco, Invitrogen Corp., Carlsbad, CA, USA), supplemented with 2% fetal bovine serum (Gibco, Invitrogen Corp., Carlsbad, CA, USA) and 1% penicillin-streptomycin (Sigma, St. Louis, MO, USA). The cells were cultured in standard cell culture flasks and were passaged as a subconfluent monolayer.

To visualize cells within the dual-layer scaffolds after seeding, the MLE-12 cells were transfected with a murine stem cell leukemia virus-based retroviral vector expressing GFP (MSCV-VSV-G-GFP), as described previously (11). The transfection was carried out 3 times and fluorescence-activated cell sorting (FACS) was employed to evaluate the transfection and to pool the GFP-labeled MLE-12 cells in suspension (data not shown).

Dual-layer scaffolds for organ fabrication

We developed a novel dual-layer scaffold for tissue engineering providing a microfabricated vascular supply that allows for the requirements for metabolic support and waste product removal (Fig. 1). The scaffold is made of polydimethylsiloxane (PDMS) (Sylgard 184; Dow Corning Corp., Midland, MI, USA) and measures about 70 mm × 30 mm × 7 mm. It consists of two compartments, separated by a 10 µm thick highly porous polycarbonate (PC) membrane (Isopore Membrane Filters; Millipore, Billerica, MA, USA). The pore size of 0.1 µm (porosity 5-20%) allows diffusion of oxygen and nutrients from one compartment to the other, but blocks cell migration.

The parenchymal compartment consists of a large chamber which provides space for cells to proliferate and for tissue to form. Its sinusoidal shape provides structural

integrity of the parenchymal compartment and facilitates even distribution of seeded cells. The surface area of the parenchymal chamber is 0.00089 m^2. The design of a parenchymal compartment with a rectangular geometry and an array of support posts and its successful utilization for hepatic organ fabrication has been reported previously by our group (12). Due to the manufacturing process, this design only provides a limited depth of less than 100 µm and failed to support a homogeneous cell distribution within the compartment. Other than minimal oxygen diffusion through the walls of the PDMS scaffold (13), its primary supply of nutrients and oxygen is achieved through the adjacent vascular compartment. The latter provides a branching network of capillary-like channels with widths ranging from 35 µm to 2650 µm. The vascular network has a uniform depth of 100 µm, spreads out in two dimensions and covers an area of 0.00088 m^2. It was designed using a computational algorithm as reported in detail before (14). The fractal design of the microvascular network and subsequent implementation in a microfabricated scaffold provide uniform laminar flow across the vascular network with physiological wall shear stress values (12).

The design of the computer generated model of the vascular network was translated onto 100 mm diameter silicon wafers using lithographic techniques and patterned into the wafer's surface using isotropic reactive ion etching to generate 100 µm deep channels for subsequent replica molding. The surface was then coated with a fluoropolymer to ease the subsequent de-molding of PDMS sheets. PDMS silicone polymer and a curing agent were mixed in a 10:1 ratio and then cast on the templates to a depth of 4 mm. Curing for 12 hours at 60°C caused polymerization. The PDMS molds were gently peeled off the silicon master in preparation for dual-layer scaffold assembly.

A PC membrane was attached to the patterned vascular PDMS molds using silicone glue (3140; Dow Corning Corp., Midland, MI, USA). The PDMS molds were aligned to achieve a maximal overlap of vascular and parenchymal compartments and form an interface of around 0.0008 m^2. Using an oxygen plasma bonding technique, an irreversible link was generated between the micromolded PDMS layers to form sealed chambers. Both compartments provide separate inlets and outlets of silastic tubing (Dow Corning Corp., Midland, MI, USA), equipped with Luer fittings. The dual-layer scaffolds were sterilized with ethylene oxide at 25°C and allowed to degas for 24 hours before use.

Prior to the seeding procedure, the parenchymal chamber of the dual-layer scaffolds was coated with laminin (Sigma, St. Louis, MO, USA). The parenchymal chamber was filled with 150 µg/mL laminin and incubated for 24 hours at room temperature to allow uniform deposition of laminin on the inner surface of the chambers.

Cell seeding

The MLE-12 cells were seeded into 12 of the dual-layer scaffolds using a novel seeding protocol. First the cells were dynamically cultured on polystyrene microcarrier beads (Biosilon; Nunc, Naperville, IL, USA) using a rotational oxygen-permeable bioreactor system as previously described (15). Five thousand microcarrier beads were added to a cell suspension of 5×10^6 cells in 40 mL of cell culture medium and mixed gently. After 48 to 72 hours of dynamic culture at a rotation of 6 rpm, the beads showed homogeneous cell attachment under phase contrast and fluorescent microscopy. Approximately 1000 loaded microcarrier beads were resuspended in 300 µL cell culture medium – a concentration which proved to be beneficial for the spatial distribution of particles within the compartment and the exact volume of the parenchymal compartment. This suspension was carefully injected into a parenchymal compartment of the dual-layer scaffold using a standard syringe, thereby avoiding the diffusion of loaded particles out of the chamber outlets. In order to achieve stable culture conditions within the dual-layer scaffold from the time of seeding, medium flow through the vascular compartment was established 12 hours before initiating the seeding procedure.

Conditioning

A syringe pump (Harvard Apparatus, Holliston, MA, USA) generated the continuous perfusion rate of 0.5 ml/h cell culture medium through the microvascular networks. The flow rate of 0.5 ml/h generated a flow of 0.082 ml/h within the smallest channels and was found to be beneficial as compared to 0.25 ml/h or 1.0 ml/h in terms of proliferation of MLE-12 cells within the dual-layer scaffolds, as previously described by our group on a similar device for hepatic tissue engineering (12). The effluent was collected in a sealed waste container (Fig. 2). The entire assembly was placed inside an incubator and maintained in a humidified atmosphere of 5% CO_2 at 37°C for up to 7 days.

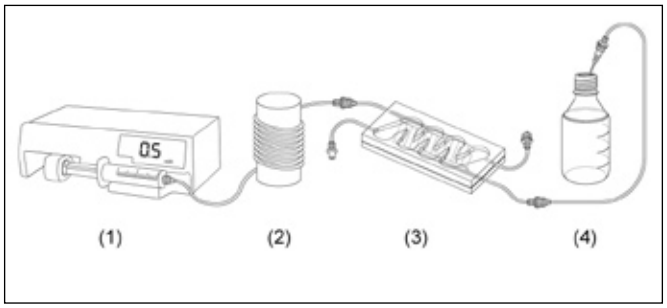

Fig. 2 - *Schematic representation of the dual-layer culture system in PDMS. Culture medium is delivered by a syringe pump **(1)** through silastic tubing via a custom-made oxygenator **(2)** to the vascular network in a microfabricated dual-layer microsystem **(3)**. It provides separate inlets and outlets for the vascular compartment and the parenchymal compartment. The effluent is collected in a waste container **(4)**.*

Evaluation

Using upright and inverted fluorescence microscopy (Eclipse TE 600 and Eclipse TE 2000U; Nikon, Tokyo, Japan) the dual-layer microsystems were evaluated every 24 hours and cell growth and proliferation in the parenchymal chambers were analyzed. The GFP-labeled MLE-12 cells could be visualized using FITC filtered light (488 nm). Six digital images were documented of the cells in randomly chosen areas of the parenchymal compartments of each dual-layer device at a magnification of 40x and 100x. Employing morphometric image analysis (Universal Imaging Corp., Downingtown, PA, USA) on the digital images, indirect evaluation of cell growth and proliferation was achieved by determination of the area covered with cells.

Conventional cell counting within the dual-layer scaffolds was not feasible as the scaffolds could not be disassembled and the cells could not be completely separated from the scaffold. Cell proliferation was thus monitored via the DNA content using the Qiagen DNeasy tissue kit (Qiagen Inc., Valencia, CA, USA). The total cellular DNA in the dual-layer scaffolds was purified using a silica-gel-membrane technology. The cells were digested by injecting 20 mg/mL proteinase K into the parenchymal compartment of the dual-layer scaffold and incubation at 70°C for 10 minutes. To meet the range of optimum yield of the DNeasy procedure (<40 pg DNA/column), the lysate was partitioned into two or four DNeasy columns, based on the expected DNA content. Within the DNeasy column the DNA is selectively bound to the DNeasy membrane. The extracted DNA was eluted in water and its absorbance was measured at 260 nm and 280 nm (A_{260} and A_{280}) with a spectrophotometer (DU-65; Beckman Coulter, Fullerton, CA, USA). The corresponding amount of cells was determined with the standard curve.

To evaluate cell viability, the cells were stained with a viability/cytotoxicity assay (Molecular Probes, Eugene, OR, USA) according to the protocol of the manufacturer. Twelve digital images of the cells were documented in randomly chosen areas of the parenchymal compartments of each dual-layer device at a magnification of 40x and 100x. Using morphometric image analysis, the area of vital cells relative to the total area of cells was estimated and cell viability was calculated.

For scanning electron microscopy, the samples of the parenchymal compartments were fixed in 10% buffered formalin (Fisher Scientific, Pittsburgh, PA, USA). Fracture sections were generated by breaking the specimens in liquid nitrogen. A coating with gold palladium of 100 Å was applied by sputter coating (Cressington Scientific Instruments, Watford, UK). Pictures were obtained at 5 kV acceleration with magnifications of 150x to 5000x (JSM-5910; JEOL, Peabody, MA, USA).

Functional immunostaining of the MLE-12 cells was conducted for the surfactant associated proteins B and C (SP-B, SP-C). Samples for the immunostaining fixed with 4% paraformaldehyde (Sigma, St. Louis, MO, USA). Rabbit anti-mouse proSP-B and proSP-C (AB-3430 and AB-3428; Chemicon, Temecula, CA, USA) were used as primary antibodies in the concentration of 1:500. As a secondary antibody, a biotinylated goat anti-rabbit IgG (Sigma, St Louis, MO, USA) was used in 1:100. The Alexa Fluor 594 streptavidin conjugate (Molecular Probes, Eugene, OR, USA) was employed to specifically detect the biotinylated targets in a concentration of 10 µg/mL. A nuclear counterstain with 10 µg/mL DAPI was performed.

Statistical analysis

The experiments were run at least three times with results reported as the mean +/– standard deviation. Student's t-test was performed using SigmaPlot 9.0 (Systat Software Inc., Point Richmond, CA, USA) and $p < 0.05$ was considered significant.

RESULTS

The MEMS manufacturing process reliably produced microfabricated dual-layer scaffolds with an integrated

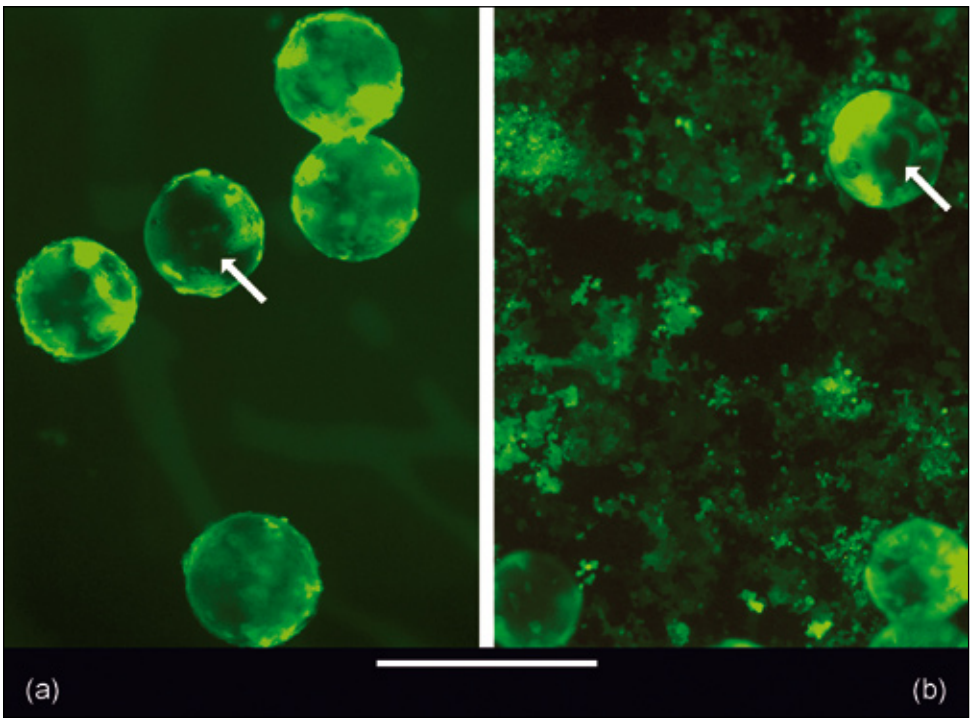

Fig. 3 - *MLE-12 cells within the parenchymal compartment. At the time of seeding* ***(a)****: cells are present only on the surface of the microcarrier beads. After 7 days* ***(b)****: cells are growing on the surface of the microcarrier beads and on the surrounding polycarbonate membrane. The arrows indicate microcarrier beads. Original magnification 40x; scale bar = 500 µm.*

vascular network modeled on the pulmonary microvasculature and a parenchymal compartment separated by a semi-permeable membrane that met the quality control release criteria. Twelve scaffolds were assembled and evaluated for their ability to sustain cell proliferation, viability and function with continuous medium flow.

The parenchymal compartments were seeded with MLE-12 cells using 1000 loaded microcarrier beads corresponding to 0.33 ± 0.01 × 10^3 cells as determined by the DNA content. This seeding procedure resulted in the delivery of confluently attached cells in the process of cell proliferation. The cells proliferated from the surface of the beads onto the surrounding membrane and quickly expanded to a confluent layer on the floor of the parenchymal chamber, as documented using fluorescent microscopy.

Morphometric image analysis of GFP-labeled cells in the parenchymal compartments of the dual-layer microsystems showed consistent cell proliferation from the surface of the microcarriers onto the PC membrane (Fig. 3) and by day 7 the cells covered 87.5 % ± 8.3 % of the surface within the parenchymal compartment (Fig. 4a). As the cell culture of GFP-labeled MLE-12 cells still contained a fraction of unlabeled cells after FACS, these results were thought to underestimate cell proliferation, as was confirmed by the viability/cytotoxicity assay. Cells could only be observed by fluorescence for up to 3 days in dual-layer microsystems with static culture conditions (negative control).

Quantitative DNA analysis showed cell proliferation at an average of 1.30 ± 0.62 × 10^6 cells until confluency was reached at day 7. This implies a fourfold increase of cells within the parenchymal chambers during the experimental period (Fig. 4b). The DNA measurements within the controls with static culture conditions showed a decline below the level of accurate reading (<10 % of baseline).

The viability/cytotoxicity assay showed the parenchymal chambers of the dual-layer devices being covered by confluent cell layers after 7 days in most experimental samples. The majority of the cells showed bright green fluorescence, representing viable cells with intact plasma membranes. Only a small number of spots of red fluorescence indicating the nuclei of dead cells were detected (Fig. 5a). The average viability of the cells was determined as 96.7% ± 1.8%. The parenchymal chambers of the negative control scaffolds appeared empty, without either living or dead cells. Only rare artifactual green fluorescent spots were found on a red background indicating background staining (Fig. 5b).

The SEM pictures depicted cells proliferating within the parenchymal compartment after 7 days of conditioning. Microcarrier beads were observed on the PC membrane

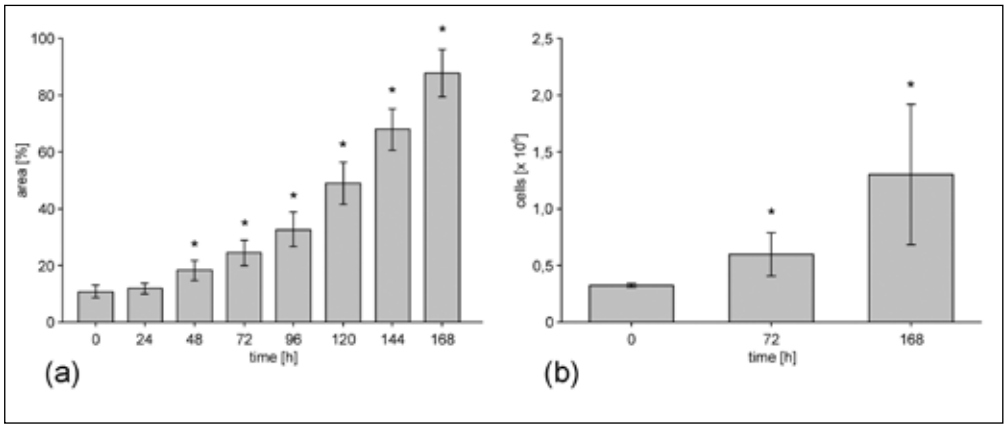

Fig. 4 - *Morphometric image analysis of GFP-labeled cells show continuous cell proliferation in the parenchymal compartments as demonstrated by the area covered with cells* **(a)**. *Quantitative DNA analysis confirmed continuous cell proliferation within the dual-layer scaffolds, showing a fourfold increase of cells during the experimental period* **(b)**.

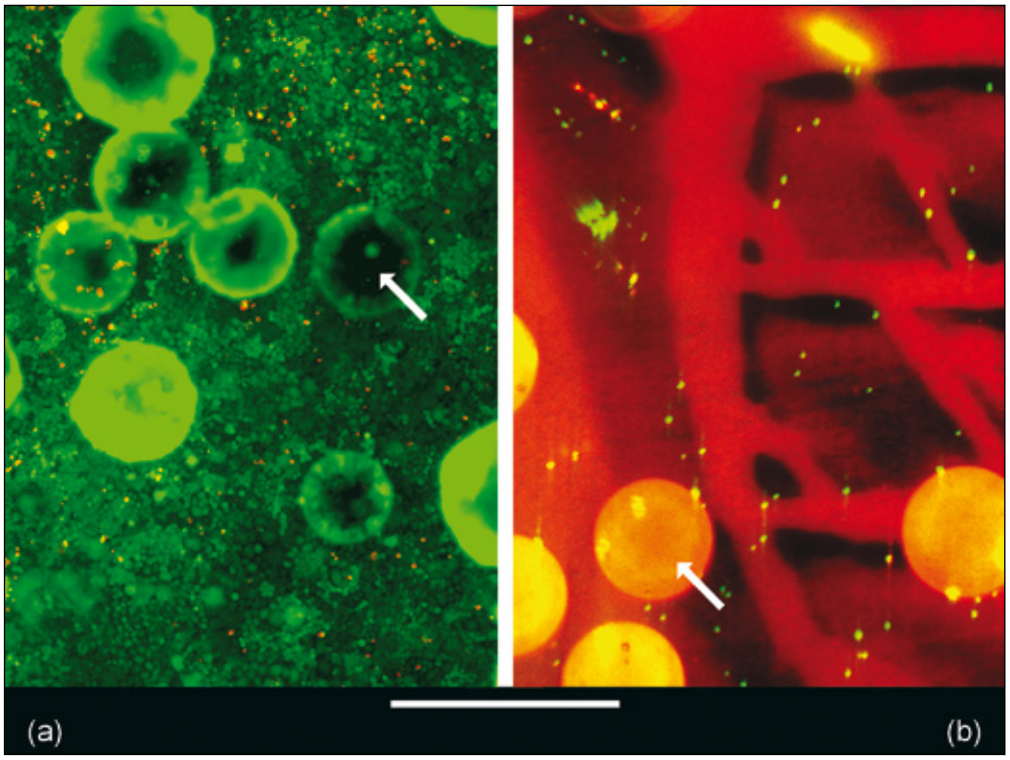

Fig. 5 - *Viability/cytotoxicity assay results: green fluorescence representing viable cells as opposed to red fluorescence indicating the nuclei of dead cells. MLE-12 cells within the parenchymal compartment show an average viability of 96.7% ± 1.8%* **(a)**, *as opposed to the negative control devices* **(b)**. *The arrows indicate microcarrier beads. Original magnification 40x; scale bar = 500 µm.*

with cells attached to their surface. The cells on the basal parts of microcarrier beads proliferated from the surface of the beads onto the membrane and formed a cell layer spreading out over the underlying membrane. A confluent cell layer was present over most of the specimens (Fig. 6).

MLE-12 cells stained positively for the surfactant associated proteins B and C. Functional immunostaining of membrane samples showed bright fluorescence throughout the cytoplasm of the cells. The nucleus of each cell was identified by DAPI counterstain (Fig. 7).

DISCUSSION

Due to the large dimensions of the human lung and the related challenges of providing a sufficient cell supply with oxygen and nutrients within the engineered tissue and to the complexity of pulmonary tissue and the variety of different cell types present in a functional lung, progress in the area of pulmonary tissue engineering has been slow. The adult human lung has a volume of up to 6000 mL. It contains about 300 × 10^6 alveoli and provides a total sur-

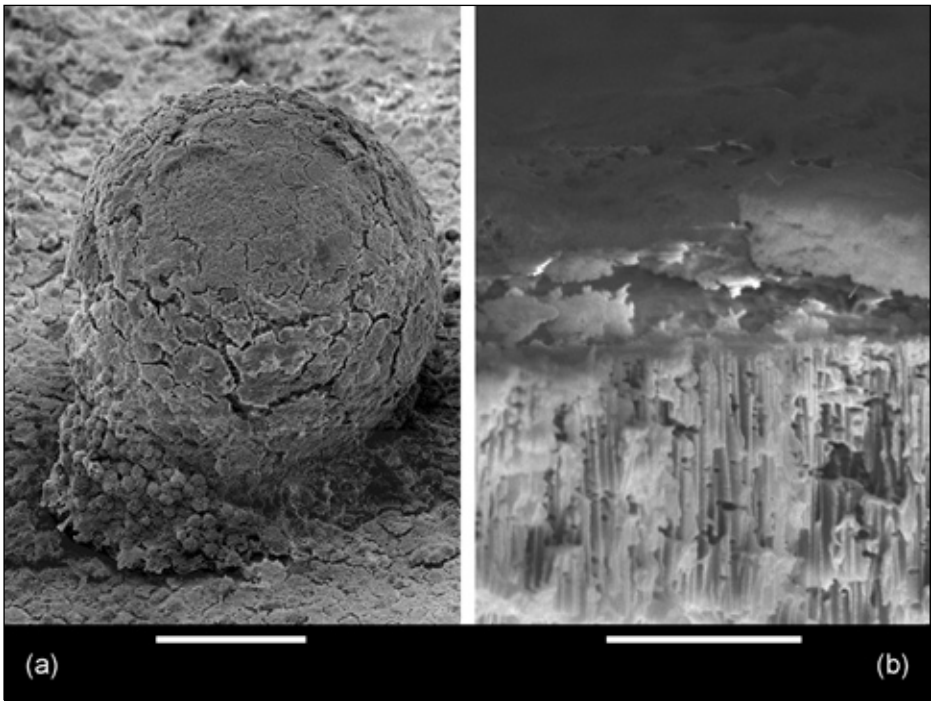

Fig. 6 - *Scanning electron micrographs: MLE-12 cells growing on the surface of a microcarrier bead and the surrounding polycarbonate membrane within the parenchymal compartment **(a)**. (original magnification 150x; scale bar 100 µm) The cross-section shows the ultrastructure of the polycarbonate membrane and cells attached to the parenchymal surface of the membrane **(b)**. Original magnification 5000x; scale bar = 5 µm.*

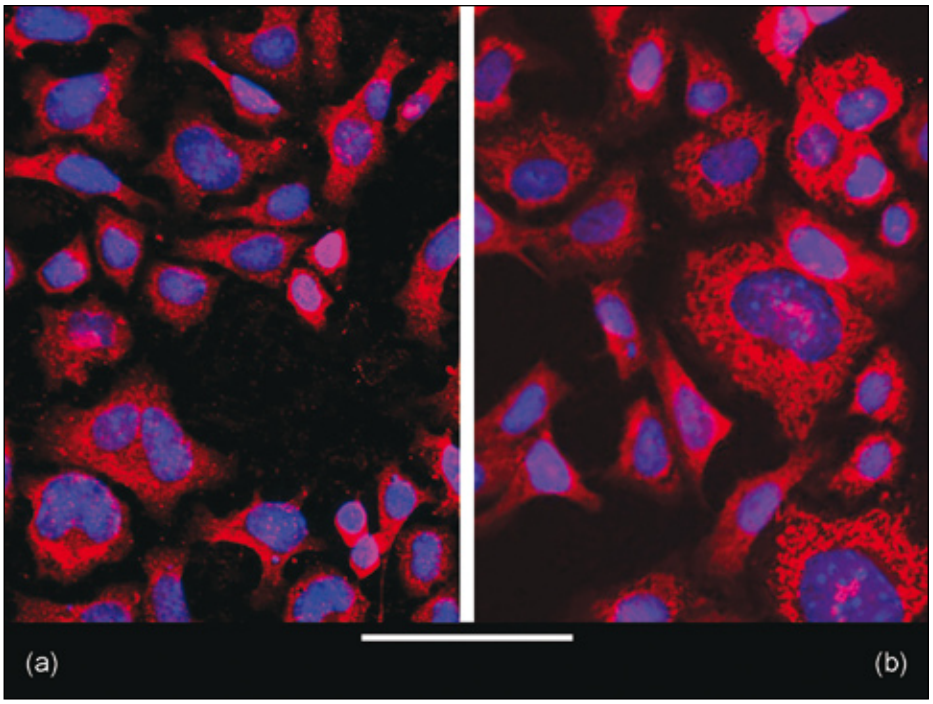

Fig. 7 - *proSP-B **(a)** and proSP-C **(b)** staining of MLE-12 cells growing in the parenchymal compartments of the dual-layer scaffolds, DAPI counterstain (original magnification 400x; scale bar=50 µm); most of the cells have been detached by the staining procedure.*

face area of up to 100 m^2 as a blood-air interface for gas exchange. This functional part of the alveolar wall consists of an alveolar epithelium which shares its basement membrane with the vascular endothelium of the adjoining pulmonary capillary.

We sought to develop a dual-layer microsystem that mimics this basic building plan of alveolar tissue. It provides a scaffold for cell proliferation and a pre-established supporting vascular bed to form vascularized alveolar tissue. The vascular network was molded in biocompatible

PDMS to entirely support an adjacent parenchymal chamber. By continuous perfusion with culture medium through the vascular compartment of the dual-layer scaffold, a sufficient exchange of nutrients, oxygen and waste products for cell proliferation in the parenchymal chamber was established. The results obtained validate the microfabricated dual-layer scaffold for pulmonary tissue engineering, demonstrating its ability to effectively support long-term alveolar epithelial cell survival, proliferation, and function. While these results are encouraging, challenges remain in developing an implantable tissue engineered lung.

The MEMS process technology has found a wide array of applications for tissue engineering, its main potential being the exquisite control of geometric features at the submicron level. This level of precision adds new dimensions of control for the guidance of tissue formation, allowing interaction with cells and tissues on their own size scale (16, 17). In this work we used MEMS technologies to create a pre-established vasculature for a dual-layer scaffold. A process methodology combining soft lithography in silicon and polymer replica molding was employed to fabricate channels with a topology that approximates a native vascular bed at micron-scale precision in PDMS. To design the vascular network we employed a computational algorithm that takes into account the non-Newtonian rheology of blood and its particulate nature, and simulates blood flow in microvascular networks at physiological levels of flow, pressure and resistance. This algorithm allows for the dynamic evolution of a branching microvascular design by adaptation of the vascular structure in response to alterations of the homeostatic state within the branching vascular network (13). The vascular network consists of a main channel branching out through 5 generations and progressively decreasing in width to the smallest capillary-like channel of 35 μm. Subsequently, these channels converge on the outflow side to form a single channel leading to the outlet. The vascular dimensions approximate the physiological morphometry of human pulmonary microvasculature, which is composed of 15 orders of arteries; the smallest artery in the lung is 20 μm in width.

In order to meet the dimensions of the native organ, several strategies have been discussed to scale up the tissue engineered constructs. The review by Borenstein et al gives a detailed overview of different approaches to fabricate three-dimensional scaffolds for tissue engineering (18). Kaihara et al formed tissue constructs by folding monolayers of hepatocytes into a compact 3-dimensional configuration and by rolling flat sheets of cells into a 3-dimensional cylinder (19). King et al described the assembly of three-dimensional scaffolds with an integrated network of vascular channels for tissue engineering by stacking large numbers of polymer films together (20). A single planar scaffold of the presented dual-layer design provides a surface area for gas exchange of 0.0008 m^2. Taking into account the anatomical dimensions, the combination of 125,000 of such units would be required to obtain the aspired surface area of 100 m^2, which appears to be rather unrealistic. Hoganson et al, however, estimated a surface area for gas exchange of less than 3 m^2 to be sufficient to meet the demands of a resting adult, based on a rate of gas transfer for O_2 of 0.17 ml/min and for CO_2 of 0.16 ml/min in similar dual-layer scaffolds (21). By further improvement of the scaffold microstructure, it should be feasible to achieve these functional requirements.

The dual-layer scaffolds described in this report are fabricated of PDMS, a transparent, flexible, non-toxic and chemically inert elastomer with excellent characteristics for cell culture. To modify the hybrid microsystems for *in vivo* applications, not only biocompatible but also biodegradable materials will be required. The transfer of the processing methodology reported here to biodegradable materials has been demonstrated and is currently being applied to a wide range of cell culture systems in our laboratory (20).

An immortalized epithelial cell line derived from murine pulmonary adenocarcinoma (MLE-12), a model of type II pneumocytes, was used in this pilot study. The alveolar epithelium consists of type I and II pneumocytes, pulmonary macrophages and other cell lineages. Only up to 7% of the alveolar wall is covered by the type II pneumocytes, whereas the type I pneumocytes account for most of the surface area. Type I pneumocytes form the functional air-blood interphase in the alveolus. They have lost the ability to replicate and in the case of injury are replaced by type II pneumocytes that differentiate into type I cells and thus represent the progenitor cells for the type I pneumocytes. Furthermore, type II pneumocytes feature the exclusive ability to produce and store surfactant. While recognizing the importance of type I pneumocytes, we focus on the culture of functional type II pneumocytes in this study, as they represent the endogenous progenitors of the alveolar epithelium and synthesize surfactant. Harvest and growth of primary alveolar epithelial cells are associated with numerous difficulties, but ultimately a solution needs to be found that circumvents the use of an immortalized cell line.

For proof-of-concept pilot studies, however, MLE-12 cells can be very useful, because they retain important phenotypic, morphologic and functional characteristics of type II pneumocytes. MLE-12 cells show a typical polygonal epithelial cell morphology, contain microvilli and cytoplasmic multivesicular bodies, grow adherently, produce cell type specific proteins (SP-B and SP-C), and can be passaged up to 40 times without losing these characteristics (10).

Several recent studies have addressed the issue of cell sources for tissue engineering of pulmonary tissue. Mixed populations of primary cells from pulmonary tissue samples have been successfully used for engineering lung tissue. Mondrinos et al developed three-dimensional alveolar-like constructs in Matrigel from mixed fetal pulmonary cells (22). Sugihara et al described the isolation of primary type II pneumocytes from pulmonary tissue samples of Wistar rats and the formation of globular structures in a collagen gel matrix (23). Cortiella et al reported the isolation of somatic stem cells from adult mammalian lung tissue, the differentiation into several lung specific cell types, and the tissue formation on two different polymeric scaffolds (24). The ability of bone marrow-derived progenitor cells to form alveolar epithelial cells is a controversial topic that currently under discussion (25). Several studies provide proof of the ability of embryonic stem cells to generate airway epithelial cells (26, 27). Coraux et al reported the formation of a fully differentiated airway epithelium from embryonic stem cells after seeding on porous Millicell-HA membranes and conditioning at the air-liquid interface for 15 days (28).

Microcarrier-based cell culture systems have been shown to support extensive cell expansion of anchorage-dependent cells *in vitro* (29). Microcarriers provide a large surface area for cell growth at low shear conditions. We used Biosilon microcarriers as vehicles for the cell transfer into the parenchymal compartments. Hence, the seeding procedure did not involve the use of trypsin, an enzyme harmful to cell membranes that potentially lowers cell viability. To promote reliable cell expansion over the parenchymal chamber surfaces, continuous perfusion of the vascular compartment during transfer of loaded beads was found to be essential. The flow rate of 0.082 ml/h within the smallest channels of the vascular network was established using a flow of 0.5 ml/h. The computed shear stress ranged from 0.520 dynes/cm^2 within the inlet channel to 0.075 dynes/cm^2 in the smallest channels. Perfusion of the dual-layer scaffold was realized in a single pass flow system, as opposed to the circulating flow loops used in various bioreactors by other groups. Single-pass flow assures stable composition of the culture medium, while intrinsic growth factors secreted by the proliferating cells are discarded. The decline of nutrients and accumulation of metabolic waste within the circulating medium can result in failure using flow loop systems. Due to the slow flow rate of cell culture medium within the vascular channels, the single-pass flow is thought to be superior to sustain cell survival within the dual-layer scaffold.

In conclusion, we developed a novel dual-layer scaffold for the tissue engineering of alveolar tissue. It provides a pre-established microvascular network which is capable of sustaining viable alveolar epithelial cells *in vitro*. Pneumocyte-like cells were shown to proliferate and to function for at least 7 days. These results represent another step toward the development of a biocompatible tissue engineered lung.

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Conflict of interest statement: I, Clemens S. Fritsche herewith confirm that none of the authors have any financial arrangements with any of the companies whose equipment or products were used in this study.

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