JOURNAL OF TISSUE ENGINEERING AND REGENERATIVE MEDICINE
J Tissue Eng Regen Med (2010).
Published online in Wiley InterScience (www.interscience.wiley.com) **DOI:** 10.1002/term.277

RESEARCH ARTICLE

Developing a tissue-engineered model of the human bronchiole

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Abstract

Scientists are always looking for new tools to better mimic human anatomy and physiology, especially to study chronic respiratory disease. Airway remodelling is a predominant feature in asthma and occurs in conjunction with chronic airway inflammation. Both the inflammatory and repair processes alter the airway wall which is marked by anatomical, physiological and functional changes. A tissue-engineered model of bronchiole remodelling presents a novel approach to investigating the initiation and progression of airway remodelling. By developing a unique bioreactor system, cylindrical-shaped bronchioles constructed from well-characterized human lung primary cells have been engineered and examined with a much greater control over experimental variables. We have grown human bronchioles composed of fibroblasts, airway smooth muscle cells, small airway epithelial cells and extracellular matrices. The various cell types are in close proximity to one another for cell–cell signalling and matrix interactions. The cylindrical geometry of the tissue applies radial distension for mechanotransduction and the air interface provides a natural environment for the epithelial cells. Optimal cell density, extracellular matrix concentration and media composition were determined. Immunohistochemistry verified bronchiole phenotypic stability. Quiescence was gauged by protein expression which verified a change in phenotype after the initial fabrication stage and implementation of the air interface. A fabrication timeline was devised for repeatable bronchiole fabrication and to understand tissue contraction and cell-seeding duration. The stability of the bronchiole structures and their cellular composition lends these bronchioles to study cell–cell interactions and remodelling events while maintaining *in vivo* geometrical dimensions and relationships.

Received 8 July 2009; Accepted 25 February 2010

Keywords tissue engineering; bioreactor; bronchioles; phenotype; mechanotransduction; lung fibroblasts; bronchiole epithelial cells; airway smooth muscle cells

1. Introduction

Although substantial progress has been made in the study of airway remodelling, the initiation and progression of chronic respiratory disease is not well understood, due to its dynamic nature *in vivo*. A tissue-engineered model of the bronchioles is a potentially powerful approach to studying airway remodelling, as cell–cell and cell–matrix interactions can be considered. Indeed, several models of the airway wall have been developed to investigate epithelial–stromal communication (Tschumperlin and Drazen, 2001; Agarwal *et al*., 2003; Choe *et al*., 2006), but none have incorporated the native cylindrical geometry of the *in vivo* bronchiole nor both fibroblasts and smooth muscle cells. Furthermore, tissue engineering has recently been proposed as an important avenue to understanding tissue physiology, as opposed to solely developing tissues for implantation or replacement (Griffith and Swartz, 2006).

As an experimental model to investigate airway remodelling, we have developed a bioreactor system to fabricate cylindrical airways, which maintains the bronchioles under mechanical stimulation and humidified

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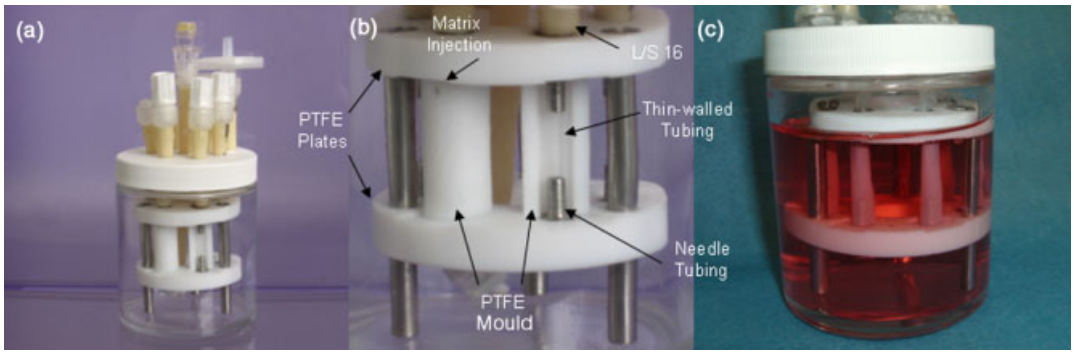

Figure 1. (a) Bioreactor insert in place. (b) The bioreactor insert shows a complete PTFE mould unit and a mould unit with half of the mould removed. The moulds are held in place by grooves in the parallel PTFE plates. The thin-walled silicone rubber tubing is inserted through the needle tubing, through the PharMed L/S 16 tubing, and connected to a 3/32 inch connector. (c) Once the tissue matrix solidifies, the moulds are removed and the chamber is filled with medium

air flow, and allows us to manipulate the growth environment. The overall intent is to develop a tissue-engineered bronchiole model of airway remodelling that approximates the behaviour of native tissue. This model system may advance understanding of the cumulative effects of individual factors associated with remodelling of human bronchioles.

Native bronchioles are affected by many factors. Lung fibroblasts, smooth muscle cells and epithelial cells are influenced by cell–cell signalling and interactions with the extracellular matrix. Stimulation of one cell type has been found to influence the behaviour of other cells types that are in close proximity (Zhang *et al*., 1999). The extracellular matrices bind soluble regulatory molecules that also mediate cell behaviour. Mechanical forces exerted on the matrix and cells during respiration influence pathophysiological conditions (Hirst *et al*., 2000; Swartz *et al*., 2001; Black *et al*., 2003). Cytoskeletal-mediated contraction of the airway is equilibrated dynamically, affecting the adaptability of the airway smooth muscle in response to mechanical changes (An and Fredberg, 2007). Shear stress and pressure exerted by air flow through the lumen also influence cell behaviour (Liu *et al*., 1999).

As part of a novel approach to model the human bronchiole, we have developed an *in vitro* model that mimics bronchiole wall physiology. The engineered bronchioles are composed of a collagen scaffold containing embedded lung fibroblasts. The exterior surface is surrounded by multiple layers of airway smooth muscle (ASM) cells, and the inner surface (lumen) is lined with bronchial epithelial cells with an air interface. The human airway cells are in close proximity to one another to promote cell–cell communications. The bioreactor environment can be manipulated to focus on various aspects of airway remodelling, such as subepithelial fibrosis (Woodruff and Fahy, 2002), smooth muscle hyperplasia and hypertrophy (Hirst, 1996) and epithelial cell metaplasia (Woodruff and Fahy, 2001; Doherty and Broide, 2007), all of which are key components of airway remodelling.

2. Materials and methods

2.1. Bioreactor design and features

The bioreactor is constructed using polytetrafluoroethylene (PTFE), stainless steel, and glass, based on their biocompatibility (Figure 1a). The tissue-engineered bronchioles are vertically supported by a unit constructed of two plates separated by stainless steel rods (Figure 1b). Scored stainless steel needle tubing is inserted into the parallel PTFE plates to act as grips for the contracting tissue (Figure 1c). Thin-walled silicone rubber tubing is threaded through the needle tubing (Figure 1c). Bisected, cylindrical PTFE tissue moulds insert into grooves on the top and bottom PTFE plate. The PTFE mould produces the tube-like shape of the airway (Figure 1c), while the thin-walled silicone rubber tubing creates the lumen of the bronchiole and also exerts dilatory forces on the engineered bronchiole when air is pulsed through the system. The glass outer housing (W216 904, Wheaton) of the bioreactor has a maximum volume of 120 ml.

One of the unique features of the bioreactor system is the ability to mechanically stimulate the engineered bronchiole through radial distension. The bioreactor applies mechanical stimulation by pulsing air through thin-walled silicone tubing. The thin-walled silicone rubber tubing (2.4 mm i.d. and 3 mm o.d.; Saint Gobain Performance Plastics, Beaverton, MI, USA) has a 0.6 mm wall thickness. which allows for radial distension. PharMed L/S 18 tubing is loaded into the head of a peristaltic pump (Masterflex L/S model 7553-80, Cole-Parmer; Figure 2a). Six PharMed L/S 13 tubes (06 485-13, Cole-Parmer) connect to six ports in the lid of the bioreactor (Figure 2a). The lid also has a vent port with 0.2 μm filter (02 915-08, Cole-Parmer) for pH stability and an injection port (2N3399, Baxter; Figures 1a, 2a). The injection port is used for medium exchange, sample withdrawal and supplement input.

Radial distension of the bronchioles is controlled by connecting the pump-tubing network to the six ports in the lid (Figure 1a). Once connected, the system is air-tight

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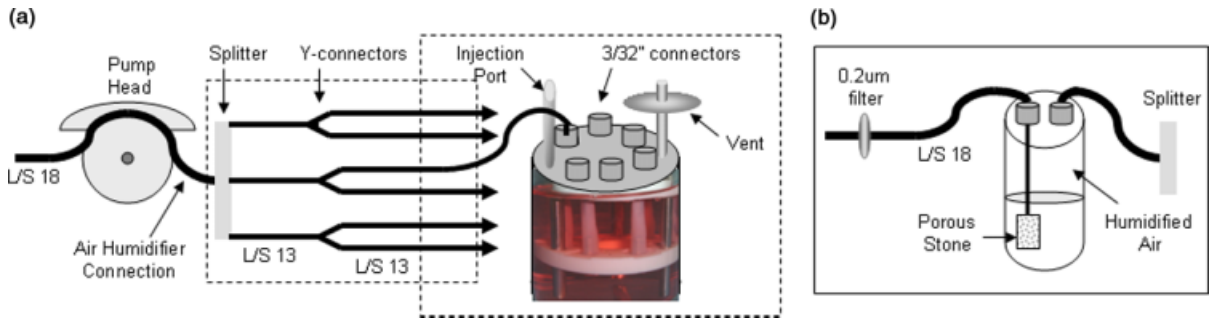

Figure 2. (a) The bioreactor is connected to the pump system via PharMed tubing to dilate the bronchioles during the contraction phase. (b) The air humidifier is connected to flowing air through the engineered bronchioles, creating an air interface. This system provides essential mechanical stimulation to the tissue

and pulses the thin-walled tubing. The expansion and relaxation of the tubing mimics adult quiet breathing. The resistance to air flow and viscous resistance to movement are nearly negligible. The engineered bronchiole is pulsed at a rate of 15 pulses/min, with a radial distension of approximately 2% and distension velocity of 0.015 mm/s.

The bioreactor system can also supply humidified air flow to the epithelialized lumen of the tissues (Figure 2b), once the luminal silicone tubing is removed. Room air is filtered with a 0.2 μm PTFE filter and then passes through a porous stone submerged in a bottle of sterile normal saline for humidification. A dew loop in the L/S 18 and a port in the three-way splitter traps extra moisture and allows for the removal of condensation.

2.2. Tissue-engineered bronchiole fabrication

The tissue-engineered bronchioles in this study are composed of three primary human lung cell types and a collagen matrix scaffold. The human primary cells (Lonza, formerly Clonetics) include normal human lung fibroblasts (CC2512), airway smooth muscle cells (CC2576) and small airway epithelial cells (CC2547). The cells are expanded in their respective medium: FGM Bullet kit (CC3131), SmGM-2 Bullet kit (CC3182) and SAGM Bullet kit (CC3118). The fabrication of the bronchioles occurs as a step-wise process. The complete process takes 28 days to create a phenotypically stable bronchiole.

First, the cylindrical-shaped bronchioles are fabricated by embedding fibroblasts (passages 1–6, 2×10^5 cells/ml matrix) in a collagen I matrix. The collagen matrix (BD Biosciences, Bedford, MA, USA) is prepared on ice by mixing 5× Dulbecco's modified Eagle's medium (DMEM; Sigma D5523, pH adjusted to 7.4) and 10× reconstitution buffer (2.2 g sodium bicarbonate, 4.77 g HEPES, 95 ml nanopure de-ionized water, and 5 ml 1 N sodium hydroxide) and then adding the type I collagen (Agarwal *et al*., 2003). The ratio of components is 20 : 10:65 w/v, respectively, with the remaining 5% consisting of fibroblasts and FGM.

Fibroblasts are suspended in a small volume of FGM and pipetted into the chilled collagen matrix and then mixed. The chilled fibroblast–collagen solution is pipetted into a PTFE mould, and the tissue solidifies during a 2 h incubation at 37 °C, after which the moulds are removed. The reactor is filled with FGM [2% fetal bovine serum (FBS)].

During the ensuing 24 h, the tissue contracts around the silicone rubber tubing. ASM cells (passages 1–5, $\sim 6 \times 10^6$ cells/reactor) are dynamically seeded onto the periphery of the collagen bronchiole constructs by stirring the SmGM-2 at 150 rpm (Figure 3). After 48 h, the medium is changed to remove any unattached ASM cells. Once the ASM cells are seeded, the airways are pulsed to mechanically stimulate circumferential ASM alignment. The tissue is maintained in FGM and stirred at 50 rpm to facilitate oxygen, pH and nutrient distribution. One-half of the FGM is refreshed every third day in the bioreactor. Large-volume exchange is necessary due to the high density of ASM cells and fibroblasts ($\geq 7 \times 10^6$ cells/reactor). ASM death occurred when the FGM was refreshed at longer intervals.

In the final cell seeding step, the lumen of the bronchiole is epithelialized. The epithelial cells (passages 1–3, 3.5×10^5 cells/tissue) are suspended in 100 μl proliferative medium (bronchiole epithelial medium with single quots, 25 ng/ml epithelial growth factor (EGF), retinoic acid 5×10^{-8} M) (Gray *et al*., 2001) and injected into the lumen of the bronchiole. Twelve hours after static seeding of the epithelial cells, the medium is gently circulated through the lumen to remove the cells that did not adhere. The system is not pulsed during epithelial cell seeding. Proliferative medium is injected (200 μl) into the lumen twice daily for 3 days and then substituted with quiescent medium (50% LHC basal, 50% DMEM, 0.5 ng/ml EGF) for 4–7 days (Gray *et al*., 2001). After 7–10 days post-epithelial seeding, the epithelial cells form a monolayer with an established basement membrane and the quiescent medium is removed (Figure 3).

After epithelial seeding and culture, the epithelial cells are exposed to air. The air humidifier is connected to the pump system, allowing humidified air flow through the epithelialized lumen. During epithelial cell seeding, the periphery of the tissue is maintained in FGM because a small amount of serum is necessary to support the ASM and fibroblasts. Figure 3 expresses the fabrication steps

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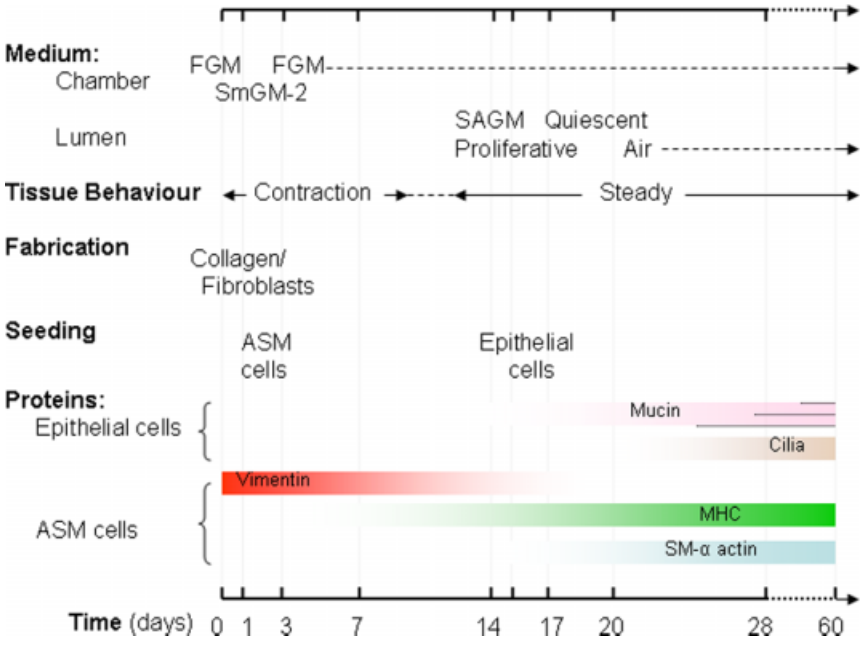

Figure 3. The tissue is fabrication by embedding fibroblasts in collagen I on day 0. The ASM are dynamically seeded and mechanical stimulation is applied on day 2. The tissue contracts over the next 7–10 days and then the epithelial cells are seeded on day 14. As the tissue is modified by the cells and stabilizes, the phenotypic gene expression alters (denoted by shading and protein name) and cell functionality changes

in chronological order, including medium use, seeding timing and tissue behaviour.

2.3. Tissue-engineered bronchiole stimulation

The bioreactor system stimulates the tissue-engineered bronchioles in two ways. First, mechanical stimulation is applied through the radial distension of the tissue construct during the contractile phase. Second, humidified air flow through the epithelialized lumen of the bronchiole also causes a slight distension in the radial direction. These force applications and the geometry of the engineered bronchiole may provide a better understanding of the effect of mechanotransduction on cell behaviour.

Mechanical stimulation is applied to the contracting tissues during the first 13 days after tissue fabrication (contraction phase, Figure 3). The fibroblasts embedded in collagen matrix contract around the silicone rubber tubing. During the initial mechanical stimulation phase, the thin-walled silicone rubber tubing is pulsed at a rate of 15 pulses/min with a radial distension of approximately 2% and distension velocity of 0.015 mm/s. The diameter of the bronchiole increases by about 60 μm, which causes biaxial (circumferential and axial) forces to act on the cells. Mechanical stimulation is secondarily applied to the engineered bronchioles by flowing humidified air through the epithelialized lumen.

Although mechanical stimulation was applied to determine whether the engineered bronchioles could be exposed to radial distension during the contraction phase (days 1–14), these bronchioles were not pulsed with physiologically normal air flow after the epithelial monolayer was formed. The bronchioles were pulsed with near-static humidified air with pressure not greater than 4 mmHg.

2.4. Tissue-engineered bronchiole phenotype analyses

The engineered bronchioles were sampled at 7, 14, 28 and 60 days post-fabrication. Immunohistochemistry was performed in order to assess changes in the tissue through protein expression. The engineered bronchioles were fixed in 10% neutral buffered formalin, graded ethanol dehydrated, embedded in paraffin and then sectioned (5 μm thick). Fluorescent staining was accomplished by blocking with 2% goat serum for 30 min at room temperature, primary antibody application for 1 h, three 1 min washes in PBS and then application of the secondary antibody for 30 min. ASM cells were labelled using primary antibodies against smooth muscle α-actin (1 : 100; DAKO, M0851) and smooth muscle myosin heavy chain (1 : 100; DAKO, M3558) with Alexa Fluor 488 (Invitrogen, A21121) secondary, and vimentin (1 : 200; DAKO, M7020) with Alexa Fluor 594 secondary (Invitrogen, A21135). Fibroblasts were labelled for FITC-conjugated β-tubulin (1 : 200; Sigma, T4026) with DAPI (1 : 2500; Invitrogen, D1306) nuclear labelling. The epithelial cells were labelled for cytokeratin-19 (1 : 100; Sigma, C6930) and collagen IV (1 : 500; Sigma, C1926) with TRITC (1 : 100; Sigma, T2659) secondary; and β-tubulin and mucin (1 : 100; Abcam, ab7874) with Alexa Fluor 488 secondary and DAPI nuclear stain. The fluorescently labelled airway cross-sections were documented using a Zeiss Axiovert 200 microscope with a digital camera.

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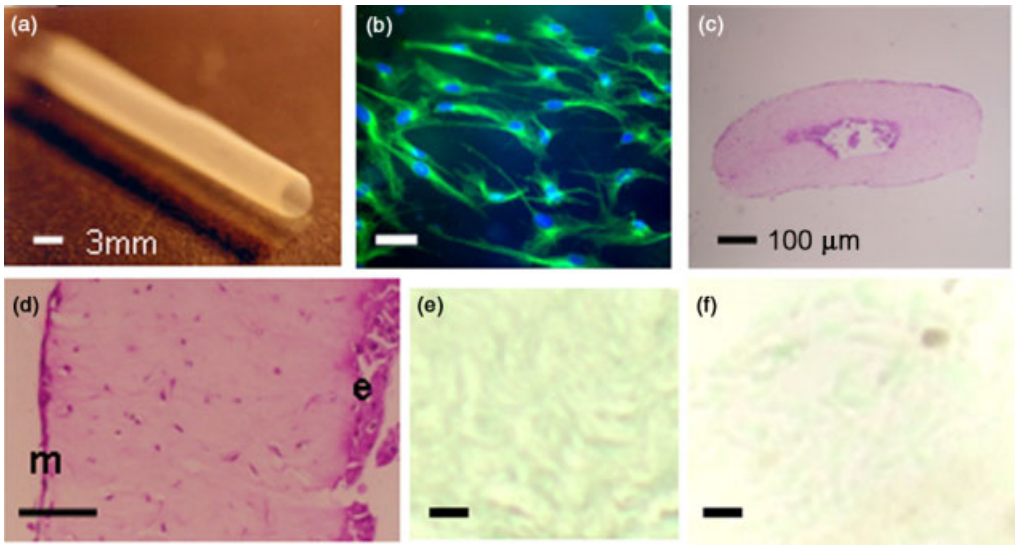

Figure 4. (a) The cylindrical bronchiole is 3 mm diameter and 3 cm long. (b) Tissue strength is provided by the fibroblasts in the collagen construct. (c, d) The ASM cells (m), fibroblasts and epithelial cells (e) are in close proximity to promote cell–cell communication. (e) The fibroblasts do not extensively proliferate or (f) undergo significant apoptosis. Bar = 10 µm unless indicated otherwise

Bright-field microscopy was performed for macroscopic observations. Haematoxylin and eosin (H&E) was used to view collagen fibres and the structure of the engineered bronchioles. Apoptosis and proliferating cell nuclear antigen (PCNA) were performed on the fibroblasts for cell viability and proliferation. For apoptosis, an ApopTAG kit (S7100, Chemicon) was used. Proliferating cells were identified with a primary antibody against PCNA (1 : 300; DAKO) and secondary antibody conjugated with horseradish peroxidase, using a VectaStain ABC Elite kit and DAB (PK-6102 and SK-4100, Vector Laboratories).

3. Results

3.1. Bioreactor design and feature optimization

The biomaterials for construction of the bioreactor system were chosen to promote ease of sterilization and low cost, minimize the quantity of biological agents and provide long-term use without defects.

The current version of the bioreactor is very user-friendly (Figure 1). The bioreactor insert can be completely disassembled for cleaning or part replacement and easily reassembled. The flint glass chamber is economical and can also be replaced. The bioreactor insert is assembled with the PTFE moulds in place and then the entire reactor is sterilized. This minimizes the risk of contamination.

The pump system affords the most unique attribute of the bioreactor system (Figure 2a). It supplies the engineered bronchioles with pulsed air to mechanically stimulate the tissue during the contractile phase and distributes humidified air to the epithelialized lumen during the differentiation phase (Figure 2b). The peristaltic pump was selected for its slow rotation (1–100 rpm) and pump head selection for the number of rollers. The PharMed L/S 18 tubing has a 7.9 mm inner diameter (i.d.) that forces a large volume of air into the three-way splitter and through the L/S 13 tubing (0.8 mm i.d.). Pump speed was varied to pulse the thin-walled silicone rubber tubing 15 times/min. The roller positions in the pump head and the diameter of the tubing allowed for a 2% radial distension, which increased the diameter of the bronchiole by 60 µm (from 3 mm to approximately 3.06 mm).

3.2. Determination of tissue fabrication parameters

Fabrication of the tissue-engineered airways involved parameter optimization of matrix concentration, fabrication mechanics, cell density and seeding methods, medium composition and cell phenotype. Proper proportioning of these parameters was necessary for the creation of a stable engineered bronchiole (Figure 4a).

3.2.1. Matrix composition

The matrix concentration was determined by modifying a pre-existing protocol (Agarwal *et al.*, 2003). Utilizing 5 mg/ml collagen for the cylindrical bronchiole maintained a tubular shape after the PTFE mould was removed. Lower concentrations of collagen tended to deform or tear during mould removal.

3.2.2. Cell seeding density

Optimal seeding densities were determined to achieve the goal of a monolayer of epithelial cells and a multilayer of ASM cells. ASM cell-seeding density was initially estimated by calculating the exterior surface area of

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the engineered bronchiole and estimating the number of ASM cells to cover the surface with three layers of cells. Seeding densities of 0.5, 1, 2, and 3 million cells/bronchiole were investigated ($n = 10$, using three lots of ASM cells). ASM cells were dynamically seeded by suspending the cells in SmGM-2. After 48 h, the entire medium volume was removed and a cell count was done. For 0.5×10^6 cell-seeding density/bronchiole, no viable ASM cells were remaining in the medium, indicating that all of the cells adhered to the exterior surface of the bronchiole. For 1×10^6 ASM cells, there were $>0.5 \times 10^6$ ASM cells remaining $9 \pm 1\%$ of the time. For 2×10^6 ASM cells/bronchiole seeding density, $31 \pm 2\%$ of the time, there were $>0.5 \times 10^6$ ASM cells/bronchiole that did not adhere to the exterior of the engineered bronchiole. For 3×10^6 cells, there were $>0.5 \times 10^6$ ASM cells remaining more than $83 \pm 5\%$ of the time. Therefore, 1×10^6 ASM cells/engineered bronchiole was considered optimal.

Epithelial cells were statically seeded into the lumen of the bronchiole. The cell density was determined by calculating the surface area of the inner diameter of the tissue construct. A monolayer of cells was deemed the fastest way to prepare the lumen, rather than waiting for the epithelial cells to proliferate and form a monolayer. The seeding time was determined by seeding the cells and then gently flushing the bronchiole after 6, 8, 12, 18 and 24 h ($n = 6$ for three lots of SAEC). Cell viability was checked by placing the flushed SAGM kit into a flask and observing the number of cells to adhere. At 6 h, $\geq 2 \times 10^5$ epithelial cells were flushed from the lumen $73 \pm 5\%$ of the time. At 8 h, $\geq 1 \times 10^5$ epithelial cells were flushed from the lumen $41 \pm 4\%$ of the time. At 12 h, $18 \pm 2\%$ of the time, $<1 \times 10^5$ epithelial cells were removed from the bronchiole. At 18 and 24 h, $<1 \times 10^5$ epithelial cells were flushed from the bronchiole $12 \pm 2\%$ of the time but cell viability decreased by $33 \pm 5\%$. The best seeding time was 12 h. Therefore, 3.5×10^5 epithelial cells were suspended in 100 µl proliferative medium and injected into the lumen of the engineered bronchiole and incubated for 12 h.

3.2.3. Medium composition

Since the bronchiole wall diameter was 200 µm or less, the medium in the chamber likely affects all three cell types by diffusion of soluble factors. The initial fabrication step was conducted using FGM. The fibroblasts were embedding in collagen matrix and after the airway had solidified FGM was added to the chamber. The tissue contracted in the first 24 h and then the ASM cells were seeded. For ASM cell seeding, the chamber was filled with SmGM-2 for 48 h while attaching to the periphery of the bronchiole. After 48 h, the SmGM-2 was replaced with FGM. This was done because the fibroblast medium has a lower serum concentration (2%) and different growth factors (eliminates EGF), which allowed the tissue to stop contracting and stabilize within 10 days.

The epithelial cells were very sensitive to medium composition. It has been shown (Gray *et al.*, 2001) that a high EGF concentration helps to establish an epithelial monolayer. After 3 days of exposure to 25 ng/ml EGF (proliferative medium), the concentration was decreased to 0.5 ng/ml EGF (quiescent medium) for 4–7 days (Figure 3). These manipulations allowed the epithelial cells to form a tight monolayer, produce a basement member and present proper morphology.

3.3. Tissue-engineered bronchiole fabrication

At the time of fabrication, the bronchiole wall thickness of the collagen–fibroblast construct is 1 mm. Over the first 5–7 days after fabrication, the fibroblasts branch (Figure 4b) and adhere to the collagen I fibres, causing the tissue wall to thin, thereby forming a 'lamina propria' of 100–200 µm thickness (Figure 4c). The thinness of the bronchiole wall is important for the diffusion of mediators between the different cell types (Figure 4d). It was determined by PCNA staining that the fibroblasts' proliferation was negligible within the first 60 days (Figure 4e). Moreover, the fibroblasts remain viable in the collagen I matrix, with little to no evidence of apoptosis (Figure 4f). Since the engineered bronchiole contracted and then stabilized, and the fibroblasts exhibited quiescent behaviour (branching, negligible proliferation and apoptosis), the seeding density of 2×10^5 fibroblasts/ml tissue matrix was deemed adequate (Agarwal *et al.*, 2003).

The ASM cells were dynamically seeded on the periphery of the engineered bronchiole, forming a multi-layer of cells. ASM cells were not seeded until the collagen–fibroblast construct contracted around the silicone rubber tubing (24 h) to prevent ASM contamination of the airway lumen.

The fabrication timeline for bronchiole contraction was integral to establish this model. Preliminary trials were conducted at 5, 7, 10, 14, 21 and 28 days to determine the fabrication timeline. The contraction period of 5–7 days for the bronchiole to decrease from 1000 to 100–200 µm in thickness was repeatable 98% ±1 ($n = 10$, three lots of ASM, one lot of NHLF). The wall diameter did not change from day 10 to day 14, indicating that the tissue construct had stabilized. The fibroblasts and ASM cells did not cause tissue contraction after day 10, which was crucial for the removal of the thin-walled silicone rubber tubing and seeding of the epithelial cells in the bronchiole lumen. When epithelial cells were seeded before the bronchiole wall was finished contracting (during days 2–4), the epithelial cells sloughed off of the wall. due to compression of the monolayer.

3.4. Tissue-engineered bronchiole analyses

Preliminary trials were conducted at 5, 7, 10, 14, 21 and 28 days to determine the fabrication timeline and to select sampling times ($n = 6$ bronchioles). Subsequently, tissue characteristics were determined at 7, 14, 28 and

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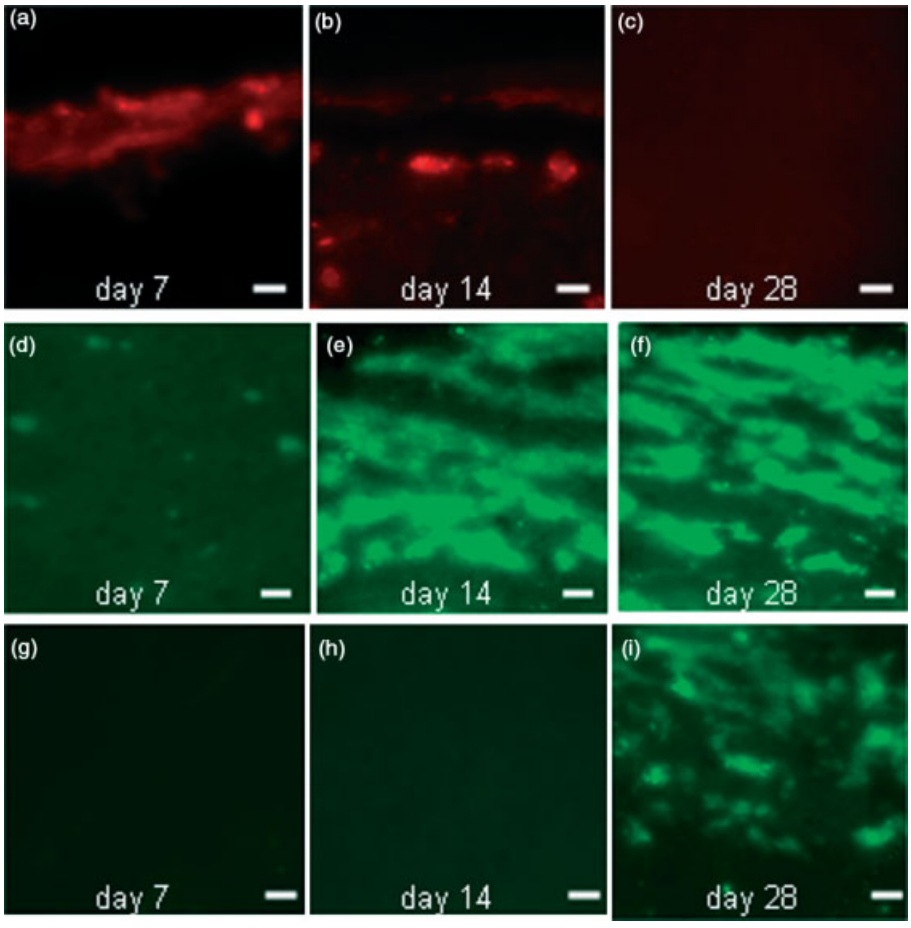

Figure 5. The ASM layer labels for (a–c) vimentin during the first 7–14 days, showing a synthetic phenotype that recedes by day 28. An increase in (d–f) MHC and (g–i) α-actin expression, indicating a change from synthetic to contractile phenotype. Bars = 10 μm unless indicated otherwise

60 days. Each bronchiole comprises 1 cm of testable tissue (Figure 4a).

Immunohistochemistry provided information regarding protein expression and location (Figure 3), to evaluate the phenotype of the fibroblasts, ASM cells and epithelial cells. ASM cell phenotype was evaluated by the presence of contractile proteins. The intensity of vimentin was more pronounced during the first 10 days (Figure 5a–c), as opposed to day 28. Vimentin expression indicates that the ASM cells are functioning synthetically. As the ASM cells establish themselves on the tissue construct, the cells made the transition from the synthetic to the contractile phenotype, as shown by the increased expression of smooth muscle myosin heavy chain (MHC) after day 14 (Figure 5d–f). Smooth muscle α-actin intensity for the ASM layer remained constant from day 7 to day 14, but increased slightly by day 28 (Figure 5g–i).

The small airway epithelial cells were seeded in the lumen of the tissue to create an epithelialized bronchiole. The cells were seeded on day 14, which was after the construct had finished contracting. When epithelial cells were seeded 3–9 days post-fabrication, the epithelial cells were compressed and shed from the luminal surface. The epithelial cells stained positively for the epithelial-specific marker cytokeratin-19 (Figure 6a). The presence

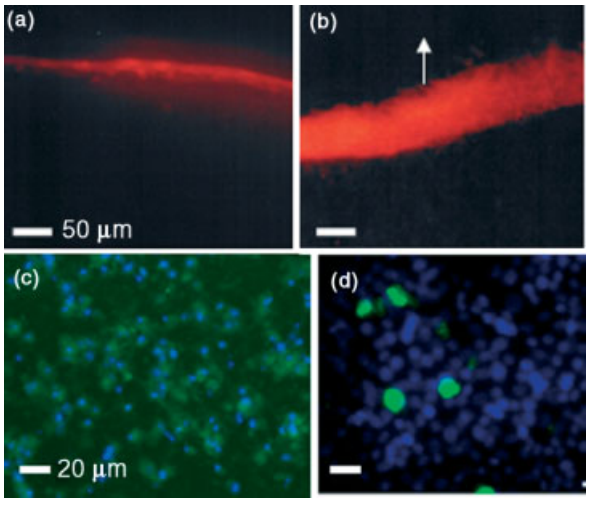

Figure 6. The epithelium is positive for (a) K-19 and establishes a basement membrane of (b) collagen IV (arrow denotes epithelial cell side of membrane). When air-lifted for 7–14 days, the epithelial cells produce (c) cilia (green) and (d) mucus (green) by day 28

of K19 implies that differentiation is not being inhibited by retinoic acid. At low quiescent retinoic acid concentrations EGF can suppress mucin, while higher concentrations

DOI: 10.1002/term

of retinoic acid override EGF and increase mucous differentiation (Denning and Verma, 2001; Gray *et al*., 2001). Collagen IV labelling showed that the epithelial cells synthesized a basement membrane of collagen IV (Figure 6b) over the collagen I tissue construct. Once the SAGM was removed from the lumen of the bronchiole around day 21, humidified air flowed at a nearly static rate through the lumen of the engineered bronchiole. The air interface promoted the production of cilia (Figure 6c) and mucin (Figure 6d) by day 28 post-fabrication, when luminal pressures up to 4 mmHg were applied. The cells remained viable as long as the air flow did not dry the cells.

4. Discussion

A bioreactor system for culturing bronchiole tissues, which comprise three different cell types, has been described. Human lung fibroblasts, airway smooth muscle cells and bronchiole epithelial cells can be grown in close proximity to one another in the same culture environment and exhibit evidence of proper cellular behaviour. Tissue fabrication protocols have been established and engineered bronchiole stability has been shown through phenotypic analyses, both protein expression and morphology, and prolonged culture times of 60 days (Figure 3). The stability of the bronchiole structures and their cellular composition allows these constructs to be used to study cell–cell interactions and airway remodelling events while maintaining *in vivo* geometrical dimensions and relationships.

Currently, treatments for asthma focus on the underlying airway inflammatory and constrictive processes. Although bronchodilators, anti-inflammatories and long-acting β_2 agonists can improve lung function, these medications only act to relieve, prevent and control symptoms, respectively (Kumar, 2001). Neither the initiation and progression of airway remodelling nor its contribution to irreversible airway obstruction in asthma is well defined. Biopsies almost always reveal airway remodelling associated with asthma (Woodruff and Fahy, 2001); however, it is not always clinically demonstrated (Beasley *et al*., 2002). The severity of asthma varies so greatly that with its onset, the clinical evidence of remodelling can occur after only a few months or as much as several decades later (Beasley *et al*., 2002). A tissue-engineered bronchiole model of airway remodelling may lead to understanding that could produce therapeutic agents to inhibit or control airway remodelling.

Immunohistological and morphological evidence, bolstered by extensive culture protocol optimization, supports a two-phase fabrication protocol of 28 days. Bronchiole construction and ASM cell seeding during the first 48 h of tissue fabrication, followed by a 7 day contraction period, appeared optimal for producing the bronchiole construct. Epithelial cell seeding too early in the fabrication process caused compression of the monolayer with ongoing tissue contraction, thus leading to epithelial sloughing. Epithelial cells seeded at day 14 proved effective to maintain cellular viability and allow for subsequent differentiation. Once the epithelial cells formed a monolayer and established a basement membrane, the cells were exposed to humidified air flow; after which they produced cilia and mucus.

Since cells can be easily manipulated *in vitro* to produce behaviour that is not like cellular behaviour under *in vivo* conditions, phenotypic modulation was deemed important to create a viable bronchiole that can be used as a model of airway remodelling. Determination of phenotypic behaviour is also informative to analyse cell–cell interactions, since the fibroblasts, airway smooth muscle cells and epithelial cells are within 200 μm of one another (Figures 3, 4c). ASM cell expression of vimentin, myosin heavy chain and α-actin changed during the first 28 days of tissue differentiation. By day 28, cell viability and markers of differentiation were stable through day 60.

Preliminary studies are currently under way to determine the parameters to induce airway remodelling. Various studies have shown that remodelling can be induced using growth factors (Stewart *et al*., 1994; Vignola *et al*., 1997; Shute, 2001; Chu *et al*., 2004), cytokines (De *et al*., 1992; Bonner and Brody, 1995; Cohn *et al*., 1998; Grunig *et al*., 1998; Wills-Karp *et al*., 1998; Renauld, 2001; Elias *et al*., 2003) and chemokines (Noveral and Grunstein, 1992; Holgate *et al*., 2003). Manipulation of the growth environment provides a format to utilize these triggers. Supplementation of the growth environment of the bioreactor may improve our understanding of remodelling events associated with ASM, fibroblasts and epithelial cells. This model can be tailored to study individual components of remodelling such as subepithelial fibrosis, smooth muscle hyperplasia and hypertrophy, and epithelial cell metaplasia. By doing a piece-wise investigation of airway remodelling, we may arrive at a better understanding of the individual contributors that initiate and cause the progression of remodelling associated with chronic respiratory disease.

Acknowledgement

A grant from the National Heart, Lung, and Blood Institute (to Ruth L. Kirschstein) National Research Service Award for Individual Postdoctoral Fellows (No. F32 HL-69646) is gratefully acknowledged.

References

Agarwal A, Mih J, George S. 2003; Expression of matrix proteins in an *in vitro* model of airway remodeling in asthma. *Allergy Asthma Proc* **24**(1): 35.

An S, Fredberg J. 2007; Biophysical basis for airway hyperresponsiveness. *Can J Physiol Pharmacol* **85**(7): 700–714.

Beasley R, Page C, Lichtenstein L. 2002; Airway remodeling in asthma. *Clin Exp All Rev* **2**: 109.

Black JL, Burges JK, Johnson P. 2003; Airway smooth muscle – its relationship to the extracellular matrix. *Respir Physiol Neurobiol* **137**: 339.

Bonner JC, Brody AR. 1995; Cytokine-binding proteins in the lung. *Am J Physiol* **268**(6, pt 1): L869–878.
Choe M, Tomei A. Swartz M. 2006; Physiological 3D tissue model of the airway wall and mucosa. *Nat Protoc* **1**(1): 357–362.
Chu HW, Balzar S, Seedorf GJ, *et al*. 2004; Transforming growth factor-β2 induces bronchial epithelial mucin expression in asthma. *Am J Pathol* **165**(4): 1097–1106.
Cohn L, Tepper JS, Bottomly K. 1998; Cutting edge: IL-4-independent induction of airway hyperresponsiveness by Th2, but not Th1, cells. *J Immunol* **161**: 3813–3816.
De S, Zelazny ET, Souhrada JF, *et al*. 1992; IL-1β and IL-6 induce hyperplasia and hypertrophy of cultured guinea pig airway smooth muscle cells. *J Appl Physiol* **78**: 1555.
Denning MF, Verma AK. 2001; The mechanism of the inhibition of squamous differentitation of rat tracheal 2C5 cells by retinoic acid. *Carcinogenesis* **15**(3): 503–507.
Doherty T, Broide D. 2007; Cytokines and growth factors in aiway remodeling in asthma. *Curr Opin Immunol* **19**: 1–5.
Elias JA, Lee CG, Zheng T, *et al*. 2003; Interleukin-13 and leukotrienes: an intersection of pathogenetic schema. *Am J Respir Cell Mol Biol* **28**: 401.
Gray T, Nettesheim KJS, Nettesheim P. 2001; Regulation of mucus differentiation and mucin gene expression in the tracheobronchial epithelium. *Toxicology* **160**: 35–46.
Griffith L, Swartz M. 2006; Capturing complex 3D tissue physiology *in vitro*. *Nat Rev Mol Cell Biol* **7**: 211–224.
Grunig G, Warnock M, Wakil AE, *et al*. 1998; Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**: 2261.
Hirst S. 1996; Airway smooth muscle cell culture: application to studies of airway wall remodelling and phenotype plasticity in asthma. *Eur Respir J* **9**(4): 808–820.
Hirst SJ, Twort C, Lee TH. 2000; Differential effects of extracellular matrix protein on human airway smooth muscle cell proliferation and phenotype. *Am J Respir Cell Mol Biol* **23**: 335.
Holgate ST, Peters-Golden M, Panettieri RA, *et al*. 2003; Roles of cysteinyl leukotrienes in airway inflammation, smooth muscle function, and remodeling. *J Allergy Clin Immunol* **111**(1): S18.
Kumar R. 2001; Understanding airway wall remodeling in asthma: a basis for improvements in therapy? *Pharmacol Therapeut* **91**: 93–104.
Liu M, Tanswell A, Post M. 1999; Mechanical force-induced signal transduction in lung cells. *Am J Physiol* **277**(4, pt 1): L667–683.
Noveral JP, Grunstein MM. 1992; Role and mechanism of thromboxane-induced proliferation of cultured airway smooth muscle cells. *Am J Physiol* **263**: L555.
Renauld J-C. 2001; New insights into the role of cytokines in asthma. *J Clin Pathol* **54**: 577.
Shute JK. 2001; Growth factor–extracellular matrix interactions in bronchial tissue in asthma. In *Airway Remodeling*, Howarth PH, Wilson JW, Bousquet J, *et al*. (eds). Marcel Dekker: New York; 245–257.
Stewart AG, Grigoriadis G, Harris T. 1994; Mitogenic actions of endothelin-1 and epidermal growth factor in cultured airway smooth muscle. *Clin Exp Pharmacol Physiol* **21**: 277.
Swartz MA, Tschumperlin DJ, Kamm RD, *et al*. 2001; Mechanical stress is communicated between different cell types to elicit matrix remodeling. *Proc Natl Acad Sci USA* **98**(11): 6180.
Tschumperlin DJ, Drazen JM. 2001; Mechanical stimuli to airway remodeling. *Am J Respir Crit Care Med* **164**: S90.
Vignola AM, Chiappara G, Chanez P, *et al*. 1997; Growth factors in asthma. *Monaldi Arch Chest Dis* **52**(2): 159–169.
Wills-Karp M, Luyimbazi J, Xu X, *et al*. 1998; Interleukin-13: central mediator of allergic asthma. *Science* **282**: 2258.
Woodruff P, Fahy J. 2001; Asthma: prevalence, pathogenesis, and prospects for novel therapies. *J Am Med Assoc* **286**(4): 395–398.
Woodruff PG, Fahy VJ. 2002; Airway remodeling in asthma. *Semin Respir Crit Care Med* **23**(4): 361–367.
Zhang S, Smartt H, Holgate ST, *et al*. 1999; Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an *in vitro* co-culture model of airway remodeling in asthma. *Lab Invest* **79**(4): 395–405.

DOI: 10.1002/term